YEAR 2020: NEW TRENDS IN PHARMACOLOGICAL TREATMENTS FOR OSTEOARTHRITIS

EDITED BY: Antonella Fioravanti, Raquel Largo, Serena Guiducci, Andrea Bernetti, Sara Cheleschi, Sara Tenti and Per-Johan Jakobsson

PUBLISHED IN: Frontiers in Pharmacology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88976-232-3
DOI 10.3389/978-2-88976-232-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

YEAR 2020: NEW TRENDS IN PHARMACOLOGICAL TREATMENTS FOR OSTEOARTHRITIS

Topic Editors:
Antonella Fioravanti, Institue of Rheumatology-Azienda Ospedaliera Senese
Raquel Largo, Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain
Serena Guiducci, University of Florence, Italy
Andrea Bernetti, Sapienza University of Rome, Italy
Sara Cheleschi, University Hospital of Siena, Italy
Sara Tenti, University of Siena, Italy
Per-Johan Jakobsson, Karolinska Institutet (KI), Sweden

Citation: Fioravanti, A., Largo, R., Guiducci, S., Bernetti, A., Cheleschi, S., Tenti, S., Jakobsson, P.-J., eds. (2022). Year 2020: New Trends in Pharmacological Treatments for Osteoarthritis. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-232-3

Table of Contents

frontiers | Frontiers in Pharmacology

EDITORIAL
published: 03 May 2022
doi: 10.3389/fphar.2022.892934

Editorial: Year 2020: New Trends in Pharmacological Treatments for Osteoarthritis

A. Fioravanti, S. Tenti and S. Cheleschi*

Rheumatology Unit, Department of Medicine, Surgery and Neuroscience, Azienda Ospedaliera Universitaria Senese, Siena, Italy

Keywords: osteoarthritis, pharmacological treatment, chondrocytes, hyaluronic acid, disease modifying osteoarthritic drugs

Editorial on the Research Topic

Year 2020: New Trends in Pharmacological Treatments for Osteoarthritis

INTRODUCTION

Osteoarthritis (OA) is the most common rheumatic condition over the world and its prevalence is rising due to the increasing obesity and life span of the general population. It is estimated that the number of people affected by this condition will increase by approximately 50% over the next 20 years (Hunter and Bierma-Zeinstra, 2019; Long et al., 2022).

OA has a remarkable impact on functional ability and quality of life and it is one of the major causes of disability; the Global Burden of Diseases Study 2019 estimates that years lived with disability (YLD) due to OA increased of 114.5% from 1990 to 2019 (GBD 2019 Diseases and Injuries Collaborators, 2020). Unfortunately, its etiology is only partly understood and multiple factors ranging from aging to biomechanical stimuli contribute to the development and progression of the disease (Ren et al., 2020).

Growing evidence shows that OA is a complex condition, in which the whole joint is involved; degradation of articular cartilage, subchondral sclerosis, and hyperplasia of synovial tissue are hallmarks of OA (Goldring and Goldring, 2016). Destructive processes of articular cartilage play a pivotal role in the development and progression of the disease resulting from an imbalance between catabolic and anabolic events (Goldring and Goldring, 2016). However, the exact mechanism that drives OA is still poor understood, even if, it is assumed that different mediators contribute to the pathogenesis of the disorder (Cheleschi et al., 2018; Zheng et al., 2021); this circumstance poses a challenge for its management. In fact, current pharmacological treatments are mostly related to the relief of the symptoms, whereas disease-modifying OA drugs (DMOADs) (aimed at reducing symptoms in addition to slowing or stopping the disease progression) are not actually available (Latourte et al., 2020). The discovery of the crucial pathways characterizing OA could offer new opportunities to identify compounds potentially able to reduce or stop the disease progression (Latourte et al., 2020). The purpose of this Research Topic was to provide an overview of emerging preclinical (*in vivo*, animal models, and *in vitro*) and clinical studies testing different approaches for the treatment of OA.

OVERVIEW OF THE RESEARCH TOPIC

The present special collection includes ten Original Articles, five narrative Reviews and one Systematic Review providing new insights on current and future therapeutic options for OA.

Edited and reviewed by:
Dieter Steinhilber,
Goethe University Frankfurt, Germany

****Correspondence:***
A. Fioravanti
fioravanti7@virgilio.it

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *09 March 2022*
Accepted: *28 March 2022*
Published: *03 May 2022*

Citation:
Fioravanti A, Tenti S and Cheleschi S (2022) Editorial: Year 2020: New Trends in Pharmacological Treatments for Osteoarthritis. Front. Pharmacol. 13:892934. doi: 10.3389/fphar.2022.892934

A large contribution has been provided on pre-clinical researches. In a study on human OA chondrocytes, Sanchez et al. investigated the effects of Zeel T (Ze14), a multicomponent medicinal product composed of plant and organ extracts, known for its symptomatic effects in observational clinical studies. The authors showed that Ze14 significantly inhibited cartilage degradation, reducing metalloproteinases expression, and promoted chondrogenesis (Sanchez et al.).

Along the same line, Baek et al. observed the protective effects of 3′-Sialyllactose, a compound derived of human milk, against the oxidative stress and inflammation processes induced by IL-1β, in SW1353 chondrocytic cells.

Selonsertib (Ser), an inhibitor of Apoptosis Signal-regulated kinase-1 (ASK1), has been studied both in *in vitro* and in *in vivo* models by Yan et al. The results of the study showed that Ser markedly prevented the IL-1β-induced inflammatory reaction, cartilage degradation and cell apoptosis in rat chondrocytes; besides, intra-articular (i.a.) injection of Ser, in rat OA model, significantly alleviated the progression of the disease (Yan et al.).

Furthermore, the pharmacological activity of seven commercially available mixtures of avocado/soybean unsaponifiables (ASUs) were studied by Lambert et al. on human OA chondrocytes cultured in alginate beads. The authors demonstrated the inhibitory effect of the mixture of PIASCLEDINE-ExpASU® on pro-inflammatory and pro-catabolic factors.

Ma et al. observed that vanillic acid, a monomer derived from chinese herbal medicines, was able to target NLRP3 inflammasome reducing synovitis in a rat model of knee OA.

The unique contribution as systematic review and meta-analysis has been provided by Sumsuzzman et al., analyzing the available experimental data on animal models on the use of melatonin for the treatment of OA. The authors placing particular emphasis on the effects of exogenous melatonin in preventing OA pathogenesis through the regulation of circadian rhythms and anabolic/anticatabolic balance.

Furthermore, the review by Zhang et al. gives new information about the role of icariin in knee OA. Icariin is a flavonoid compound from the traditional Chinese medicine and it is known for its clinical efficacy in the treatment of bone and joint diseases. This review article confirms the potential role of icariin in alleviating knee OA, through the inhibition of inflammation, cartilage breakdown and extracellular matrix degradation.

One narrative review focused on recent developments of agents for the treatment of OA, providing a general overview on potential DMOADs, as lorecivivint, MIV-711 and spirifermin, and new therapeutic option for pain relief. Indeed, the most recent clinical trials and preclinical studies investigated a variety of possible therapeutics targeting of different underlying mechanisms, as inflammation, cellular senescence, cartilage metabolism, subchondral bone remodeling and peripheral nociceptive pathways. However, long-term randomized clinical trials are needed to confirm the safety and the efficacy of these novel pharmacological agents for OA (Cai et al.).

Another narrative review discussed the current evidence on the efficacy and safety of the i.a. therapy for thumb-base OA (TBOA). The authors presented literature data about i.a. corticosteroids, which remained a mainstay of therapy, i.a. hyaluronic acid and emerging i.a. agents, as platelet-rich plasma (PRP) or mesenchymal-derived stem cell populations. Despite, the i.a. therapy represents an attractive strategy for the local treatment of TBOA, within the multidisciplinary approach for the management of hand OA, the current evidence remains equivocal, mainly due to the heterogeneity among the conducted studies (Tenti et al.).

A mini review dealt with the articular and extra-articular effects of glucosamine sulfate in the treatment of OA. In particular, it was discussed the efficacy of glucosamine sulfate on OA pain and its potential structure-modifying effect in patients with knee OA. Furthermore, the authors focused on the protective role of glucosamine sulfate on the cardiovascular mortality, probably due to the modulation of the O-GlcNAcylation pathway (Conrozier and Lohse).

An opinion article by Scanu et al. provided a brief overview on the immunological events associated to OA and on the current and future therapeutics for OA. In addition, the authors described the role of balneotherapy in OA prevention and treatment with a particular highlight on the immunomodulatory properties of mineral waters.

Finally, Negrini et al. presented the case of two patients (one 85-year-old patient with severe functional impairment and one active 59-year-old patient) with knee OA treated with PRP injections, coupled with a post-treatment home-based rehabilitation program, consisting in a series of exercise to be performed at home, during the 5 days following PRP for two consecutive weeks. This therapeutic approach resulted safe and well tolerated and led to a significant improvement of pain and function, especially in the older patient. The authors concluded stating that the obtained results motivated them to plan further studies based on the same program, with the implementation of telemedicine and biomechanical evaluation to enhance compliance, efficacy, and outcomes.

The search for the pathological processes of bone formation and remodeling, and its implication in the pathogenesis of osteoporosis was performed in three original articles. Among them, Zhang et al. explored the role of Juglanin, a natural compound derived from the *crude Polygonum aviculare*, in RAW 246.7 macrophage cell line and in ovariectomized mice. The authors showed that Juglanin suppressed osteoclastogenesis inhibiting the expression of receptor activator of nuclear factor-κB ligand (RANKL) and NF-κB signaling pathway (Zhang et al.).

In a similar manner, other authors (Sun et al., 2021) reported that total flavonoids of rhizoma drynariae promotes mineralization of bone graft and differentiation of osteoblasts in a dose-dependent manner in osteoblasts cultures and in Sprague-Dawley rats, partly related to the activation of Wnt/β-catenin signaling pathway.

The role of the insulicolide A, a natural nitrobenzoyl sesquiterpenoid derived from marine fungus, on RANKL stimulated osteoclastogenesis *in vitro* and on LPS induced osteolysis on mice model *in vivo* was investigated by Tan et al.

The obtained results showed the inhibition of osteoclastogenesis and indicates that insulicolide A may have potential for the treatment of osteoclast related diseases such as osteoporosis or bone metastasis.

SUMMARY

Through this Research Topic efforts have been made in order to better understand the underlying pathophysiological mechanisms of OA and to investigate the potential effectiveness of a variety of natural and pharmacological agents. Furthermore, this special provides an update about some OA pathogenetic processes, as "anabolic and catabolic imbalance" and discusses the current evidence in the treatment of the disease, with a particular focus on i.a. therapy and glucosamine. This Research Topic demonstrates the growing interest in pre-clinical and clinical research in the field of OA, despite unmet needs remain in therapeutic area.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Cheleschi, S., Giordano, N., Volpi, N., Tenti, S., Gallo, I., Di Meglio, M., et al. (2018). A Complex Relationship between Visfatin and Resistin and microRNA: An *In Vitro* Study on Human Chondrocyte Cultures. *Int. J. Mol. Sci.* 19, 3909. doi:10.3390/ijms19123909

GBD 2019 Diseases and Injuries Collaborators (2020). Global burden of 369 Diseases and Injuries in 204 Countries and Territories, 1990-2019: a Systematic Analysis for the Global Burden of Disease Study 2019. *Lancet* 396, 1204–1222. doi:10.1016/S0140-6736(20)30925-9

Goldring, S. R., and Goldring, M. B. (2016). Changes in the Osteochondral Unit during Osteoarthritis: Structure, Function and Cartilage-Bone Crosstalk. *Nat. Rev. Rheumatol.* 12, 632–644. doi:10.1038/nrrheum.2016.148

Hunter, D. J., and Bierma-Zeinstra, S. (2019). Osteoarthritis. *Lancet* 393, 1745–1759. doi:10.1016/S0140-6736(19)30417-9

Latourte, A., Kloppenburg, M., and Richette, P. (2020). Emerging Pharmaceutical Therapies for Osteoarthritis. *Nat. Rev. Rheumatol.* 16, 673–688. doi:10.1038/s41584-020-00518-6

Long, H., Liu, Q., Yin, H., Wang, K., Diao, N., Zhang, Y., et al. (2022). Prevalence Trends of Site-specific Osteoarthritis from 1990 to 2019: Findings from the Global Burden of Disease Study 2019. *Arthritis Rheumatol.* 1, 1. Online ahead of print. doi:10.1002/art.42089

Ren, Y., Hu, J., Tan, J., Tang, X., Li, Q., Yang, H., et al. (2020). Incidence and Risk Factors of Symptomatic Knee Osteoarthritis Among the Chinese Population: Analysis from a Nationwide Longitudinal Study. *BMC Public Health* 20, 1491. doi:10.1186/s12889-020-09611-7

Sun, W., Li, M., Xie, L., Mai, Z., Zhang, Y., Luo, L., et al. (2021). Corrigendum: Exploring the Mechanism of Total Flavonoids of Drynariae Rhizoma to Improve Large Bone Defects by Network Pharmacology and Experimental Assessment. *Front. Pharmacol.* 12, 739503. doi:10.3389/fphar.2021.739503

Zheng, L., Zhang, Z., Sheng, P., and Mobasheri, A. (2021). The Role of Metabolism in Chondrocyte Dysfunction and the Progression of Osteoarthritis. *Ageing Res. Rev.* 66, 101249. doi:10.1016/j.arr.2020.101249

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 27 January 2021
doi: 10.3389/fphar.2020.596230

Juglanin Inhibits Osteoclastogenesis in Ovariectomized Mice via the Suppression of NF-κB Signaling Pathways

Fangxue Zhang[1†], Xiaowei Huang[2†], Yuhan Qi[3†], Zhi Qian[1], Shuo Ni[1], Zeyuan Zhong[1], Xu Zhang[1], Dejian Li[1,4] and Baoqing Yu[1*]*

[1]Department of Orthopedics, Shanghai Pudong Hospital, Fudan University Pudong Medical Center, Shanghai, China, [2]Department of Orthopedics, The First Affiliated Hospital of Soochow University, Suzhou, China, [3]Department of Plastic Surgery, First Affiliated Hospital of Nanchang University, Nanchang, China, [4]Department of Oral & Cranio-Maxillofacial Surgery, Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine, National Clinical Research Center for Oral Diseases, Shanghai Key Laboratory of Stomatology & Shanghai Research Institute of Stomatology, Shanghai, China

OPEN ACCESS

Edited by:
Raquel Largo,
Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain

Reviewed by:
Soon Yew Tang,
University of Pennsylvania, United States
Luigi Brunetti,
University of Studies G. d'Annunzio Chieti and Pescara, Italy

***Correspondence:**
Dejian Li
18211330002@fudan.edu.cn
Baoqing Yu
doctorybq@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 18 August 2020
Accepted: 23 December 2020
Published: 27 January 2021

Citation:
Zhang F, Huang X, Qi Y, Qian Z, Ni S, Zhong Z, Zhang X, Li D and Yu B (2021) Juglanin Inhibits Osteoclastogenesis in Ovariectomized Mice via the Suppression of NF-κB Signaling Pathways. Front. Pharmacol. 11:596230. doi: 10.3389/fphar.2020.596230

Bone metabolism is a physiological process that involves both osteoblasts and osteoclasts. Pathological changes of osteoclasts are commonly seen in osteoporosis diseases. Juglanin is a natural compound, reported to have an inhibitory effect on inflammation, oxidative stress and cancer progression. The purpose of this study is to explore the role that Juglanin plays on the osteoclast functions and underlying signaling pathways. *In vitro* study demonstrated that Juglanin had negative influence on osteoclastic differentiation by suppressing the transcription activity of osteoclastogenesis-related genes and proteins. To determine the underlying mechanism, Western blot was employed to show that Juglanin could significantly have negative effect on the phosphorylation of P50, P65, I-κB , ultimately suppressing the expression and transcriptional activity of nuclear factor of activated T cells (NFATc1). *In vivo* Juglanin treatment attenuate bone reducing in mice with removed ovary through suppressing osteoclast functioning. Taken together, our study demonstrated that in the molecular mechanism, JUG inhibited the expression of receptor activator of nuclear factor-κ B ligand (RANKL) induced NF - κ B signaling pathway, thus may play a vital part in preventing postmenopausal osteoporosis.

Keywords: juglanin, osteoclastogenesis, NF-κB pathways, ovariectomized-mice, RANKL

INTRODUCTION

Osteoporosis is the disease with high incidence rate which is manifested by bone loss and bone microstructure reducing, resulting in impaired rigidity as well as increased risk of fracture (Ensrud and Crandall, 2017; Leutner et al., 2019). Postmenopausal osteoporosis (PMO) is a typical orthopedic disease caused by excessive activation of osteoclasts (Ensrud and Crandall, 2019). Stepan et al. (Curr Osteoporos Rep, 2019) reported that estrogen can play an anti-osteoporotic effect by promoting the secretion of osteoprotegerin (OPG) in osteoblast and inhibit the formation of osteoclasts (Allison and McNamara, 2019; Stepan et al., 2019). On the other side, it was also observed that the osteoclastogeneis process was over-activated after menopause, thus leading to net bone loss and increased risk of osteoporosis (Levin et al., 2018; Kanis et al., 2019).

Osteoclast belongs to the monocyte-macrophage family and is the key member in bone metabolism (Madel et al., 2019). Several cytokines are responsible for osteoclastogenesis (Lorenzo, 2017). RANK (NF-κB receptor activator) and its ligand RANKL initiate the signaling for osteoclast formation (Park et al., 2017; Choi et al., 2018; Funakubo et al., 2018; Ikebuchi et al., 2018), recruiting TNF receptor related factors 6 which further stimulate the phosphorylation of down-stream transcription factors (Sambandam et al., 2016; Wu et al., 2016; van Dam et al., 2019). It has been shown that selective inhibition of NF-κB and MAPK pathways can reduce osteoclast formation (Yao et al., 2017; Kim et al., 2018).

Juglanin (JUG) is a natural compound derived from the *crude Polygonum aviculare*. It has been reported the inhibitory effect on inflammation, oxidative stress and cancer progression. According to previous studies, juglanin prevents hepatitis through inflammation suppression by inactivating TLR4/NF-κB signaling pathway (Chen et al., 2017; Sun et al., 2017; Hou et al., 2018; Zhang and Xu, 2018; Chen et al., 2019). In addition, JUG has been explored in the occurrence of human breast cancer through apoptosis, by inducing reactive oxygen species (ROS) production in cells (Sun et al., 2017). However, the effect of JUG on osteoclastogenesis remains unknown. Therefore, in our research, we studied the role JUG play in the process of osteoclastogenesis, and the potential mechanism of JUG on RANKL-treated osteoclasts. In addition, a mouse model of bone loss was built by ovariectomy (OVX) to validate its effectiveness *in vivo*.

MATERIAL AND METHODS

Reagents and Antibodies

The JUG with a purity of more than 98% was obtained from Nancheng biochemistry (Shanghai, China) and dissolved in DMSO as for storing with concentration of 1 μmol/L and stored at −20°C. Further dilution was carried out in culture medium for cells and PBS medium for animals. Primary antibodies against NFATc1 (#8032, CST) and V-ATPase-d2 (#ABS1677, Sigma) were acquired from Zhongshantech (Wuhan, China). Primary antibodies for P50 (#13586, CST), P-P50 (#4806, CST), P65 (#4764S, CST), P-P65 (#3033, CST), and IκB (9242, CST), P- IκB (#2859, CST) were delivered by Cell Signaling biology (TA, United States). MMT kit was bought from Tengyi Technology (Nantong, China).

Cell Culture, Cell Viability Assay and Osteoclastogenesis Assay

As mentioned in previous studies (Sambandam et al., 2016; Wu et al., 2016; van Dam et al., 2019), in order to extract bone marrow mononuclear cells (BMM), C57BL/6 mice between 4 to 6 week-old were executed and the lower limbs were dissected. 1 ml syringe was used to wash the cells out of the bone cavity of femoral and tibia. The cells were then co-cultured by 30 ng/ml M-CSF (Macrophage Colony stimulating Factor) for 24 h. Removing the non-adherent cells, the attached cells were kept until the cells reached 80% of confluency. For cell viability determination, BMMs were plated on 96-well plates with a concentration of 10,000 cells/well. MTT assay was then performed to detect JUG cytotoxicity. After a 24-h incubation, different concentrations of JUG (0, 10, 20, 40, 80, 160, 320, 640 μmol/L) was then added and cultured with the cells for 72 h (Chen et al., 2018b; Maridas et al., 2018). In order to differentiate osteoclasts, the cells were stimulated with 30 ng/mL M-CSF, 50 ng/ml RANKL and different concentrations of JUG (0, 20, 40 or 80 μmol/L). Changing the medium regularly until osteoclasts formed and matured and fixing the cells with 4% paraformaldehyde for 20 min, then the staining process was conducted. The stained cells with no less than 3 nuclei were scored.

F-Actin Ring Formation Assay

RAW 246.7 cells were stimulated with JUG of different doses for 5 days as well as 50 ng/ml RANKL. Then a 0.25 percent Triton X-100 was used to penetrate the cell membrane. The cells were blocked in 3% BSA. After blocking, the f-actin ring was labeled with Rhodamine coupled phalloidin (Eugene, Oregon, USA), and the nucleus of osteoclasts was labeled with DAPI (Chen et al., 2018a).

Determination of Absorption Pit

Bone resorption is the most important function of osteoclasts. The test of osteoclast absorption function is the gold standard of osteoclast examination. In this study, the absorption pit assay was used to evaluate the bone resorption function. BMMs were plated into 6-well plates at a density of 8 × 10^4 cells/well and cultured with 30 ng/ml M-CSF for 3 days, then stimulated with 30 ng/ml M-CSF and 50 ng/ml RANKL for 5 days until osteoclast formation. Osteoclasts were then implanted into 96-well plates, each with bone slices. After co-culture with osteoclasts for 48 h, hematoxylin staining was performed to detect the absorption pit (Chen et al., 2018b).

Immunofluorescence Staining

Immunofluorescence was used to evaluate the nuclear translocation of p65. Briefly, BMMs were fixed with 4% PFA, then, washing with Triton X-100 to facilitate staining, followed by incubated with anti-p65 antibody, goat anti-mouse IgG antibody, the result was observed by microscope.

Real-Time PCR

Real-time quantitative polymerase chain reaction (qRT-PCR) is used to quantify the mRNA expression of osteoclastogenesis related genes. Using TRIzol reagent, the total RNA of RAW 264.7 was extracted in a 6-well plate, followed by reversely transcribing to cDNA. The sequences of the primers used are: cathepsin K(CtsK) (forward: 5-GGGAGAAAAACCTGAAGC-3'; reverse: 5′-ATTCTGGGGACTCAGAGC-3′); c-Fos (forward: 5′- GCGAGCAACTGAGAAGAC - 3′, reverse: 5′- TTGAAA CCCGAGAACATC-3′); TRAcP(forward: 5′-TGTGGCCATCTT TATGCT-3'; reverse:5′GTCATTTCTTTGGGGCTT-3′); MMP-9 (forward: AGTTTGGTGTCGCGGAGCAC; reverse: TACATG AGCGCTTCCGGCAC), GAPDH (forward: AACTTTGGCATT GTGGAAGG; reverse:ACACATTGGGGGTAGGAACA). The parameters of RT-PCR was set according to previously published papers (Tan et al., 2017).

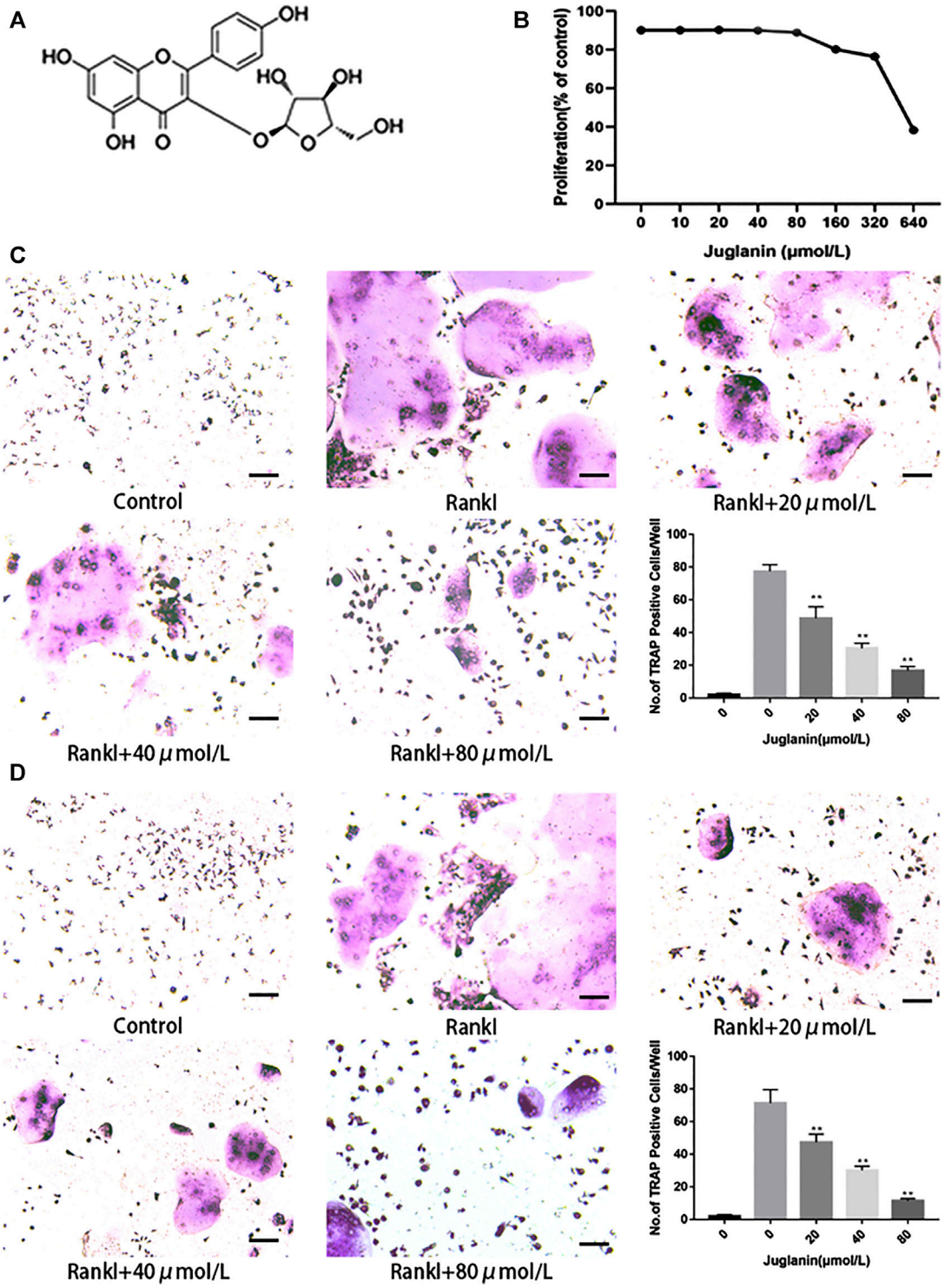


FIGURE 1 | JUG inhibits osteoclastogenesis *in vitro*. All the experiments were performed six times and the average was taken. **(A)** Chemical structure of JUG **(B)** MTT analysis of JUG cytotoxicity in BMSCs. **(C)** Formation of TRAP-positive cells from BMMs and quantification of osteoclast **(D)** Formation of TRAP-positive cells from RAW264.7 cells and quantification of osteoclast. Scale bar 100 μm. Data are presented as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the control group. n = 3

Western Blot Analysis

Cells were lyzed to obtain total protein content in freshly prepared frozen radioimmunoprecipitation assay buffer (RIPA). After quantification by the BCA method, the standardized protein samples were separated by sodium dodecyl sulfate sodium polyacrylamide gel (SDS PAGE) and transferred to

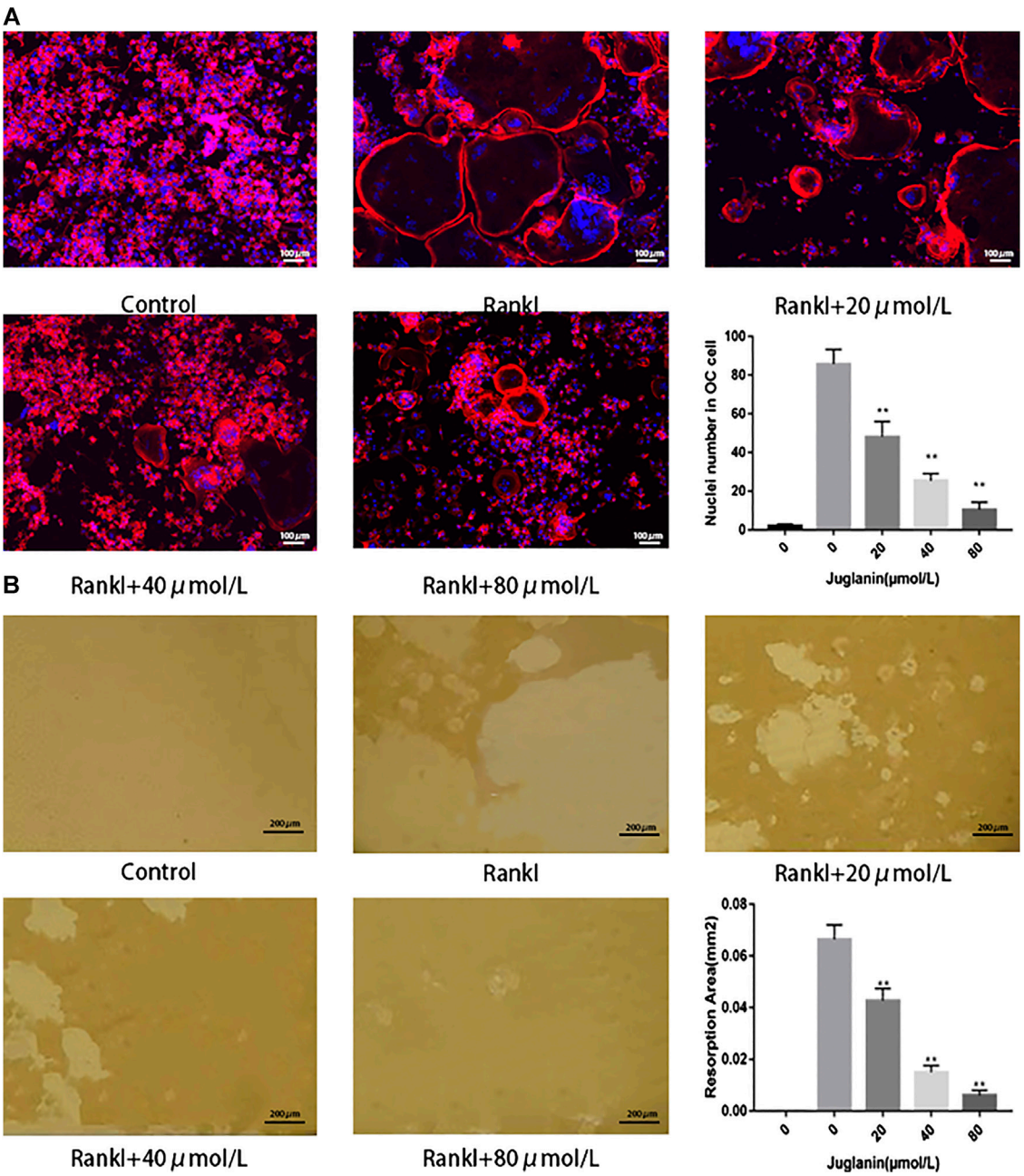


FIGURE 2 | JUG inhibits osteoclasts function. **(A)** F-actin staining of osteoclasts (RANKL-induced BMMs) and quantification of the actin ring **(B)** Pits formation assay of osteoclasts and quantification of resorption area. Scale bar 100 μm. Data are presented as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the control group. n = 3

nitrocellulose membrane. Then the membrane was blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h, and then incubated with diluted primary antibody overnight at 4 °C (Abcam, Cambridge, MA). After washing 3 times with Tris-buffered saline (TBST), it was incubated with IgG monoclonal antibody for 12 h at 4 °C. Using INTAS Science Imaging (Göttingen, Germany), the signal development film was incubated with the ECL matrix solution for 1 min for visualization.

Establishment of OVX Mice Model

Thirty 5-week-old female C57BL/6 mice were divided into sham group, OVX group and OVX + JUG (10 mg/kg) group with10 in each group. Mice in OVX group and OVX + JUG (10 mg/kg) group underwent bilateral ovariectomy and salpingectomy. After OVX surgery, the mice received no treatment and had recovery time of one week. After that, the OVX + JUG (10 mg/kg) group was given a JUG

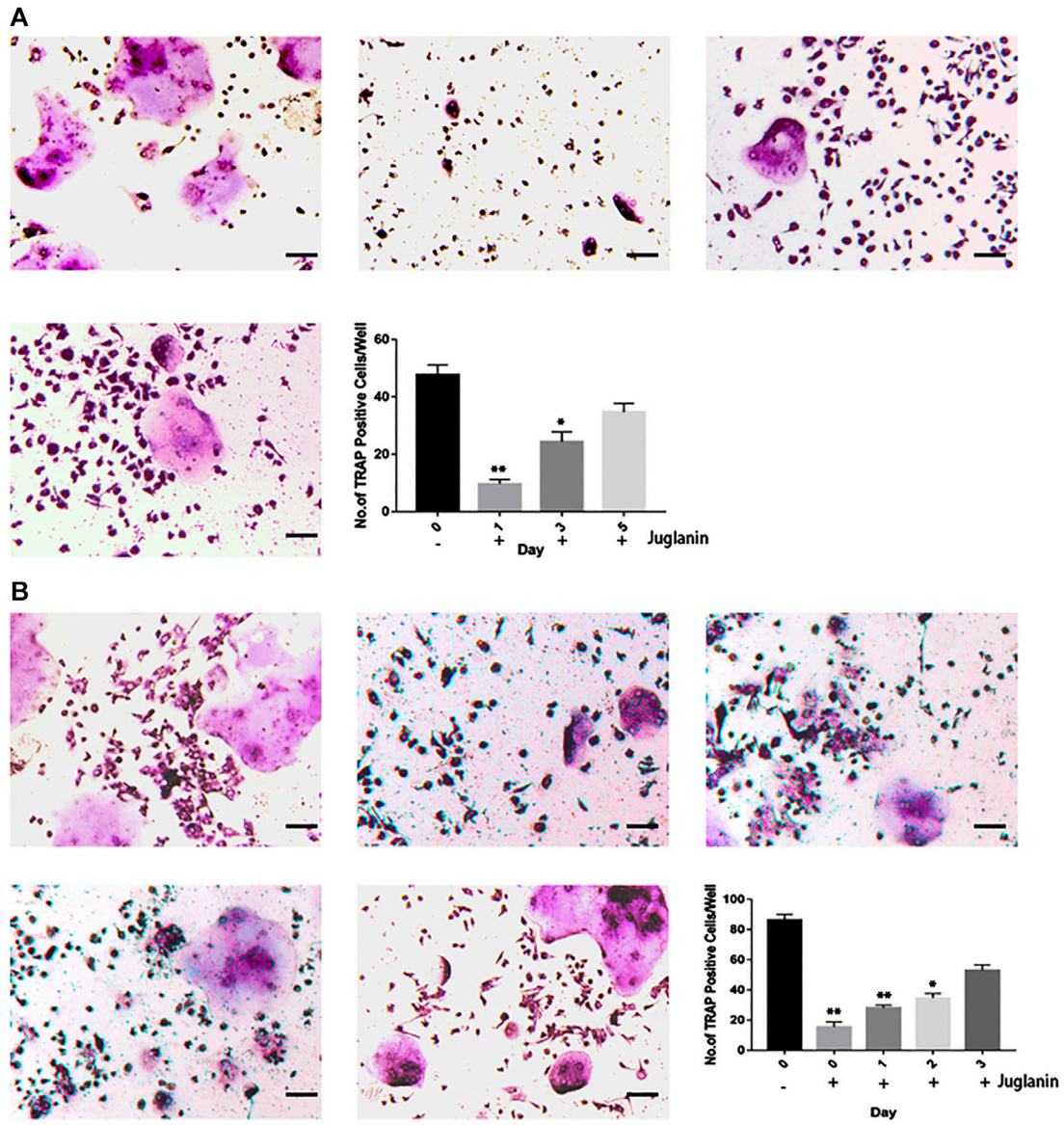


FIGURE 3 | JUG inhibits RANKL-induced osteoclast formation at the early stage. **(A)** Effect of JUG on RANKL-induced BMMs differentiation at different stage **(B)** Effect of JUG on RANKL-induced RAW264.7 cell differentiation at different stages. Scale bar 100 μm. Data are presented as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the control group. n = 3

intraperitoneal injection every two days. All animals were sacrificed 9 weeks after the operation. Femur specimens were taken for microCT scanning and histological staining (Zhou et al., 2016).

Luciferase Reporter Gene Assay

Luciferase Reporter gene assay was used to detect JUG's effect on NF—B or NFATc1. The experimental procedure has been described in previous study (Singh et al., 2012; Zeng et al., 2016). RAW 264.7 cells were stably transfected with either an NF-κB-responsive luciferase construct or an NFATc1-responsive luciferase reporter construct (Wang et al., 2003; van der Kraan et al., 2013). Cells were lyzed and luciferase test substrates (Promega, Madison, WI, USA) were mixed into the samples. BMG Polar Star Optima Luminescent Reader (BMG, Germany) was used to detect fluorescent luminescence. Luciferase activity represented NF—B and NFAT activity.

Histological Examination and Miro-CT Scanning

The femoral specimens were incubated with 4% paraformaldehyde for 4 days for tissue fixation and with 10% tetracycline-EDTA for

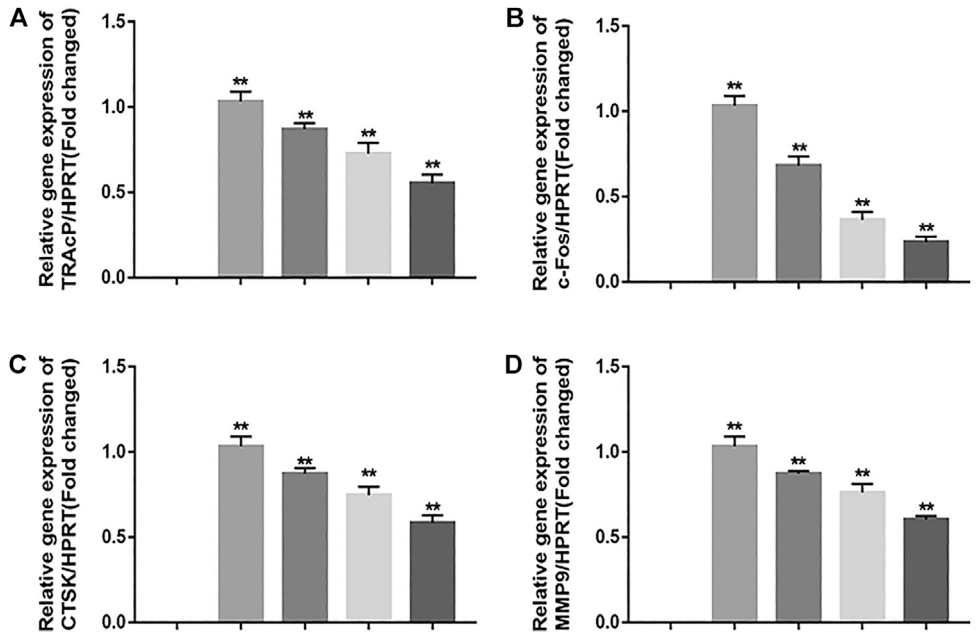


FIGURE 4 | JUG suppresses osteoclastogenic gene expression. **(A–D)** Real-time PCR analysis showing that down-regulates the expression of osteoclastogenic genes c-Fos, TRAcP, MMP9 and CTSK. The expression levels of these genes were normalized to the expression of HPRT. Data are presented as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the control group. n = 3

3 weeks for decalcification. The distal femur was cut into 4 mm sections for H&E and TRAP staining. Scanning HE stained sections with Aperio Scanscope, histomorphometric parameters were recorded and analyzed. TRAP staining was utilized to determine the number and morphology of osteoclasts in each section. Micro-CT (Siemens, Germany) was employed to scan 100 slices of each bone growth plate. The image data of bones and trabecular bone were analyzed by Mimics 15.0 software (Matralise, Belgium).

Statistical Analysis

The results presented are representative of at least three independent experiments and are expressed as mean ± standard error of the mean (SEM). Student's t test and couple ANOVA to a post-hoc test is used to determine the statistical significance between the intervention group and the control group. The one-way analysis of variance model is used to compare multiple groups. The significance level is set to 0.05.

RESULTS

Juglanin had Limited Effect on Differentiation and Mineralization of Osteoblasts *in vitro*

ALP assay and alizarin red staining assay showed that JUG (80 μmol/L) had no inhibitory or promotive effect on BMSCs differentiation (**Supplementary Figures S1A,B**). Additionally, the cytotoxicity of JUG (10, 20, 40, 80, 160, 320, 640 μmol/L) on BMSCs, as demonstrated in **Figure 1B**, was evaluated *in vitro* by an MTT assay. No significant difference was observed in terms of cell viability after treatment of 48 h with various concentrations of JUG aforementioned. Thus, the results indicated that JUG had no detrimental impact on two types of cells with concentration no more than 80 μmol/L.

JUG Negatively Affected RANKL-Induced Osteoclastogenesis in RAW264.7 and BMMs

Two types of cell modes, RAW264.7 and BMMs, were employed to test the impact of Juglanin on osteoclastogenesis *in vitro*. As observed in TRAP staining, the addition of RANKL can effectively induce osteoclast differentiation and formation, whereas the increase of JUG concentration negatively correlated to TRAP-positive multinucleated osteoclasts (>3 nuclei) both in RAW264.7 and BMMs, as demonstrated in **Figures 1C,D**.

JUG Inhibited F-Actin Ring Formation

It was found that rings and multiple intact nuclei emerged after stimulation by RANKL, while the size of F-actin ring and number of nuclei was reduced under JUG treatment (**Figure 2A**). The results aforementioned indicated that JUG had negative impact on RANKL-induced F-actin formation.

JUG Suppressed Bone Resorption by Osteoclasts

The capability to absorb bone is the most important function for osteoclasts to exert the physiological role in bone remodeling. As a result, the pit formation assay to determine the resorptive

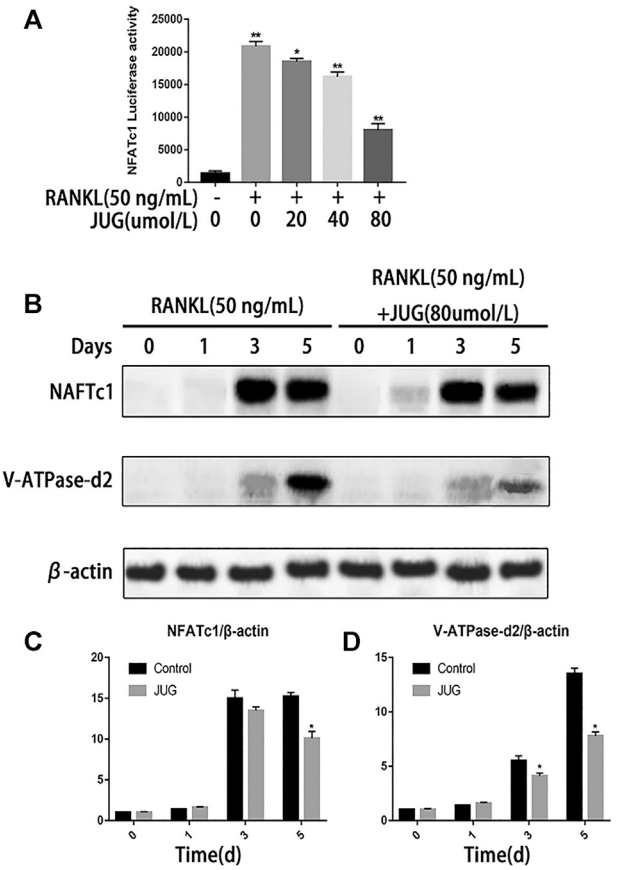


FIGURE 5 | JUG suppresses NFATc1 activity and down-regulates the expression of related protein. **(A)** luciferase reporter gene assay was performed to detect transcriptional activity of NFATc1. **(B)** Representative images of western blots demonstrating the effect of JUG on the expression of NFATc1 and related proteins V-ATPase d2, induced by RANKL on the indicated days. **(C,D)** Relative results were expressed by the ratio of the amount of NFATc1 and related proteins V-ATPase d2 determined by image J. Data are presented as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$. n = 3

function of osteoclasts was conducted as a golden standard to evaluate the function of osteoclasts. In the present study, osteoclasts were cultured onto bone slices and then intervened with JUG. The results showed that the standardized resorption area was negatively correlated with the concentration of JUG and the control group, with no osteoclasts induced, demonstrated none resorption area, as illustrated in **Figure 2B**.

JUG Inhibited Osteoclastogenesis in Early Stage

To identify the timing of osteoclastogenesis affected after the JUG treatment, BMMs were treated with JUG from day 0 to day 5 (**Figure 3A**) and RAW 264.7 cells from day 0 to day 3 (**Figure 3B**). The JUG treatment mainly inhibited osteoclast differentiation on the day1. The results indicated that JUG inhibited the RANKL-mediated osteoclast differentiation in an early stage.

Real-Time PCR Demonstrated Decreasing in Osteoclastogenic Gene Expression

In order to exam the impact of JUG on osteoclastogenic gene expression, the expression level of c-Fos and other osteoclast-related genes which are essential for osteoclast formation and resorptive function were determined using real-time PCR analysis. It was clearly illustrated that the addition of JUG suppressed the osteoclastogenic gene expression in a dose-dependently, as shown in **Figure 4**.

Western Blot Analysis Revealed Reducing Expression of NFATc1 and Related Protein

NFATc1 is regarded as the key transcription factors which plays an important part in signal transduction of osteoclastogenesis. Luciferase reporter gene assays were employed to assess NFATc1 transcriptional activity. As demonstrated in **Figure 5A**, JUG significantly inhibited the NFATc1 expression induced by RANKL. Also, western blot analysis revealed the similar tendency of V-ATPase-d2 expression at day 3 and day 5 which was a downstream protein in NFATc1 pathway as shown in **Figure 5B**. Thus, the expression of V-ATPase-d2 was down-regulated accordingly (**Figure 5C,D**).

JUG Down-Regulation of NF-κB Signaling Pathway

Phosphorylation of the NF-kB protein complex under JUG treatment of different concentrations were tested using western blot analysis. It was found that the phosphorylation of IκBα, P50 and P65 was significantly down-regulated 30 min or 60 min after the JUG treatment with the concentration of 80 μmol/L, as demonstrated in **Figures 6B–E**. With the stimulation of RANKL, p65 was phosphorylated and translocate to the nucleus. Without the RANKL, p65 was mainly located in the cytoplasm (**Figure 6F**). What's more, luciferase reporter gene assays revealed similar results as western blot analysis did (**Figure 6A**).

Therapeutic Effect of JUG on OVX Mice Model

The OVX mice model was built to simulate PMO conditions. Based on the model, the therapeutic effect of JUG to prevent bone loss was studied. H&E (**Figure 7C**) staining and mica-CT (**Figure 7A**) were employed to determine changes of trabecular bone structure. In comparison with solvent control group, oral administration of JUG reduced the bone loss, with augmentation in BV/TV and Tb.N and decreasing in Tb. Sp. (**Figure 7B,D**) However, it is interesting to note that the Tb.Th remained basically the same level among all three groups. Furtherly, TRAP staining (**Figure 7E**) of decalcified distal femoral sections confirmed the micro-architecture changes of trabecular bone assessed through micro-CT.

DISCUSSION

Bone metabolism is a physiological process that involves both osteoblasts and osteoclasts. Pathological changes of osteoclasts

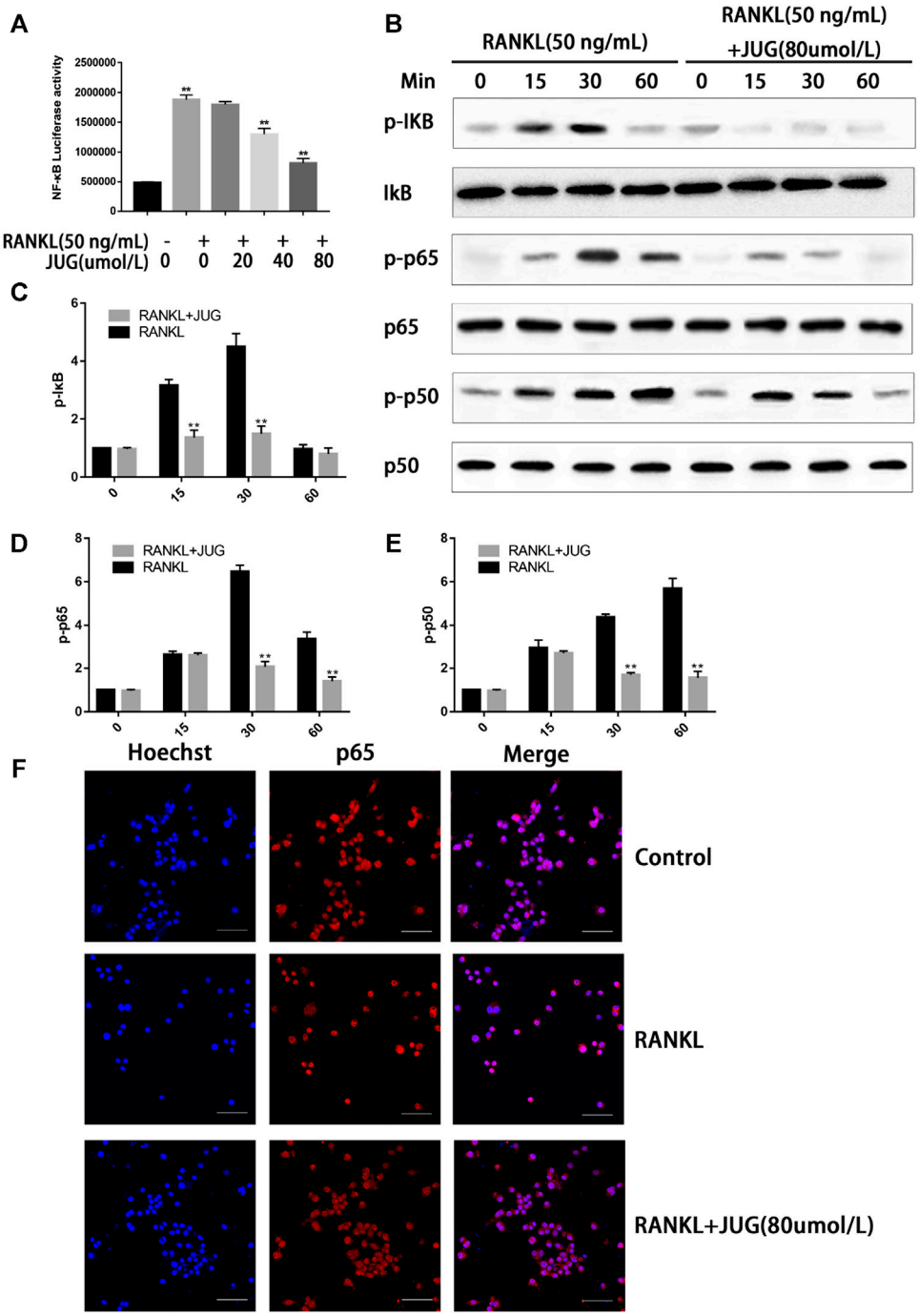


FIGURE 6 | JUG suppresses the NF-κB signaling pathway. The ratio of the fluorescence intensity was quantified by ImageJ software **(A)** luciferase reporter gene assay showing that JUG suppresses the RANKL-induced transcriptional activity of NF-κB. **(B)** Western blot of phosphorylation of IκB, p65, p50 **(D)** Representative image of a western blot demonstrating the effect of JUG on IκB degradation and phosphorylation of p65 and p50 at the indicated times **(F)** JUG inhibits RANKL-induced P65 nuclear translocation. Scale bar 50 μm. Data are presented as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$. n = 3

are commonly seen in osteoporosis diseases. Therefore, it may be a novel and efficient strategy to find natural osteoclasts inhibitors. Many natural substances and their derivatives have potential value to function as osteoclasts inhibitors. Juglanin (JUG) is a natural compound derived from the *crude Polygonum aviculare*. It has been reported the inhibitory effect on inflammation, oxidative stress. In addition, JUG has been explored in the occurrence of human breast cancer through apoptosis, by inducing ROS production in cells. Considering its role. However, there is

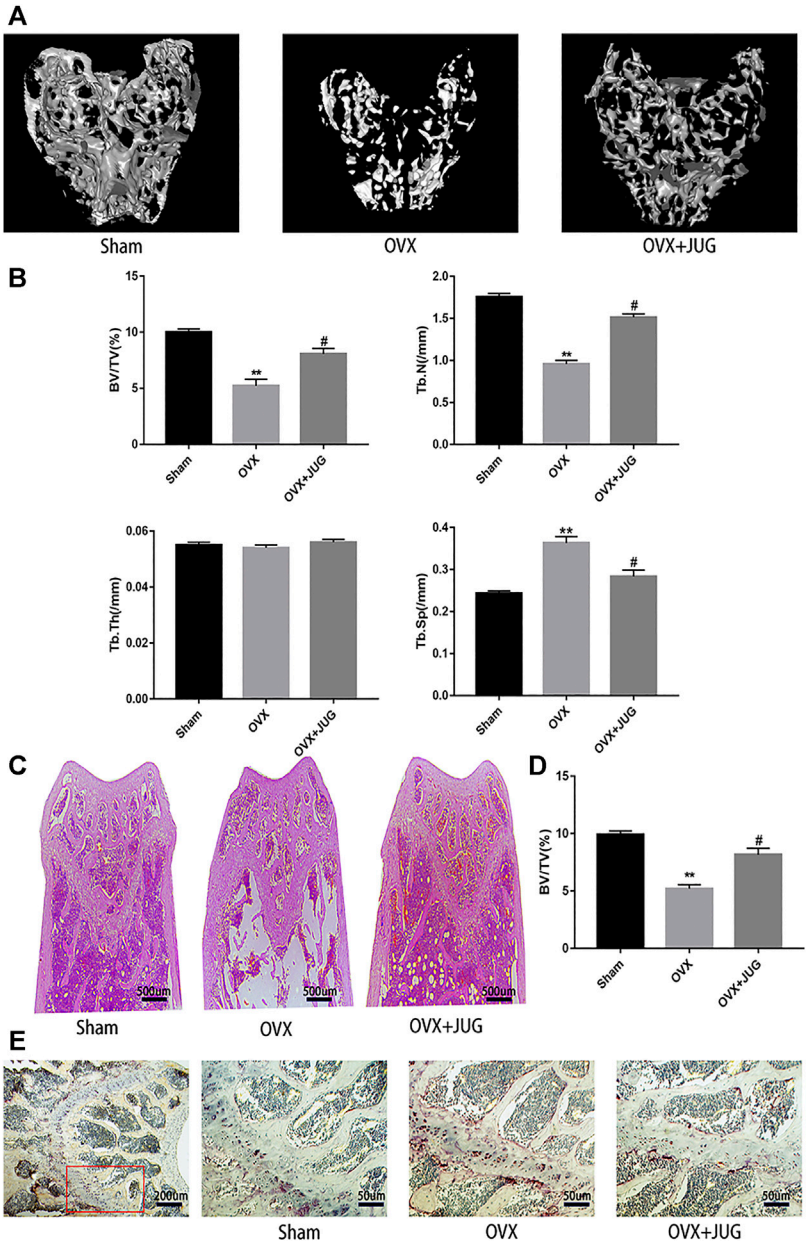


FIGURE 7 | JUG ameliorates ovariectomy-induced bone loss *in vivo*. **(A)** Micro CT analysis of the distal femur from sham, OVX, and OVX + JUG group **(B)** Bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) were analyzed with micro-CT Skyscan CTAn software. **(C–D)** HE staining of distal femoral and quantification of BV/TV **(E)** TRAP-stained histologic distal femur from sham, OVX and OVX + JUG group.

very little information about the mechanism of JUG on bone activity or related literature reports. As a result, we aim to investigate the potential inhibitory effect of JUG on onsteoclastogenesis in this study.

Pre-experimental results found that 80 μmol/L of JUG can significantly inhibit osteoclast differentiation. After An MTT assay was performed to detect JUG cytotoxicity, we selected 20, 40 and 80 μmol/L dose concentrations for this experiment. The results of this experiment showed that the inhibitory effect of JUG is positively correlated with its concentration. We also found that the effect is mediated through suppressing NF-κB transcription which is a transcription factor in the RANKL/RANK signaling pathway. (Chambers, 2000; Zhao et al., 2007). NF-κB signaling pathway is the most important signaling pathway for osteoclast differentiation and maturation induced by RANKL.

NFATc1 and c-Fos are important transcription factors for osteoclastogenesis. NFATc1 is essential for regulating the process of osteoclast differentiation by controlling osteoclast-related genes, and c-Fos is an important cytosine

promoting NFATc1 expression. The results in our study showed that JUG can negatively affect the expression of C-Fos/NFATc1 gene in RANKL-induced osteoclasts. In the later stage of osteoclastogenesis, NFATc1 acts as the terminal transcription factor to regulate the expression of osteoclast-related genes CTSK and TRAP. Previous study reported that over-expression of cathepsin K can lead to loss of bone mass. In this study, JUG can reduce the expression of CtsK gene in osteoclasts induced by RANKL. TRAP is an iron-binding protein, which is highly presented in the generation of osteoclasts and induced osteoclast differentiation. In our study, JUG can also inhibit the expression of TRAP gene in osteoclasts induced by RANKL. Taken together, the binding of RANK to RANKL activates IKK by recruiting the molecule TRAF6. After phosphorylation, IKK degrades IκBα and releases NF-κB (P65) which then enters the nucleus, activates a series of gene expressions that promote osteoclast differentiation, and ultimately promotes osteoclast differentiation. In our research, it was found that the JUG inhibited RANKL induced IκB, P50 and P65 phosphorylation, thus confirming that JUG inhibited osteoclastogenesis through down-regulating NF-κB signaling pathway.

Osteoblast is also a key member in bone metabolism. Therefore, we also studied the effect of JUG on osteoblasts. However, JUG had no inhibitory or promotive effect in terms of ALP staining and Alizarin red staining. In general, JUG has demonstrated no significant effect differentiation and mineralization of osteoblasts.

In vivo model showed that JUG prevented bone loss in ovariectomized mice. The number of trap-positive cells around trabecular of distal femur decreased significantly after JUG treatment. The number of osteoclasts in the JUG treatment group was significantly lower. Also, micro-CT showed that after JUG treatment, the percentage of trabecular bone and the number of trabecular bones in OVX mice increased significantly, and the trabecular bone space decreased significantly. Collectively, JUG has demonstrated great potential in preventing osteoporosis.

In conclusion, JUG has negative influence on RANKL induced osteoclast formation *in vitro* and prevent bone loss in mice model. In the molecular mechanism, the inhibition is mediated by NF - κ B signaling pathway.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Shanghai Pudong Hospital, Fudan University Pudong Medical Center, No.2800 Gongwei Road, Huinan Town, Pudong new area, Shanghai City, China.

AUTHOR CONTRIBUTIONS

FZ, XH and YQ contributed equally in this work. ZQ, and SN were responsible for performing the analyzing of the data. FX were responsible for drafting the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (81971753), The Outstanding Clinical Discipline Project of Shanghai Pudong (Grant No. PWYgy 2018–09), the key Disciplines Group Construction Project of Pudong Health Bureau of Shanghai (PWZxq 2017–11), Program for Outstanding Leader of Shanghai (046).

ACKNOWLEDGMENTS

This work is supported by grants from my fellows from Zhongshan Hospital of Furan University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.596230/full#supplementary-material.

REFERENCES

Allison, H., and Mcnamara, L. M. (2019). Inhibition of osteoclastogenesis by mechanically stimulated osteoblasts is attenuated during estrogen deficiency. *Am. J. Physiol. Cell Physiol.* 317, C969–C982. doi:10.1152/ajpcell.00168.2019

Chambers, T. J. (2000). Regulation of the differentiation and function of osteoclasts. *J. Pathol.* 192, 4–13. doi:10.2302/kjm.52.1

Chen, L., Xiong, Y. Q., Xu, J., Wang, J. P., Meng, Z. L., and Hong, Y. Q. (2017). Juglanin inhibits lung cancer by regulation of apoptosis, ROS and autophagy induction. *Oncotarget* 8, 93878–93898. doi:10.18632/oncotarget.21317

Chen, X., Li, X., Zhai, X., Zhi, X., Cao, L., Qin, L., et al. (2018a). Shikimic acid inhibits osteoclastogenesis *in Vivo* and *in Vitro* by blocking RANK/TRAF6 association and suppressing NF-κB and MAPK signaling pathways. *Cell. Physiol. Biochem.* 51, 2858–2871. doi:10.1159/000496039

Chen, X., Zhang, C., Wang, X., and Huo, S. (2019). Juglanin inhibits IL-1β-induced inflammation in human chondrocyte. *Artif Cells Nanomed Biotechnol* 47, 3614–3620. doi:10.1080/21691401.2019.1657877

Chen, X., Zhi, X., Yin, Z., Li, X., Qin, L., Qiu, Z., et al. (2018b). 18β-Glycyrrhetinic acid inhibits osteoclastogenesis in vivo and in vitro by blocking RANKL-mediated RANK-TRAF6 interactions and NF-κB and MAPK signaling pathways. *Front. Pharmacol.* 9, 647. doi:10.3389/fphar.2018.00647

Choi, J. H., Jang, A. R., Kim, D. I., Park, M. J., Lim, S. K., Kim, M. S., et al. (2018). PRMT1 mediates RANKL-induced osteoclastogenesis and contributes to bone loss in ovariectomized mice. *Exp. Mol. Med.* 50, 111. doi:10.1038/s12276-018-0134-x

Ensrud, K. E., and Crandall, C. J. (2017). Osteoporosis. *Ann. Intern. Med.* 167, Itc17–itc32. doi:10.7326/AITC201708010

Ensrud, K. E., and Crandall, C. J. (2019). Bisphosphonates for postmenopausal osteoporosis. *Jama* [Epub ahead of print]. doi:10.1001/jama.2019.15781

Funakubo, N., Xu, X., Kukita, T., Nakamura, S., Miyamoto, H., and Kukita, A. (2018). Pmepa1 induced by RANKL-p38 MAPK pathway has a novel role in osteoclastogenesis. *J. Cell. Physiol.* 233, 3105–3118. doi:10.1002/jcp.26147

Hou, G. R., Zeng, K., Lan, H. M., and Wang, Q. (2018). Juglanin ameliorates UVB-induced skin carcinogenesis via anti-inflammatory and proapoptotic effects *in vivo* and *in vitro*. *Int. J. Mol. Med.* 42, 41–52. doi:10.3892/ijmm.2018.3601

Ikebuchi, Y., Aoki, S., Honma, M., Hayashi, M., Sugamori, Y., Khan, M., et al. (2018). Coupling of bone resorption and formation by RANKL reverse signalling. *Nature* 561, 195–200. doi:10.1038/s41586-018-0482-7

Kanis, J. A., Cooper, C., Rizzoli, R., and Reginster, J. Y. (2019). Executive summary of the European guidance for the diagnosis and management of osteoporosis in postmenopausal women. *Calcif. Tissue Int.* 104, 235–238. doi:10.1007/s00223-018-00512-x

Kim, B., Lee, K. Y., and Park, B. (2018). Icariin abrogates osteoclast formation through the regulation of the RANKL-mediated TRAF6/NF-κB/ERK signaling pathway in Raw 264.7 cells. *Phytomedicine* 51, 181–190. doi:10.1016/j.phymed.2018.06.020

Leutner, M., Matzhold, C., Bellach, L., Deischinger, C., Harreiter, J., Thurner, S., et al. (2019). Diagnosis of osteoporosis in statin-treated patients is dose-dependent. *Ann. Rheum. Dis.* 78, 1706–1711. doi:10.1136/annrheumdis-2019-215714

Levin, V. A., Jiang, X., and Kagan, R. (2018). Estrogen therapy for osteoporosis in the modern era. *Osteoporos. Int.* 29, 1049–1055. doi:10.1007/s00198-018-4414-z

Lorenzo, J. (2017). The many ways of osteoclast activation. *J. Clin. Invest.* 127, 2530–2532. doi:10.1172/JCI94606

Madel, M. B., Ibáñez, L., Wakkach, A., De Vries, T. J., Teti, A., Apparailly, F., et al. (2019). Immune function and diversity of osteoclasts in normal and pathological conditions. *Front. Immunol.* 10, 1408. doi:10.3389/fimmu.2019.01408

Maridas, D. E., Rendina-Ruedy, E., Le, P. T., and Rosen, C. J. (2018). Isolation, culture, and differentiation of bone marrow stromal cells and osteoclast progenitors from mice. *JoVE* 2018, 56750. 10.3791/56750

Park, J. H., Lee, N. K., and Lee, S. Y. (2017). Current understanding of RANK signaling in osteoclast differentiation and maturation. *Mol Cells* 40, 706–713. doi:10.14348/molcells.2017.0225

Sambandam, Y., Baird, K. L., Stroebel, M., Kowal, E., Balasubramanian, S., and Reddy, S. V. (2016). Microgravity induction of TRAIL expression in preosteoclast cells enhances osteoclast differentiation. *Sci. Rep.* 6, 25143. doi:10.1038/srep25143

Singh, P. P., Van Der Kraan, A. G., Xu, J., Gillespie, M. T., and Quinn, J. M. (2012). Membrane-bound receptor activator of NFκB ligand (RANKL) activity displayed by osteoblasts is differentially regulated by osteolytic factors. *Biochem. Biophys. Res. Commun.* 422, 48–53. doi:10.1016/j.bbrc.2012.04.103

Stepan, J. J., Hruskova, H., and Kverka, M. (2019). Update on menopausal hormone therapy for fracture prevention. *Curr. Osteoporos. Rep.* 17, 465–473. doi:10.1007/s11914-019-00549-3

Sun, Z. L., Dong, J. L., and Wu, J. (2017). Juglanin induces apoptosis and autophagy in human breast cancer progression via ROS/JNK promotion. *Biomed. Pharmacother.* 85, 303–312. doi:10.1016/j.biopha.2016.11.030

Tan, Z., Cheng, J., Liu, Q., Zhou, L., Kenny, J., Wang, T., et al. (2017). Neohesperidin suppresses osteoclast differentiation, bone resorption and ovariectomised-induced osteoporosis in mice. *Mol. Cell. Endocrinol.* 439, 369–378. doi:10.1016/j.mce.2016.09.026

Van Dam, P. A., Verhoeven, Y., Trinh, X. B., Wouters, A., Lardon, F., Prenen, H., et al. (2019). RANK/RANKL signaling inhibition may improve the effectiveness of checkpoint blockade in cancer treatment. *Crit. Rev. Oncol. Hematol.* 133, 85–91. doi:10.1016/j.critrevonc.2018.10.011

Van Der Kraan, A. G., Chai, R. C., Singh, P. P., Lang, B. J., Xu, J., Gillespie, M. T., et al. (2013). HSP90 inhibitors enhance differentiation and MITF (microphthalmia transcription factor) activity in osteoclast progenitors. *Biochem. J.* 451, 235–244. doi:10.1042/BJ20121626

Wang, C., Steer, J. H., Joyce, D. A., Yip, K. H., Zheng, M. H., and Xu, J. (2003). 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibits osteoclastogenesis by suppressing RANKL-induced NF-kappaB activation. *J. Bone Miner. Res.* 18, 2159–2168. doi:10.1359/jbmr.2003.18.12.2159

Wu, Y. X., Wu, T. Y., Xu, B. B., Xu, X. Y., Chen, H. G., Li, X. Y., et al. (2016). Protocatechuic acid inhibits osteoclast differentiation and stimulates apoptosis in mature osteoclasts. *Biomed. Pharmacother.* 82, 399–405. doi:10.1016/j.biopha.2016.05.008

Yao, Z., Lei, W., Duan, R., Li, Y., Luo, L., and Boyce, B. F. (2017). RANKL cytokine enhances TNF-induced osteoclastogenesis independently of TNF receptor associated factor (TRAF) 6 by degrading TRAF3 in osteoclast precursors. *J. Biol. Chem.* 292, 10169–10179. doi:10.1074/jbc.M116.771816

Zeng, X. Z., He, L. G., Wang, S., Wang, K., Zhang, Y. Y., Tao, L., et al. (2016). Aconite inhibits RANKL-induced osteoclast differentiation in RAW264.7 cells by suppressing NF-κB and NFATc1 activation and DC-STAMP expression. *Acta Pharmacol. Sin.* 37, 255–263. doi:10.1038/aps.2015.85

Zhang, F. X., and Xu, R. S. (2018). Juglanin ameliorates LPS-induced neuroinflammation in animal models of Parkinson's disease and cell culture via inactivating TLR4/NF-κB pathway. *Biomed. Pharmacother.* 97, 1011–1019. doi:10.1016/j.biopha.2017.08.132

Zhao, Q., Shao, J., Chen, W., and Li, Y. P. (2007). Osteoclast differentiation and gene regulation. *Front. Biosci.* 12, 2519–2529. doi:10.2741/2252

Zhou, L., Liu, Q., Yang, M., Wang, T., Yao, J., Cheng, J., et al. (2016). Dihydroartemisinin, an anti-malaria drug, suppresses estrogen deficiency-induced osteoporosis, osteoclast formation, and RANKL-induced signaling pathways. *J. Bone Miner. Res.* 31, 964–974. doi:10.1002/jbmr.2771

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 15 February 2021
doi: 10.3389/fphar.2020.599022

Vanillic Acid Reduces Pain-Related Behavior in Knee Osteoarthritis Rats Through the Inhibition of NLRP3 Inflammasome-Related Synovitis

Zhenyuan Ma[1,2,3†], Zhengquan Huang[1,2†], Li Zhang[1,2,3], Xiaochen Li[1,2,3], Bo Xu[1,2,3], Yancheng Xiao[1,2,3], Xiaoqing Shi[1,2], Haosheng Zhang[1,2,3], Taiyang Liao[1,2,3] and Peimin Wang[1,2]*

[1]Department of Orthopedics, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China, [2]Jiangsu Province Hospital of Chinese Medicine, Nanjing, China, [3]Key Laboratory for Metabolic Diseases in Chinese Medicine, First College of Clinical Medicine, Nanjing University of Chinese Medicine, Nanjing, China

Objectives: Synovitis plays an important role in knee osteoarthritis (KOA) pain. The activation of the NOD-like receptor protein 3 (NLRP3) inflammasome in fibroblast-like synoviocytes (FLSs) promotes KOA development. In this study, we aimed to investigate whether vanillic acid (VA), a monomer derived from Chinese herbal medicines, could target NLRP3 inflammasome-related synovitis to reduce pain.

Methods: Rats in the KOA and KOA + VA groups were injected with monosodium iodoacetate (MIA) in the knee to induce KOA. From day 14, the KOA + VA group was given VA at 30 mg/kg every day via gastric intubation. FLSs were collected from the synovial tissues. We examined both the protein and gene expression of caspase-1, apoptosis-associated speck-like protein with a caspase recruitment domain (ASC), NLRP3, components of the NLRP3 inflammasome, and interleukin-1β (IL-1β) and IL-18 *in vivo* and *in vitro*.

Results: The upregulation of caspase-1, ASC, and NLRP3 in the KOA model were reduced by VA. VA also lowered the level of IL-1β and IL-18 in the KOA model. In addition, VA relieved pain-related behavior of KOA model rats and downregulated the pain mediators CGRP, NGF, and TrkA in FLSs. Interestingly, we also observed reduced synovial fibrosis in the animal experiments.

Conclusion: Our research showed that VA reduces synovitis and pain-related behaviors in a rat model of KOA, which provides the basis for further investigations into the potential therapeutic impact of VA in KOA.

Keywords: vanillic acid, knee osteoarthritis, synovial inflammation, NLRP3 inflammasome, pain 3

OPEN ACCESS

Edited by:
Raquel Largo,
Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain

Reviewed by:
Claudio Ferrante,
University of Studies G. d'Annunzio Chieti and Pescara, Italy
Roman A. Zinovkin,
Lomonosov Moscow State University, Russia

****Correspondence:***
Peimin Wang
drwpm@163.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *28 August 2020*
Accepted: *23 December 2020*
Published: *15 February 2021*

Citation:
Ma Z, Huang Z, Zhang L, Li X, Xu B, Xiao Y, Shi X, Zhang H, Liao T and Wang P (2021) Vanillic Acid Reduces Pain-Related Behavior in Knee Osteoarthritis Rats Through the Inhibition of NLRP3 Inflammasome-Related Synovitis. Front. Pharmacol. 11:599022. doi: 10.3389/fphar.2020.599022

INTRODUCTION

Knee osteoarthritis (KOA) is one of the most common chronic degenerative bone diseases and joint pain is the main clinical symptom that needs to be solved urgently (Litwic et al., 2013). The occurrence and development of KOA are associated with all the tissues that make up the knee joint, such as the synovium, cartilage, subchondral bone, and subcondylar fat pads (Muratovic et al., 2019). KOA also leads to the alteration of the whole joint structure (Akhbari et al., 2020). Symptoms in KOA are also somewhat related to structure. As an immune-related disease, the inflammation of the synovium in KOA plays a vital role and leads directly to the related pain (Atukorala et al., 2013). Recent studies have revealed that the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome is the beginning of inflammatory cascade amplification and is strongly correlated to hepatitis, pneumonia, nephritis, and other types of chronic aseptic inflammation (Ferrucci and Fabbri, 2018). NLRP3 is a molecular platform activated by caspase-1, which interacts with apoptosis-associated speck-like protein with a caspase recruitment domain (ASC) and procaspase-1, to assemble the NLRP3 inflammasome. Subsequently, the activated NLRP3 inflammasome cleaves procaspase-1, which leads to the maturation and secretion of interleukin- (IL-) 1β and IL-18 (Martinon et al., 2002). Our previous study showed that activation in KOA fibroblast-like synoviocytes (FLSs) promoted the development of synovitis (Zhao et al., 2018; Zhang et al., 2019).

Among many pain-related factors, the nerve growth factor (NGF) is a key regulator of KOA pain. High expression of NGF in the synovial fluid and cartilage of KOA patients has been widely reported. NGF antibodies have shown potential in the treatment of KOA pain. A similar situation occurs in tropomyosin receptor kinase A (TrkA), a high-affinity NGF receptor (Pecchi et al., 2014). Meanwhile, pain is associated with calcitonin gene-related peptide (CGRP), which is expressed in the sensory neurons that dominate the synovium. Studies have shown that CGRP levels from the KOA synovium are significantly increased and are highly correlated with KOA peripheral sensitization (Arnalich et al., 1994). In summary, NGF, TrkA, and CGRP are associated with pain, and changes in their expression can be used as biomarkers in KOA pain studies.

Chinese herbal medicine used in the clinical treatment of KOA has a long history. "Sanse powder," which is created from eleven substances found in Chinese materia medica (CMMs) (Wu et al., 2020), *Forsythia suspensa*, *Glycyrrhiza uralensis*, *Salvia miltiorrhiza*, *Gentiana macrophylla*, *Chaenomeles sinensis*, *Strychnos nux-vomica*, *Ligusticum striatum* Hort, *Curcuma longa*, *Paeonia lactiflora*, *Notopterygium* root, and *Saposhnikovia divaricata*, is a classic prescription widely used, not only to relieve synovitis but also to effectively relieve KOA pain. We performed a high-performance liquid chromatography analysis on "Sanse powder" to determine the effective components, of which vanillic acid (VA) is one (Wu et al., 2020). VA is a well-known flavonoid that is rich in nuts, fruits, and herbs. In recent years, VA has been mentioned in some studies as having anti-inflammatory and analgesic effects (Wu et al., 2020). However, the mechanisms by which VA affects the inflammation of KOA and its effects on pain are still poorly understood. Whether VA can affect the NLRP3 inflammasome has not been described in detail. Therefore, in this study, we observed the intervention effects of VA on NLRP3 inflammasome activation, synovitis, and KOA pain-related behaviors/mediator *in vivo* or *in vitro*.

MATERIAL AND METHODS

Reagents

Vanillic acid was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The primers were supplied by Sangon Biotech (Shanghai, China). The enzyme-linked immunosorbent assay (ELISA) kits for IL-1β and IL-18 were supplied by Invitrogen (Life Technologies Corp. California, United States). Fetal bovine serum (FBS), bovine serum albumin (BSA), Dulbecco's Modified Eagle's Medium (DMEM), TRIzol, and 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were purchased from Gibco (Life Technologies Corp., California, United States). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Antibodies against NLRP3, ASC, caspase-1, CGRP, NGF, TrkA, and type I collagen were purchased from Abcam (Cambridge, United Kingdom). Monoidoacetate acid, type I collagenase, and dimethylsulfoxide (DMSO) were all obtained from Sigma (St Louis, USA). All other chemicals were of reagent grade. Goat anti-rabbit IgG H&L (HRP) and Picro Sirius Red Stain kit were also supplied by Abcam (Cambridge, United Kingdom).

Rat KOA Model and Experimental Design

Twenty-four 3-month-old male Sprague-Dawley rats, weighing 280–320 g (provided by Beijing Vital River Laboratory Animal Technology Co., Ltd.), were fed in specific pathogen-free animal facilities under standard conditions (temperature 21 ± 1 °C, 50–80% relative humidity) with a 12:12 h light-dark cycle. Animal management was done in accordance with the Animal Care and Use Protocol approved by the Animal Care and Use Committee of our institution (201810A001). All animals were experimented on according to the National Institute of Health Animal Care and Use Guidelines. Rats were randomly divided into three groups: control (n = 8), KOA (n = 8), and KOA + VA (n = 8). The KOA and KOA + VA rats were injected with monosodium iodoacetate (MIA) in the knee to induced KOA (Rannou et al., 2016). Our previous data showed that the knee joint diameter was significantly larger than the control at the 14th day (Li et al., 2020). Therefore, from day 14, the KOA + VA group was given VA at 30 mg/kg (dissolved in 0.9% saline) every day via gastric intubation. On day 56, all rats were sacrificed by anesthesia and blood was collected. Knee joint tissues were collected for further experiments. We chose the VA dose based on a previous study published by another team (Huang et al., 2019). Animal number was estimated using PASS. When $\alpha = 0.05$, the power = 0.9, the minimum detected difference is 1, and the standard deviation is 0.5, so the number should be ≥6 when the power reaches 0.9. Therefore, eight rats in each group were used. The

animal experiments complied with the relevant regulations for animal experiments of the Ethics Committee at our institution.

Paw Withdrawal Experiments on a Cold Plate

Before scarification, the rats were placed on a cold glass surface to determine their paw withdrawal time. The rats were briefly placed on a temperature-adjustable cold plate (0 ± 1 °C, 35,150,001, Ugo Basil SLR, Italy) and covered with an organic cylinder. We recorded the time between the start and the limb moving off the glass plate.

Sirius Red Staining and Immunohistochemistry

The synovial tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned; then, conventional Sirius red staining was used to observe tissue changes under a light microscope. Synovial tissues were paraffin-sectioned via immunohistochemical staining. The content of collagen in each group was also detected by immunohistochemical staining.

Hematoxylin and Eosin Staining

Synovial tissues were fixed in 4% paraformaldehyde, then embedded in paraffin, and cut into thin slices for routine hematoxylin and eosin (HE) staining.

Cell Culture

Primary FLSs were isolated from rat KOA. Briefly, synovial tissues were washed 2–3 times with cold phosphate-buffered saline (PBS), minced into pieces of 2–3 mm^2, and then digested in 0.1% collagenase type II (Sigma, St. Louis, MO, USA) for 30 min. The solution was filtered through a cell strainer. After dissociation, fibroblasts were collected via centrifugation at 1,500 rpm for 4 min and cultured in DMEM supplemented with 10% fetal calf serum (FCS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; Invitrogen, CA, USA). Cells were identified as described in our previous studies (Li et al., 2020). Passages three to six of the synovial fibroblasts were used for the experiments. In the cell experiment, we used lipopolysaccharide (LPS) (5 μg/ml) to interfere with the synovial cells for 24 h to mimic a KOA environment and then added VA (5 μg/ml) to the VA + LPS group to intervene with the cells.

Cell Cytotoxicity Assay

A CCK-8 kit was used to detect the cytotoxicity of VA to FLSs. FLSs were cultured in 96-well plates. When the cell density reached 85–90%, they were treated with different VA concentrations (0, 5, 10, and 20 μg/ml) for 24 or 48 h. Then, we added 10 μL of the CCK-8 solution to each well and placed the cells in the incubator for 3 h. The optical density of the wells was detected using a 450 nm microplate spectrophotometer. All experiments were repeated three times.

Western Blotting Assay

Synovial tissues and FLSs were mixed with radioimmunoprecipitation assay (RIPA) lysate and grinded for 10–15 min. The samples were agitated on ice for 30 min and the supernatant was collected. The protein levels were quantified with a bicinchoninic acid (BCA) protein assay kit (Roche, Basel, Switzerland). Then, the protein samples were electrophoresed in sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) to separate the protein bands. Proteins were transferred from gel onto a polyvinylidene fluoride (PVDF) membrane and blocked with 5% nonfat milk for 2 h. The membrane was incubated with the primary antibody (1: 1000) at 4 °C overnight and then with the second antibody for 2 h. Bands were visualized via exposure to the electrochemiluminescence (ECL) method, and the overall gray value of protein bands (average gray value area) was quantified. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal marker. The relative protein expression was taken as the target protein gray value/ internal reference gray value.

Real-Time Quantitative Polymerase Chain Reaction

RNA was isolated from synovial tissue and FLSs with Trizol (Invitrogen, CA, United States). Reverse transcription was performed using a first-strand cDNA synthesis kit (Takara, Otsu, Japan) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed using Premix Ex Taq SYBR-Green PCR (Takara) according to the manufacturer's instructions on an ABI PRISM 7300 device (Applied Biosystems, Foster City, CA, USA).

The primer was designed and synthesized by the Shanghai Biotechnology Service Company. Primers Sequences were as follows. Caspase-1: forward, 5′-ATGGCCGACAAGGTCCTGAGG-3′and reverse, 5′-GTGACATGATCGCACAGGTCTCG-3′; NLRP3: forward, 5′-GAGCTGGACCTCAGTGACAATGC-3′and reverse, 5′-ACCAATGCGAGATCCTGACAACAC-3′; IL-18: forward, 5′-TCT GTAGCTCCATGCTTTCCG-3′ and reverse, 5′-GATCCTGGAGGT TGCAGAAGA-3′; and IL-1β: forward, 5′-ACAGCAGCATCTCGA CAAGAGC-3′ and reverse, 5′-CCACGGGCAAGACATAGGTAGC-3′; CGRP: forward, 5′-ATCTGGTCCTTCCTCACACTGTCC-3′ and reverse, 5′-TCATCCGTCTTCAGCTTGGCATTC-3′; TrkA: forward, 5′-AGGTTGAAGCCATTCTCCTG-3′ and reverse, 5′-TCTCGG TGGTGAACTTACGG-3′; NGF: forward, 5′-CCAGCCTCCACC CACCTCTTC-3′ and reverse, 5′-GCTTGCTCCTGTGAGTCCTGT TG-3′; GAPDH: forward, 5′-TTCACCACCATGGAGAAGGC-3′ and reverse, 5′-CTCGTGGTTCACACCCATCA-3′; ASC: forward, 5′-AGAGTCTGGAGCTGTGGCTACTG-3′ and reverse, 5′-ATGAGT GCTTGCCTGTGTTGGTC-3′. The PCR reactions (per well: 0.4 μL of forward and reverse primers, 10 μL 2 × ChamQ SYBR qPCR Master Mix (Low ROX Premixed), 1 μL CDNA, and 8.2 μL ddH_2O; three replicate wells) were performed using an ABI 7500 qRT-PCR system (Applied Biosystems, United States). The following reaction conditions were employed: the first stage (predenaturation), 95 °C for 30 s; the second stage (denaturation), 95 °C for 10 s and 60 °C for 30 s; the third stage (melting curve), 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s.

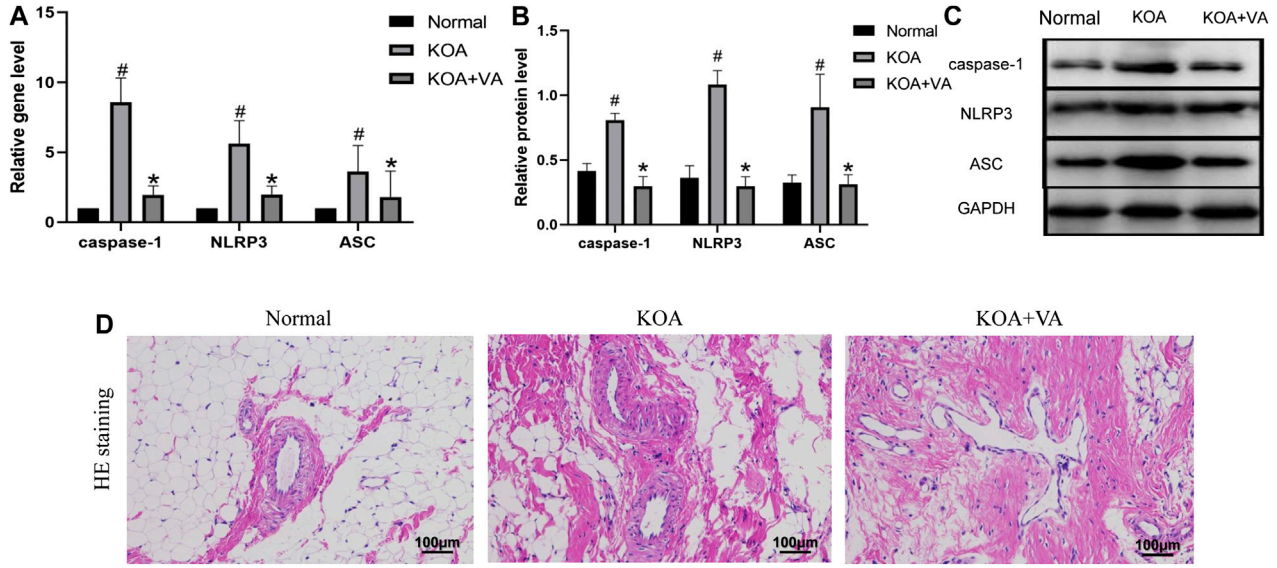


FIGURE 1 | Vanillic acid (VA) inhibits NLRP3 inflammasome activation. **(A)** Relative gene expression of caspase-1, NLRP3, and ASC in synovial tissue of rats in each group. Compared with the normal group, $p^{\#} < 0.05$; compared with KOA + VA group, $p^{*} < 0.05$. **(B and C)** Western blot analysis of the effects of VA on caspase-1, NLRP3, and ASC in rat synovial tissue. Compared with the normal group, $p^{\#} < 0.05$; compared with KOA + VA group, $p^{*} < 0.05$. **(D)** Synovial tissues of each group stained with HE, 400 ×, scale bar = 100 μm.

The relative expression of mRNA was adjusted using GAPDH as the internal reference and calculated using the method of $2^{-\Delta\Delta Ct}$.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1β and IL-18 levels in the culture media were determined using a commercially available rat IL-1β and IL-18 ELISA kit according to the manufacturer's instructions. The rat peripheral serum and cell culture supernatants were collected and centrifuged at 10,000 rpm for 20 min at 4 °C.

Statistical Analysis

The statistical analysis was performed using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Group comparisons were assessed with one-way ANOVA or two-way ANOVA with Bonferroni's *post hoc* test for comparison of multiple columns. A value of $p < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

VA Inhibits NLRP3 Inflammasome Activation *In Vivo*

To explore whether VA inhibits NLRP3 activation *in vivo*, we analyzed the expression of caspase-1, NLRP3, and ASC in the Normal group, KOA group, and KOA + VA group. The mRNA and protein expressions of caspase-1, NLRP3, and ASC (**Figures 1A–C**) in the KOA group were higher than those in the normal group ($p < 0.05$), whereas the KOA + VA group showed a reduction compared to the KOA group ($p < 0.05$). Compared with the KOA group, HE staining (**Figure 1D**) of rats treated with VA resulted in less inflammation infiltration and cell proliferation.

VA Reduces Pain-Related Behavior/Mediator in Knee Osteoarthritis *In Vivo*

To assess the effect of VA on pain-related behavior/mediator during KOA *in vivo*, we analyzed pain-related factors in the KOA and KOA + VA groups. The protein and mRNA expressions of NGF, TrkA, and CGRP in the synovial tissue of rats (**Figures 2A–C**) in the KOA + VA group were lower than those in the KOA group ($p < 0.05$). In the cold-plate paw withdrawal experiment (**Figure 2D**), compared with the blank group, the paw withdrawal time of the KOA group was significantly shortened ($p < 0.05$). In the KOA + VA group, the claw lift time was close to that of the blank group.

Interestingly, we observed the effects of VA on synovial fibrosis. In the type I collagen immunohistochemical analysis, Sirius red staining showed that the KOA group experienced a significant increase in collagen deposition, while the KOA + VA group showed a relative decrease (**Figure 2E**).

VA Reduces Expression of IL-18 and IL-1β *In Vivo*

To evaluate the effect of VA on NLRP3 inflammasome activation, we further analyzed the gene expression of IL-18 and IL-1β via real-time quantitative PCR (**Figure 3**). The expressions of IL-18 and IL-1β under VA intervention were significantly lower than

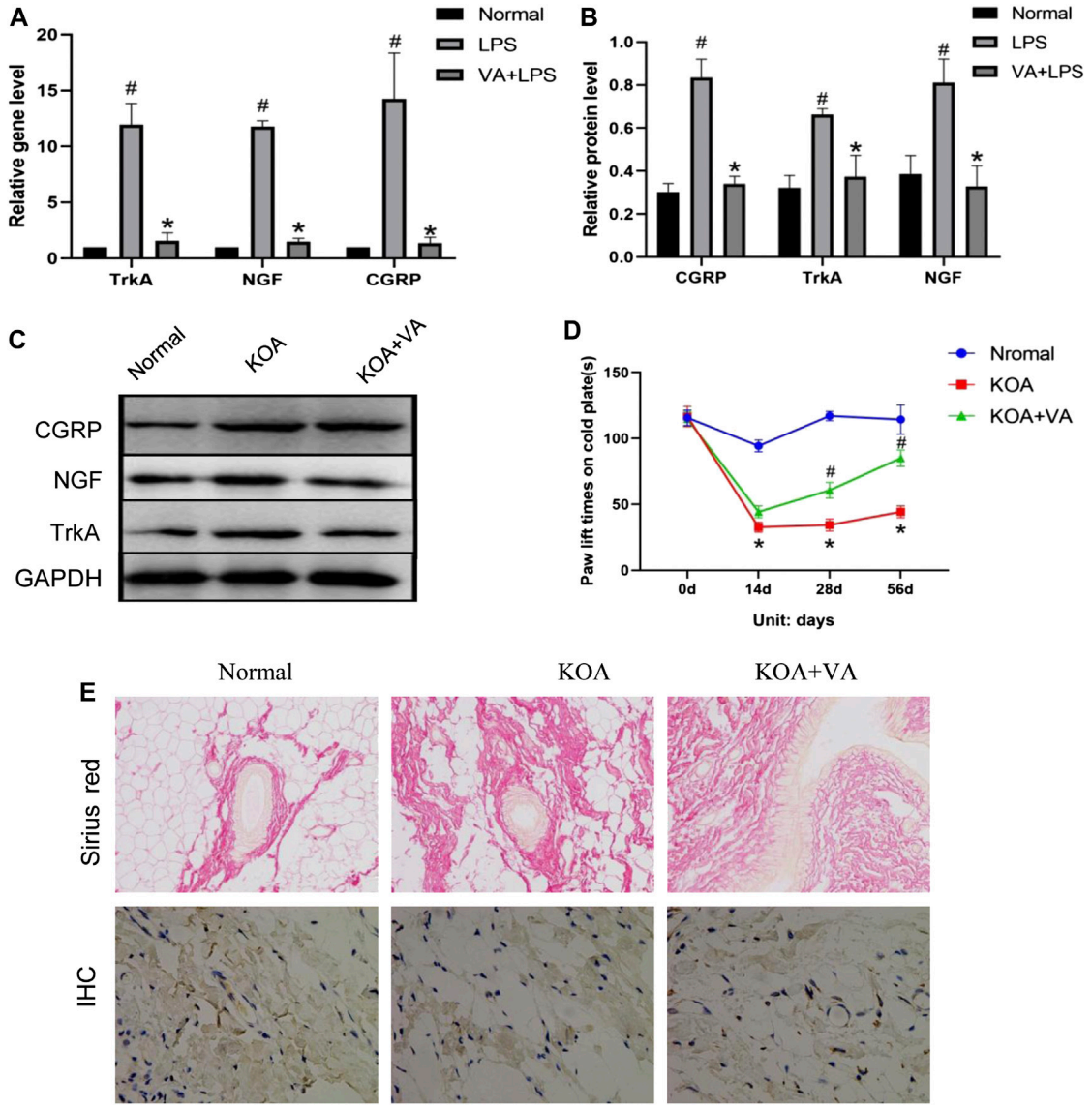


FIGURE 2 | Vanillic acid (VA) relieves pain in knee osteoarthritis. **(A)** Relative gene expression of NGF, TrkA, and CGRP in synovial tissue of rats in each group. Compared with the normal group, $p^{\#} < 0.05$; compared with KOA + VA group, $p^{*} < 0.05$. **(B and C)** Western blot analysis of the effects of VA on NGF, TrkA, and CGRP in rat synovial tissue. Compared with the normal group, $p^{\#} < 0.05$; compared with KOA + VA group, $p^{*} < 0.05$. **(D)** Statistics of paw withdrawal time on a cold plate in each group. **(E)** Sirius red staining of synovium tissue, 200x, scale bar = 100 μm. Immunohistochemical detection of type I collagen in synovium tissue, 400×, scale bar = 100 μm.

those without VA intervention ($p < 0.05$). The protein expression of these NLRP3 downstream proinflammatory factors showed the same trend.

Effects of VA on FLSs Viability

We also used CCK-8 to determine the activity of synovial cells under the treatment of different concentrations of VA *in vitro* (**Figure 4**). The results showed that the activity of synovial cells was better at a dose of 5 μg/ml, although no statistical difference was observed. We decided to use VA at the minimal concentration of 5 μg/ml for subsequent experiments.

VA Inhibits the Inflammatory Changes in FLSs

To explore the effects of VA on inflammation in FLSs, the expressions of caspase-1, NLRP3, and ASC with or without VA intervention after LPS treatment were analyzed (**Figure 5**). As shown in **Figures 5A,B**, the mRNA and protein levels of caspase-1, ASC, and NLRP3 were highly expressed in LPS-induced cells ($p < 0.05$), while VA intervention significantly prevented the upregulation of these factors ($p < 0.05$). Moreover, both the gene and protein expressions of IL-18 and IL-1β (**Figures 5C–E**) were increased in the LPS group ($p < 0.05$), whereas the expressions

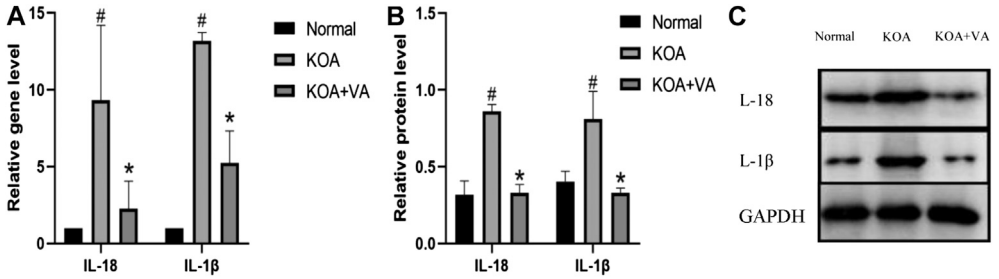


FIGURE 3 | Vanillic acid (VA) reduces the expression of IL-18 and IL-1β. **(A)** Relative gene expression of IL-18 and IL-1β in synovial tissue of rats in each group. Compared with the normal group, $p^{\#} < 0.05$; compared with KOA + VA group, $p^{*} < 0.05$. **(B and C)** Western blot analysis of the effects of VA on synovial tissue IL-18 and IL-1β in rats.

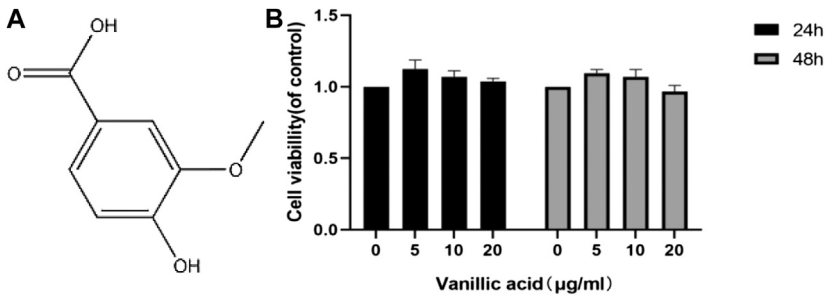


FIGURE 4 | Effects of vanillic acid (VA) on fibroblast-like synoviocytes (FLSs) viability. **(A)** Chemical structure of VA. **(B)** The cells were cultured with increasing concentrations of vanillic acid (0, 5, 10, and 20 μg/ml) for 24 h and 48 h. Cell viability was measured using the CCK-8 kit.

of these factors in the VA + LPS group were significantly lower than those in the LPS group ($p < 0.05$).

DISCUSSION

Studies have shown that VA can improve inflammation and relieve pain, but the mechanisms underlying VA treatment remain unknown. In this study, we demonstrated that VA can inhibit the activation of NLRP3 inflammasomes, which are mainly manifested through the downregulation of caspase-1, ASC, and NLRP3, thereby reducing the inflammatory state of synovitis in KOA. VA also inhibited the expression of proinflammatory factors IL-18 and IL-1β, the downstream substances released after the activation of NLRP3 inflammasomes. In addition, VA decreased both the gene and protein expression of the pain-related factors NGF, TrkA, and CGRP, alleviating pain in the KOA rats. Interestingly, we also observed an inhibitory effect of VA treatment on synovial fibrosis in rats. Immunohistochemical staining of collagen I and Sirius red staining of the synovial tissue both showed less collagen deposition after VA intervention.

KOA is a disabling disease for the elderly worldwide. In recent years, the number of osteoarthritis patients has gradually increased. The main features of KOA include synovial inflammation, cartilage destruction, and osteophyte formation. Synovitis occurs in the early stage of KOA and is associated with joint pain and stiffness in patients. For treatment, nonsteroidal anti-inflammatory drugs (NSAIDs) are usually the first-line medications in clinical guidance. These types of drugs have analgesic, antipyretic, and high-dose anti-inflammatory effects but also have many adverse side-effects, especially in the digestive system (Mobasheri, 2013; Rannou et al., 2016). Intra-articularly injected drugs, such as local anesthetics and tranexamic acid, also show cytotoxicity (Busse et al., 2019). Chinese herbal medicines, including decoctions and herbal extracts, are effective in treating KOA with few adverse effects. Therefore, finding a safe anti-inflammatory and analgesic drug among Chinese herbal medicine seems to be a feasible method. In our previous study, we conducted an active component analysis of Sanse powder, a classic prescription for KOA clinical treatment, and identified dozens of potential effector components, including VA. It has been noted that VA has anti-inflammatory and analgesic effects (Calixto-Campos et al., 2015; Lee et al., 2018). However, how VA affects the inflammation of KOA and its effects on pain are still poorly understood. Therefore, we performed this study to determine the potential mechanisms.

Synovitis is a process characterized by thickening of the synovium (hyperplasia and hypertrophy) and cellular infiltration (macrophages and lymphocytes) (Miao et al., 2010; Jorgensen and Miao, 2015). Oehler et al. (2000) reported that cell layer proliferation is the most common phenomenon among

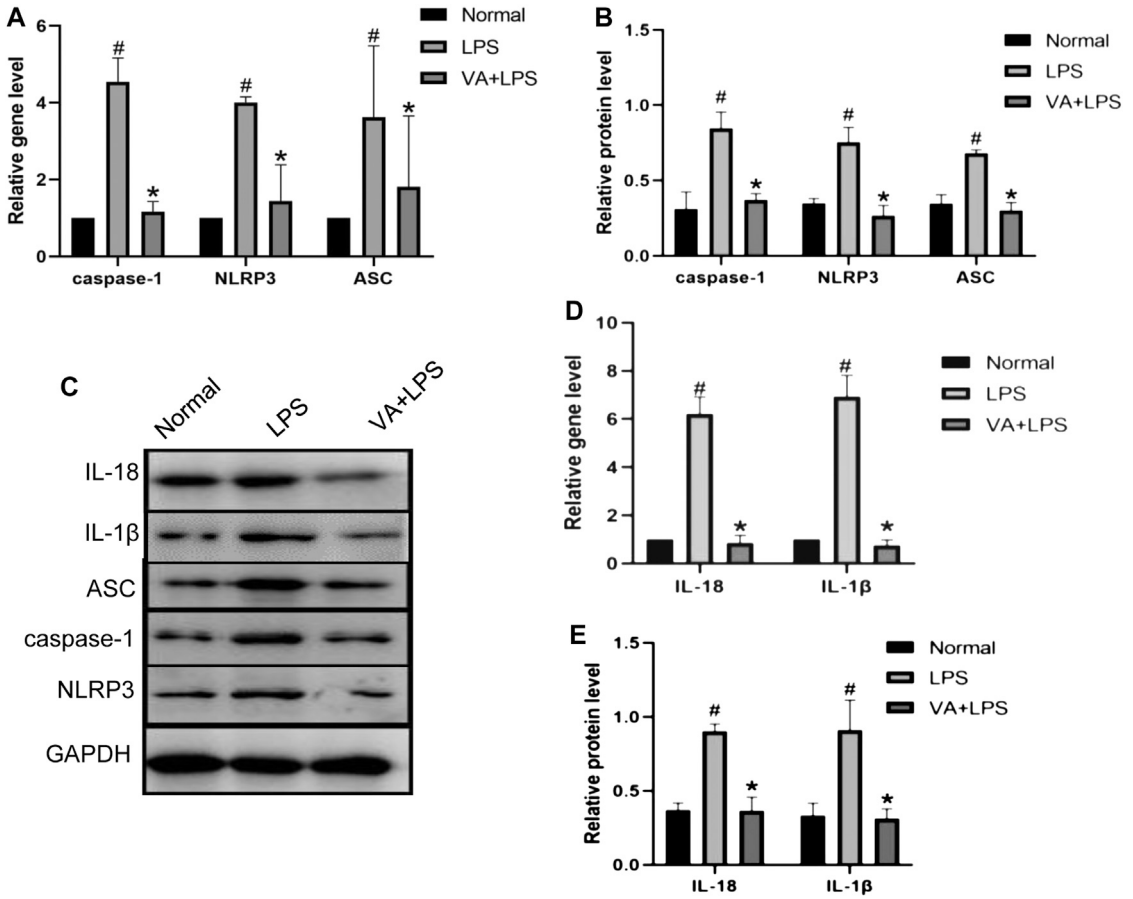


FIGURE 5 | Vanillic acid (VA) inhibits the inflammatory changes in fibroblast-like synoviocytes (FLSs). **(A)** Relative gene expression of caspase-1, NLRP3, and ASC in FLSs of each group. Compared with the normal group, $p^{\#} < 0.05$; compared with VA + LPS group, $p^{*} < 0.05$. **(B and C)** Western blot analysis of the effects of VA on procaspase-1, p10, NLRP3, and ASC in rat synovial cells in cell experiments. Compared with the normal group, $p\# < 0.05$; compared with VA + LPS group, $p^{*} < 0.05$. **(D)** Relative gene expression of IL-18 and IL-1β in rat synovial cells in cell experiments. Compared with the normal group, $p\# < 0.05$; compared with VA + LPS group, $p^{*} < 0.05$. Western blot analysis of the effects of VA on IL-18 and IL-1β in rat synovial cells in cell experiments **(C and E)**. Compared with the normal group, $p\# < 0.05$; compared with VA + LPS group, $p^{*} < 0.05$.

early synovial changes in KOA. The NLRP3 inflammasome is also associated with synovitis. The role of VA on inflammatory and oxidative stress has likewise been reported (Calixto-Campos et al., 2015). Lee et al. (2018) also showed that o-vanillic acid has the potential to treat inflammation by inhibiting macrophages. Huang et al. (2019) confirmed that VA may affect chondrocytes through the mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways. Recently, Ziadlou et al. (2020) indicated that VA could inhibit NF-κB signaling through the attenuation of the nuclear factor of the kappa light polypeptide gene enhancer in B-cell inhibitor alpha (IκBα) phosphorylation. Meanwhile, other studies have shown that IL-1β and IL-18 are key inflammatory mediators in the pathological process of synovitis (Rahmati et al., 2016). IL-1β can activate the TIR superfamily of receptors (Ziadlou et al., 2020). Moreover, VA treatment significantly inhibits IL-1β levels (Calixto-Campos et al., 2015; Ziadlou et al., 2020), which indicates that the TIR superfamily of receptors may be a target for VA and its anti-inflammatory properties. However, few studies have shown the effects of VA on NLRP3 inflammasomes. Recent studies have explained the role of NLRP3 inflammatory bodies in the inflammatory cascade. NLRP3 is a multiprotein oligomer composed of caspase-1. Caspase-1 is a speckle-like protein associated with apoptosis and contains CARD (ASC) and NOD-like receptor protein 3 (NLRP3). After activation, NLRP3 interacts with ASC, which can bridge NLRP3 to procaspase-1, and procaspase-1 can activate caspase-1. Activated caspase-1 cleaves the original forms of IL-1β and IL-18 into mature and active forms (Groslambert and Py, 2018; Krishnan et al., 2014; Lacey et al., 2018). In the present study, VA was shown to interfere with the inflammatory pathway, thereby reducing the expression of caspase-1, ASC, and NLRP3 in synovial cells in the KOA environment simulated by LPS and in the KOA model group. VA also inhibited the increases in the expression of the NLRP3 inflammatory bodies and their downstream substances, IL-1β and IL-18, suggesting that VA

inhibited the activation of NLRP3 inflammatory bodies, which is consistent with previous studies on NLRP3 inflammasome-driven inflammation (Martinon et al., 2002; Zhang et al., 2019). In this study, we showed that VA can inhibit the activation of NLRP3 inflammasomes by inhibiting the expression of caspase-1, ASC, and NLRP3 proteins, thereby reducing the inflammatory expression in KOA and exerting anti-inflammatory effects. In addition, it also reduced the expression of IL-18 and IL-1β in the downstream substances of the NLRP3 inflammasome.

Inflammation can cause nerve damage, which may be one of the causes of pain. Studies have shown that NGF, TrkA, and CGRP are closely related to OA pain (Denk et al., 2017; Schou et al., 2017). NGF is widely considered a mediator of chronic pain and is mainly expressed in the synovial fluid, OA osteochondral junction, synovium, and cartilage (Montagnoli et al., 2017; Takano et al., 2017a). Animal and population studies have shown that NGF levels increase under trauma, inflammation, and chronic pain (Collison, 2019). TrkA is a functional receptor for NGF and one of the main targets of the NGF pain signaling pathway (Sousa-Valente et al., 2018). Moreover, an increase in CGRP is closely related to the generation and maintenance of chronic pain, and CGRP's sensory neurons dominate most joint structures, including the synovium, ligaments, and subchondral bone, which contribute to peripheral sensitization and inflammation. Therefore, under OA pain sensitization, the expression of CGRP will also increase accordingly (Walsh et al., 2015; Takano et al., 2017b). At the same time, CGRP can enhance or reduce the effects of other chemicals and regulate the excitability of sensory ends after experiencing harmful stimuli (Grässel and Muschter, 2017). Therefore, we subsequently investigated whether VA inhibition in the activation of NLRP3 inflammatory bodies could affect the above mediators. We found that VA can inhibit the upregulation of NGF, TrkA, and CGRP in FLSs induced by LPS. In animal experiments, we found that oral VA also inhibited the upregulation of these pain mediators in the model rats with KOA induced by MIA. These results indicate that VA can reduce the expression of pain mediators in KOA model rats, thereby alleviating osteoarthritis pain. A previous study also showed that VA can reduce inflammation-related pain by inhibiting neutrophil recruitment, oxidative stress, cytokine production, and NF-κB activation (Calixto-Campos et al., 2015).

However, for technical reasons, our study has several limitations. For example, the specific mechanism of VA role in the pain caused by KOA inflammatory response has not been studied in detail, and no further research has been done on the observed synovial fibrosis. At the same time, only one dose of VA was used in this study. Therefore, we did not observe the long-term effects of VA on synovial inflammation. In addition, an animal model of KOA that cannot account for all related factors of human KOA was used in this study.

In summary, our data show that VA can reduce KOA synovial inflammation and inhibit NLRP3 inflammasome activation. In addition, we also observed that VA reduces pain-related behavior/mediator in knee osteoarthritis *in vivo*. Overall, VA may protect knee joints by inhibiting NLRP3 activation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Affiliated Hospital of Nanjing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

ZM, ZH, and PW participated in the design of the study and wrote the manuscript. XL, LZ, and XS analyzed the data. YX, BX, TL, and H Z performed the experiments. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (81774334) and Chinese Medicine Leading Talents in Jiangsu Province (SLJ0207).

REFERENCES

Akhbari, P., Karamchandani, U., Jaggard, M. K. J., Graça, G., Bhattacharya, R., Lindon, J. C., et al. (2020). Can joint fluid metabolic profiling (or "metabonomics") reveal biomarkers for osteoarthritis and inflammatory joint disease?: a systematic review. *Bone. Joint. Res.* 9, 108–119. doi:10.1302/2046-3758.93.BJR-2019-0167.R1

Arnalich, F., de Miguel, E., Perez-Ayala, C., Martinez, M., Vazquez, J. J., Gijon-Banos, J., et al. (1994). Neuropeptides and interleukin-6 in human joint inflammation relationship between intraarticular substance P and interleukin-6 concentrations. *Neurosci. Lett.* 170, 251–254. doi:10.1016/0304-3940(94)90331-x

Atukorala, I., Kwoh, K., Guermazi, A., Roemer, F., Boudreau, R. M., Hannon, M. J., et al. (2013). Synovitis in OA: a precursor of disease? The onset of synovitis and radiographic osteoarthritis in a incidence sub cohort of the osteoarthritis initiative (OAI). *Osteoarthritis Cartilage* 21, s41. doi:10.1016/j.joca.2013.02.105

Busse, P., Vater, C., Stiehler, M., Nowotny, J., Kasten, P., Bretschneider, H., et al. (2019). Cytotoxicity of drugs injected into joints in orthopaedics. *Bone Joint Res.* 8, 41–48. doi:10.1302/2046-3758.82.bjr-2018-0099.r1

Calixto-Campos, C., Carvalho, T. T., Hohmann, M. S., Pinho-Ribeiro, F. A., Fattori, V., Manchope, M. F., et al. (2015). Vanillic acid inhibits inflammatory pain by inhibiting neutrophil recruitment, oxidative stress, cytokine production, and NFκB activation in mice. *J. Nat. Prod.* 78, 1799–1808. doi:10.1021/acs.jnatprod.5b00246

Collison, J. (2019). Anti-NGF therapy improves osteoarthritis pain. *Nat. Rev. Rheumatol.* 15, 450. doi:10.1038/s41584-019-0266-y

Denk, F., Bennett, D. L., and McMahon, S. B. (2017). Nerve growth factor and pain mechanisms. *Annu. Rev. Neurosci.* 40, 307–325. doi:10.1146/annurev-neuro-072116-031121

Ferrucci, L., and Fabbri, E. (2018). Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. *Nat. Rev. Cardiol.* 15, 505–522. doi:10.1038/s41569-018-0064-2

Grässel, S., and Muschter, D. (2017). Peripheral Nerve fibers and their neurotransmitters in osteoarthritis pathology. *Int. J. Mol. Sci.* 18, 931. doi:10.3390/ijms18050931

Groslambert, M., and Py, B. F. (2018). Spotlight on the NLRP3 inflammasome pathway. *J. Inflamm. Res.* 11, 359–374. doi:10.2147/JIR.S141220

Huang, X., Xi, Y., Mao, Z., Chu, X., Zhang, R., Ma, X., et al. (2019). Vanillic acid attenuates cartilage degeneration by regulating the MAPK and PI3K/AKT/NF-κB pathways. *Eur. J. Pharmacol.* 859, 172481. doi:10.1016/j.ejphar.2019.172481

Jorgensen, I., and Miao, E. A. (2015). Pyroptotic cell death defends against intracellular pathogens. *Immunol. Rev.* 265, 130–142. doi:10.1111/imr.12287

Krishnan, S. M., Sobey, C. G., Latz, E., Mansell, A., and Drummond, G. R. (2014). IL-1β and IL-18: inflammatory markers or mediators of hypertension? *Br. J. Pharmacol.* 171, 5589–5602. doi:10.1111/bph.12876

Lacey, C. A., Mitchell, W. J., Dadelahi, A. S., and Skyberg, J. A. (2018). Caspase-1 and caspase-11 mediate pyroptosis, inflammation, and control of Brucella joint infection. *Infect. Immun.* 86, e00361–18. doi:10.1128/IAI.00361-18

Lee, J.-K., Lee, S., Shin, T.-Y., Khang, D., and Kim, S.-H. (2018). Anti-inflammatory effect of o-vanillic acid on lipopolysaccharide-stimulated macrophages and inflammation models. *J. Food Nutri. Res.* 6, 227–233. doi:10.12691/jfnr-6-4-4

Li, X., Mei, W., Huang, Z., Zhang, L., Zhang, L., Xu, B., et al. (2020). Casticin suppresses monoiodoacetic acid-induced knee osteoarthritis through inhibiting HIF-1α/NLRP3 inflammasome signaling. *Int. Immunopharm.* 86, 106745. doi:10.1016/j.intimp.2020.106745

Litwic, A., Edwards, M. H., Dennison, E. M., and Cooper, C. (2013). Epidemiology and burden of osteoarthritis. *Br. Med. Bull.* 105, 185–199. doi:10.1093/bmb/lds038

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell* 10, 417–426. doi:10.1016/s1097-2765(02)00599-3

Miao, E. A., Leaf, I. A., Treuting, P. M., Mao, D. P., Dors, M., Sarkar, A., et al. (2010). Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat. Immunol.* 11, 1136–1142. doi:10.1038/ni.1960

Mobasheri, A. (2013). The future of osteoarthritis therapeutics: targeted pharmacological therapy. *Curr. Rheumatol. Rep.* 15, 364. doi:10.1007/s11926-013-0364-9

Montagnoli, C., Tiribuzi, R., Crispoltoni, L., Pistilli, A., Stabile, A. M., Manfreda, F., et al. (2017). β-NGF and β-NGF receptor upregulation in blood and synovial fluid in osteoarthritis. *Biol. Chem.* 398, 1045–1054. doi:10.1515/hsz-2016-0280

Muratovic, D., Findlay, D. M., Cicuttini, F. M., Wluka, A. E., Lee, Y. R., Edwards, S., et al. (2019). Bone marrow lesions in knee osteoarthritis: regional differences in tibial subchondral bone microstructure and their association with cartilage degeneration. *Osteoarthritis Cartilage* 27, 1653–1662. doi:10.1016/j.joca.2019.07.004

Oehler, S., Fassbender, H. G., Neureiter, D., Meyer-Scholten, C., Kirchner, T., and Aigner, T. (2000). Cell populations involved in pigmented villonodular synovitis of the knee. *J. Rheumatol.* 27, 463–470.

Pecchi, E., Priam, S., Gosset, M., Pigenet, A., Sudre, L., Laiguillon, M. C., et al. (2014). Induction of nerve growth factor expression and release by mechanical and inflammatory stimuli in chondrocytes: possible involvement in osteoarthritis pain. *Arthritis Res. Ther.* 16, R16. doi:10.1186/ar4443

Rahmati, M., Mobasheri, A., and Mozafari, M. (2016). Inflammatory mediators in osteoarthritis: a critical review of the state-of-the-art, current prospects, and future challenges. *Bone* 85, 81–90. doi:10.1016/j.bone.2016.01.019

Rannou, F., Pelletier, J. P., and Martel-Pelletier, J. (2016). Efficacy and safety of topical NSAIDs in the management of osteoarthritis: evidence from real-life setting trials and surveys. *Semin. Arthritis Rheum.* 45, s18–s21. doi:10.1016/j.semarthrit.2015.11.007

Schou, W. S., Ashina, S., Amin, F. M., Goadsby, P. J., and Ashina, M. (2017). Calcitonin gene-related peptide and pain: a systematic review. *J. Headache Pain* 18, 34. doi:10.1186/s10194-017-0741-2

Sousa-Valente, J., Calvo, L., Vacca, V., Simeoli, R., Arévalo, J. C., and Malcangio, M. (2018). Role of TrkA signalling and mast cells in the initiation of osteoarthritis pain in the monoiodoacetate model. *Osteoarthritis Cartilage* 26, 84–94. doi:10.1016/j.joca.2017.08.006

Takano, S., Uchida, K., Inoue, G., Minatani, A., Miyagi, M., Aikawa, J., et al. (2017a). Increase and regulation of synovial calcitonin gene-related peptide expression in patients with painful knee osteoarthritis. *J. Pain Res.* 10, 1099–1104. doi:10.2147/JPR.S135939

Takano, S., Uchida, K., Inoue, G., Miyagi, M., Aikawa, J., Iwase, D., et al. (2017b). Nerve growth factor regulation and production by macrophages in osteoarthritic synovium. *Clin. Exp. Immunol.* 190, 235–243. doi:10.1111/cei.13007

Walsh, D. A., Mapp, P. I., and Kelly, S. (2015). Calcitonin gene-related peptide in the joint: contributions to pain and inflammation. *Br. J. Clin. Pharmacol.* 80, 965–978. doi:10.1111/bcp.12669

Wu, P., Huang, Z., Shan, J., Luo, Z., Zhang, N., Yin, S., et al. (2020). Interventional effects of the direct application of "Sanse powder" on knee osteoarthritis in rats as determined from lipidomics via UPLC-Q-Exactive Orbitrap MS. *Chin. Med.* 15, 9. doi:10.1186/s13020-020-0290-5

Zhang, L., Zhang, L., Huang, Z., Xing, R., Li, X., Yin, S., et al. (2019). Increased HIF-1α in Knee Osteoarthritis Aggravate Synovial Fibrosis via Fibroblast-Like Synoviocyte Pyroptosis. *Oxid. Med. Cell. Longev.* 2019, 6326517. doi:10.1155/2019/6326517

Zhao, L. R., Xing, R. L., Wang, P. M., Zhang, N. S., Yin, S. J., Li, X. C., et al. (2018). NLRP1 and NLRP3 inflammasomes mediate LPS/ATP-induced pyroptosis in knee osteoarthritis. *Mol. Med. Rep.* 17, 5463–5469. doi:10.3892/mmr.2018.8520

Ziadlou, R., Barbero, A., Martin, I., Wang, X., Qin, L., Alini, M., et al. (2020). Anti-inflammatory and chondroprotective effects of vanillic acid and epimedin C in human osteoarthritic chondrocytes. *Biomolecules* 10, 932. doi:10.3390/biom10060932

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 12 April 2021
doi: 10.3389/fphar.2021.609817

3′-Sialyllactose Protects SW1353 Chondrocytic Cells From Interleukin-1β-Induced Oxidative Stress and Inflammation

Ahreum Baek[1,2†], So Hee Jung[3†], Soonil Pyo[2,4], Soo Yeon Kim[5], Seongmoon Jo[2,4], Lila Kim[6], Eun Young Lee[3], Sung Hoon Kim[1] and Sung-Rae Cho[2,4,7,8]**

[1]Department of Rehabilitation Medicine, Yonsei University Wonju College of Medicine, Wonju, Korea, [2]Department and Research Institute of Rehabilitation Medicine, Yonsei University College of Medicine, Seoul, Korea, [3]Department of Rehabilitation Medicine, The Graduate School Yonsei University Wonju College of Medicine, Wonju, Korea, [4]Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea, [5]Department of Medicine, Yonsei University Wonju College of Medicine, Wonju, South Korea, [6]GeneChem Inc., Daejeon, Korea, [7]Graduate Program of Nano Science and Technology, Yonsei University College of Medicine, Seoul, Korea, [8]Rehabilitation Institute of Neuromuscular Disease, Yonsei University College of Medicine, Seoul, Korea

OPEN ACCESS

Edited by:
Sara Tenti,
University of Siena, Italy

Reviewed by:
Roman A. Zinovkin,
Lomonosov Moscow State University, Russia
Anna Scanu,
University of Padua, Italy

***Correspondence:**
Sung Hoon Kim
kimrehab@yonsei.ac.kr
Sung-Rae Cho
srcho918@yuhs.ac

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 24 September 2020
Accepted: 19 February 2021
Published: 12 April 2021

Citation:
Baek A, Jung SH, Pyo S, Kim SY, Jo S, Kim L, Lee EY, Kim SH and Cho S-R (2021) 3′-Sialyllactose Protects SW1353 Chondrocytic Cells From Interleukin-1β-Induced Oxidative Stress and Inflammation. Front. Pharmacol. 12:609817. doi: 10.3389/fphar.2021.609817

Osteoarthritis (OA) is a major degenerative joint disease. Oxidative stress and inflammation play key roles in the pathogenesis of OA. 3′-Sialyllactose (3′-SL) is derived from human milk and is known to regulate a variety of biological functions related to immune homeostasis. This study aimed to investigate the therapeutic mechanisms of 3′-SL in interleukin-1β (IL-1β)-treated SW1353 chondrocytic cells. 3′-SL potently suppressed IL-1β-induced oxidative stress by increasing the levels of enzymatic antioxidants. 3′-SL significantly reversed the IL-1β mediated expression levels of reactive oxygen species in IL-1β-stimulated chondrocytic cells. In addition, 3′-SL could reverse the increased levels of inflammatory markers such as nitrite, prostaglandin E2, inducible nitric oxide synthase, cyclooxygenase-2, IL-1β, and IL-6 in IL-1β-stimulated chondrocytic cells. Moreover, 3′-SL significantly inhibited the apoptotic process, as indicated by the downregulation of the pro-apoptotic protein Bax, upregulation of the anti-apoptotic protein Bcl-2 expression, and significant reduction in the number of TUNEL-positive cells in the IL-1β-treated chondrocytic cells. Furthermore, 3′-SL reversed cartilage destruction by decreasing the release of matrix metalloproteinases (MMP), such as MMP1, MMP3, and MMP13. In contrast, 3′-SL significantly increased the expression levels of matrix synthesis proteins, such as collagen II and aggrecan, in IL-1β-treated chondrocytic cells. 3′-SL dramatically suppressed the activation of mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways, which are related to the pathogenesis of OA. Taken together, our data suggest that 3′-SL alleviates IL-1β-induced

Abbreviations: 3′-SL, 3′-Sialyllactose; Akt, protein kinase B; DAPI, 4,6-diamidino-2-phenylindole; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; IL-1β interleukin-1β; IκBα, inhibitor of nuclear factor kappa B; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells; OA, osteoarthritis; PI3K, phosphatidylinositol-3-kinase; qRT-PCR, quantitative Real-Time Reverse Transcription polymerase Chain Reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TUNEL, terminal dUTP nick end-labeling; XO, xanthine oxidase.

OA pathogenesis via inhibition of activated MAPK and PI3K/AKT/NF-κB signaling cascades with the downregulation of oxidative stress and inflammation. Therefore, 3′-SL has the potential to be used as a natural compound for OA therapy owing to its ability to activate the antioxidant defense system and suppress inflammatory responses.

Keywords: osteoarthritis, 3′-sialyllactose, oxidative stress, inflammation, apoptosis, matrix metalloproteinases

INTRODUCTION

Osteoarthritis (OA) is a complex progressive degenerative joint disorder that accompanies cartilage degradation and physical disability (Sanchez et al., 2005; Sanchez et al., 2008; Jeon et al., 2017). The development and progression of OA are related to oxidative stress-induced cartilage damage and an imbalance between catabolic and anabolic factors in joints (Loeser, 2009; Appleton, 2018). Although the occurrence and development of OA have been studied extensively, there is currently no efficient therapy to prevent OA progression.

The accumulation of reactive oxygen species (ROS) causes an increase in oxidative stress, and these reactive products are detoxified by the anti-oxidative defensive system (Betteridge, 2000; Jones, 2008). ROS are free radicals that are mainly generated by mitochondria, in the form of non-mitochondrial membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (XO) (Turrens, 2003). Several studies have indicated that elevated oxidative stress and excessive generation of ROS are observed in OA patients (Altindag et al., 2007; Erturk et al., 2012; Altay et al., 2015).

Recent studies revealed that excessive generation of ROS occurs as OA develops, leading to increased inflammation (Bolduc et al., 2019; Xie et al., 2019; Ansari et al., 2020). Interleukin-1β (IL-1β) and IL-6 are highly upregulated in OA joints and play important roles in the pathogenesis of OA by modulating oxidative stress, apoptosis, cartilage extracellular matrix degradation extracellular matrix (ECM) synthesis, and intracellular signaling pathways in OA (Kapoor et al., 2011; Lepetsos and Papavassiliou, 2016; Collins et al., 2018). In particular, mitogen-activated protein kinases (MAPK) and phosphoinositol 3 kinase (PI3K)/protein kinase B (AKT)/nuclear factor-κ light chain enhancer of activated B cells (NF-κB) pathways play vital roles in OA pathogenesis (Ahmed et al., 2005; Lu et al., 2018; Huang et al., 2019). Thus, oxidative stress and inflammation are closely related, and their regulation should be considered as therapeutic strategies of OA.

Human breast milk contains various bioactive factors with developmental and protective functions (Gila-Diaz et al., 2019). 3′-Sialyllactose (3′-SL) contains N-acetyl-D-neuramic acid and galactose subunit of lactose (Luo et al., 2014). 3′-SL is known to regulate a variety of biological functions in immune homeostasis (Zenhom et al., 2011; Donovan and Comstock, 2016). Moreover, 3′-SL has demonstrated therapeutic effects in OA and rheumatoid arthritis by protecting cartilage degradation and modulating chemokines and cytokines, respectively (Jeon et al., 2018; Kang et al., 2018). However, the antioxidant and inflammatory activities of 3′-SL in OA remain uncharacterized.

Human SW1353 chondrocytic cells and human chondrocytes have similar phenotypes. Previous studies have shown that IL-1β can mimic the pathological microenvironment of OA chondrocytes (Jia et al., 2013; Bao et al., 2016). Herein, we investigated the antioxidant and anti-inflammatory activities and mechanisms of action of 3′-SL on IL-1β-treated human SW1353 chondrocytic cells and explored the mechanisms underlying potential therapeutic effects in OA.

MATERIALS AND METHODS

Reagents

SW1353 human chondrocytic cells were obtained from American Type Culture Collection (ATCC HTB-94; Manassas, VA, United States). Dulbecco's modified Eagle medium with high glucose medium (DMEM-HG) was obtained from Hyclone (Grand Island, NY, United States). Fetal bovine serum (FBS) was purchased from T&I (Seoul, Korea). Phosphate-buffered saline (PBS) was provided by Welgene (Daegu, Korea). Penicillin-streptomycin, trypsin-EDTA, and BCA™ Protein Assay Kit were obtained from Thermo Fisher Scientific (Waltham, MA, United States). 3′-SL was provided by GeneChem Inc (Daejeon, Korea). Recombinant human IL-1β protein, nitrite, prostaglandin E2 (PGE2), IL-1β, and IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, MN, United States). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor Bay 11-7082, dimethyl sulfoxide, and gelatin were obtained from Sigma-Aldrich (Saint Louis, MO, United States). Total antioxidant capacity (TAC) assay kit, 2′,7′-dichlorofluorescin diacetate (DCFDA)-cellular ROS assay kit, superoxide dismutase (SOD) activity assay kit, catalase activity assay kit, and oxidative stress defense cocktail were purchased from Abcam (Cambridge, MA, United States). DeadEnd™ Fluorometric Terminal dUTP Nick End-Labeling (TUNEL) System and 4,6-diamidino-2-phenylindole (DAPI; Vectorshield were purchased from Promega (Madison, WI, United States) and Vector Laboratories (Burlingame, CA, United States), respectively. TRIzol reagent, 4–12% Bis-Tris gels, 1× NuPage MES SDS running buffer, MOPS SDS running buffer, PVDF membrane, and NuPage Transfer Buffer were obtained from Invitrogen Life Technologies (Carlsbad, CA, United States). ReverTra Ace® qPCR RT Master Mix with gDNA Remover and qPCRBIO SyGreen Mix Hi-ROX were purchased from Toyobo (Osaka, Japan) and PCR BIOSYSTEMS (London, United Kingdom), respectively. Bax, Bcl-2, iNOS, COX-2, MMP1, MMP3, MMP13, collagen II, aggrecan, p-extracellular signal-regulated kinase (ERK), ERK, p-P38, P38, p-c-Jun N-terminal kinase

(JNK), JNK, p-PI3K, PI3K, p-AKT, AKT, p-inhibitor of nuclear factor kappa B (p-IκBα), IκBα, p-P65, P65, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase–conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, United States). p-PI3K antibody and enhanced chemiluminescence detection system were purchased from Cell Signaling Technologies (Beverly, MA, United States) and Amersham Pharmacia Biotech (Little Chalfont, United Kingdom), respectively.

Cell Culture

Human chondrocytic cells were maintained in DMEM-HG with 10% FBS and 1% penicillin-streptomycin at 37°C. When approximately 80% confluency was achieved, the cells were washed with PBS and harvested with 0.05% trypsin-EDTA. Next, the cells were washed, centrifuged (1,000 g, 5 min, 25°C), resuspended, and finally seeded in new plates. The medium was replaced every 2–3 days. The cells were treated with IL-1β (10 ng/ml) under hypoxic conditions for 24 h, as previously described (Baek et al., 2018a; Baek et al., 2018b) to mimic the pathological microenvironment of OA chondrocytes. Then, 50 or 100 μM of 3′-SL was added and incubation continued for 24 h. In the NF-κB inhibitor experiment, the cells were pretreated with Bay 11-7082 (10 μM), which was dissolved in dimethyl sulfoxide, before the cells were exposed to IL-1β with or without 3′-SL. The culture supernatants were harvested, centrifuged (2,000 g, 5 min, 25°C), and stored at −70°C until further analysis. The cell pellets were washed with ice-cold PBS, centrifuged (2,000 g, 5 min, 4°C), and stored at −70°C until further analysis.

Enzyme-Linked Immunosorbent Assay

Cell culture supernatants were collected, centrifuged (12,000 ×g, 5 min, 4°C), and stored at −70°C until use. The production of ROS, activities of TAC, SOD, and CAT, and the levels of IL-1β, IL-6, nitrite, and PGE2 in cell culture supernatants were measured according to the manufacturer's instructions.

Terminal dUTP Nick End-Labeling Assay

For analysis of apoptosis, SW1353 human chondrocytic cells were seeded in gelatin-coated slides in 6-well cell plates. IL-1β and 3′-SL treatments were performed as described in *Cell Culture*. The DeadEnd™ Fluorometric TUNEL System was conducted according to the manufacturer's protocol. Samples were mounted on glass slides with fluorescent mounting medium with DAPI for imaging, using the Zeiss Axio Imager M2 (Carl Zeiss, Gottingen, Germany) fluorescence microscope. The number of positively stained cells over the total number of cells per specimen field was measured, and the percentage of positive cells was calculated. Four individual specimens per group were analyzed.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA extraction from the cell pellets was carried out using the TRIzol reagent, according to the manufacturer's protocol. Total RNA was used in cDNA synthesis with ReverTra Ace® qPCR RT Master Mix with gDNA Remover. The mRNA expression of genes such as iNOS, COX-2, MMP1, MMP3, MMP13, collagen II, aggrecan, and GAPDH was profiled with qPCRBIO SyGreen Mix Hi-ROX in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). Data analysis was performed using the $2^{-\Delta\Delta CT}$ method. Primers used for qRT-PCR are described in **Table 1**.

Western Blot Analysis

To assess the oxidative stress defense WB cocktail, Bax, Bcl-2, iNOS, COX-2, MMP1, MMP3, MMP13, collagen II, aggrecan, p-ERK, ERK, p-P38, P38, p-JNK, JNK, p-PI3K, PI3K, p-AKT, AKT, p-IκBα, IκBα, p-P65, P65, and GAPDH. The proteins from cell pellets were harvested and quantified using the BCA™ Protein Assay Kit. The protein samples were denatured and separated in 4–12% Bis-Tris gels with 1× NuPage MES and MOPS SDS running buffer. Proteins were transferred onto a PVDF membrane in NuPage Transfer Buffer with methanol at 4°C. The membranes were blocked with TBS plus Tween 20 in 5% skim milk and then incubated overnight at 4°C with the following primary antibodies: oxidative stress defense WB cocktail (1:250), Bax (1:1,000), Bcl-2 (1:1,000), iNOS (1:1,000), COX-2 (1:1,000), MMP1 (1:1,000), MMP3 (1:1,000), MMP13 (1:1,000), collagen II (1:1,000), Aggrecan (1:1,000), p-ERK (1:1,000), ERK (1:1,000), p-P38 (1:1,000), P38 (1:1,000), p-JNK (1:1,000), JNK (1:1,000), p-PI3K (1:1,000), PI3K (1:1,000), p-AKT (1:1,000), AKT (1:1,000), p-IκBα (1:1,000), IκBα (1:1,000), p-P65 (1:1,000), P65 (1:1,000), and GAPDH (1:1,000). The next day, the blots were washed three times with TBS plus Tween 20 and incubated with horseradish peroxidase–conjugated secondary antibodies (1:3,000) at 25°C for 1 h. After the blots were rinsed three times, the protein was detected with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The relative expression of each protein was quantified using the internal controls (smooth muscle actin and GAPDH) or the total form of proteins (ERK, P38, JNK, PI3K, AKT, IκBα, and P65) with Multi Gauge (v3.0) software (Fujifilm, Tokyo, Japan).

Statistical Analysis

All data are expressed as mean ± standard error of the mean from at least three independent experiments. Statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) version 25.0 (SPSS, Inc, Chicago, IL, United States). To confirm statistically significant results, one-way analysis of variance, followed by a post hoc Bonferroni comparison, was conducted. Statistical significance was set at $p < 0.05$.

RESULTS

3′-Sialyllactose Suppressed Interleukin-1β-Induced Oxidative Stress in the Chondrocytic Cells

To determine whether 3′-SL has antioxidant effects, generation of ROS in the chondrocytic cells was induced by IL-1β. Treatment with

TABLE 1 | Primers used for qRT-PCR.

Gene symbol	Forward primer (5′→3′)	Reverse primer (5′→3′)
iNOS	AGGGACAAGCCTACCCCTC	CTCATCTCCCGTCAGTTGGT
COX2	CTGGCGCTCAGCCATACAG	CGCACTTATACTGGTCAAATCCC
MMP1	GGGGCTTTGATGTACCCTAGC	TGTCACACGCTTTTGGGGTTT
MMP3	CTGGACTCCGACACTCTGGA	CAGGAAAGGTTCTGAAGTGACC
MMP13	TCCTGATGTGGGTGAATACAATG	GCCATCGTGAAGTCTGGTAAAAT
COL2A	TGGACGCCATGAAGGTTTTCT	TGGGAGCCAGATTGTCATCTC
Aggrecan	GTGCCTATCAGGACAAGGTCT	GATGCCTTTCACCACGACTTC
GAPDH	AAGGGTCATCATCTCTGCCC	GTGAGTGCATGGACTGTGGT

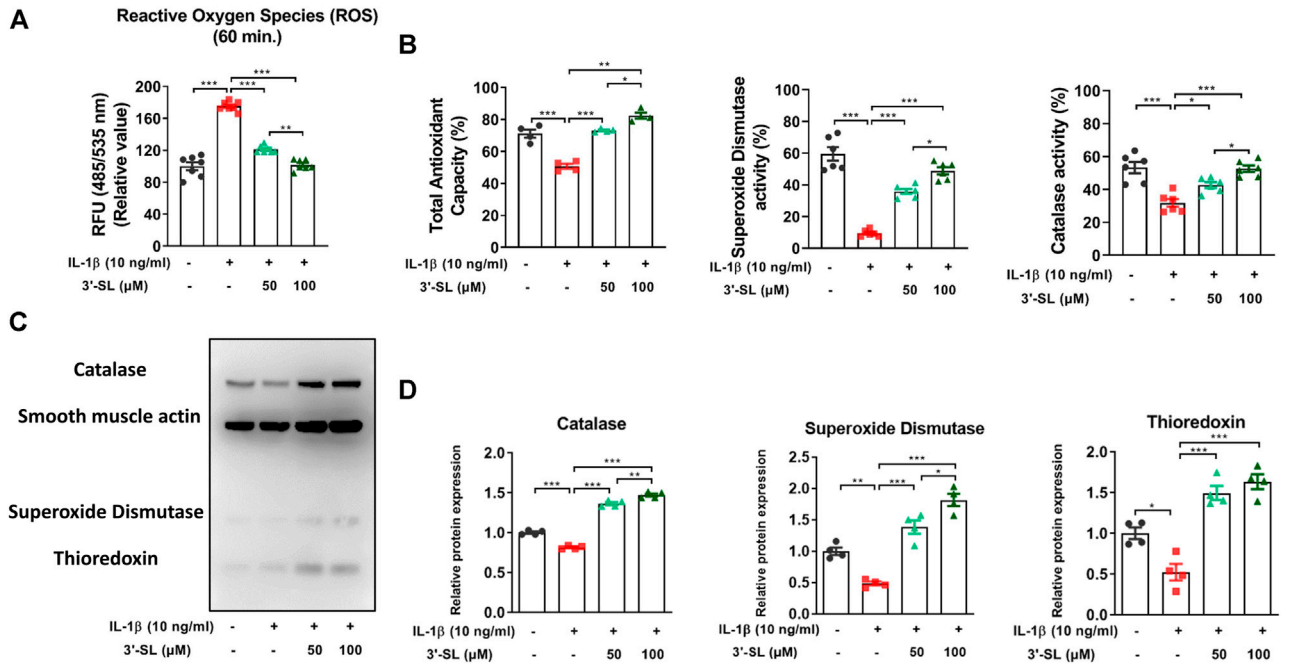


FIGURE 1 | Effect of 3′-SL on IL-1β-induced oxidative stress in the chondrocytic cells. Chondrocytic cells were stimulated with or without IL-1β (10 ng/ml) for 24 h and then treated with the different concentrations of 3′-SL (50 and 100 μM) for 24 h. **(A)** Generation of ROS was measured using the DCFDA assay. **(B)** Antioxidant enzyme activities were analyzed using specific enzyme assay kits **(C)** The oxidative stress defense (Catalase, SOD1, TRX, smooth muscle actin) western blot cocktail was determined by western blot and **(D)** quantification analysis. The values are mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. 3′-SL, 3′-Sialyllactose; IL-1β; interleukin-1β; ROS, reactive oxygen species; DCFDA, 2′,7′-dichlorofluorescin diacetate; SOD1, superoxide dismutase 1; TRX, thioredoxin.

IL-1β elevated ROS levels in the chondrocytic cells. This elevation was significantly attenuated by treatment with 3′-SL in a dose-dependent manner (**Figure 1A**, **$p < 0.01$, and ***$p < 0.001$). Next, total antioxidant capacity and antioxidant enzyme activities were examined. 3′-SL potently suppressed IL-1β-induced oxidative stress, as revealed by the significant increase in total antioxidant capacity and levels of antioxidant enzymes, such as SOD and catalase, in a dose-dependent manner (**Figures 1B–D**, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Similarly, thioredoxin, which was decreased by IL-1β, was significantly increased after 3′-SL treatment of the chondrocytic cells (**Figures 1C,D**, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Taken together, these findings suggest that 3′-SL could suppress IL-1β-induced oxidative stress in chondrocytic cells via the reduction of ROS and the upregulation of antioxidant enzyme activities.

3′-Sialyllactose Suppressed Interleukin-1β-Induced Inflammatory Response in the Chondrocytic Cells

To determine whether 3′-SL has the ability to act against the inflammatory response induced by IL-1β, the expression of inflammatory mediators was examined in chondrocytic cells. 3′-SL significantly reduced the increased mRNA and protein levels of iNOS and COX-2 (**Figures 2A–C**, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Moreover, the production of endogenous nitrite and PGE2 was upregulated when cells were treated with IL-1β (**Figure 2D**, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). However, after treatment with 3′-SL, the production of nitrite and PGE2 was significantly downregulated (**Figure 2D**, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Moreover, IL-1β treatment significantly increased IL-1β

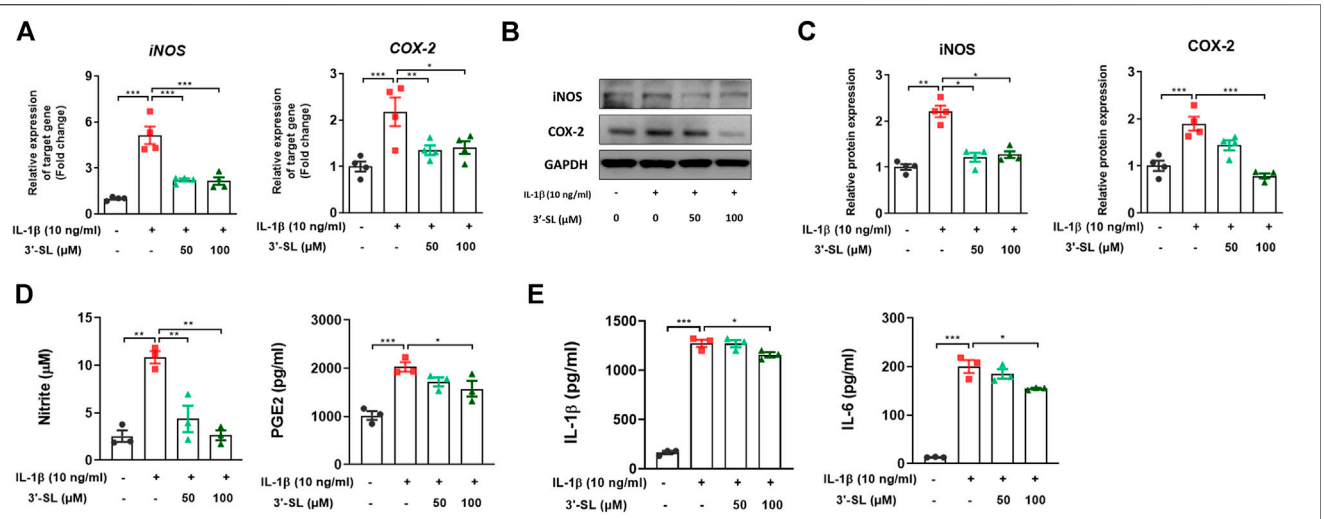


FIGURE 2 | Effect of 3′-SL on IL-1β-induced inflammatory responses in the chondrocytic cells. Chondrocytic cells were stimulated with or without IL-1β (10 ng/ml) for 24 h and then treated with different concentrations of 3′-SL (50 and 100 μM) for 24 h. **(A)** The mRNA expression levels of iNOS and COX-2 were assayed by qRT-PCR. **(B)** The protein expression levels of iNOS and COX-2 were determined by western blot and **(C)** quantification analysis. **(D)** The nitrite levels in the culture medium were assessed by the Griess reaction. The levels of PGE2 were determined using ELISA. **(E)** The levels of IL-1β and IL-6 were determined using ELISA. The values are the mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. 3′-SL, 3′-Sialyllactose; IL-1β; interleukin-1β; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2.

and IL-6 production (**Figure 2E**, $^{*}p < 0.05$, and $^{***}p < 0.001$). The increased production of these molecules was reversed by treatment with 3′-SL. Taken together, these results indicate that 3′-SL could exert anti-inflammatory properties by inhibiting the inflammatory response in chondrocytic cells.

3′-Sialyllactose Suppressed Interleukin-1β-Induced Apoptosis in the Chondrocytic Cells

To investigate whether 3′-SL could inhibit IL-1β-induced apoptosis, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and the expression of apoptosis-related proteins were examined in the chondrocytic cells. The percentage of TUNEL-positive cells was significantly upregulated in IL-1β-treated chondrocytic cells (**Figures 3A,B** and $^{***}p < 0.001$). The percentage of TUNEL-positive cells was significantly decreased after treatment with 3'-SL (**Figures 3A,B** and $^{***}p < 0.001$). Next, IL-1β significantly increased the expression of the pro-apoptotic protein Bax and decreased the expression of anti-apoptotic Bcl-2 (**Figures 3C,D**, $^{*}p < 0.05$, and $^{***}p < 0.001$). However, treatment with 3'-SL significantly reversed these alterations (**Figures 3C,D**, $^{**}p < 0.01$, and $^{***}p < 0.001$). Taken together, these results show that 3′-SL could reduce apoptosis in IL-1β-treated chondrocytic cells.

3′-Sialyllactose Suppressed Interleukin-1β-Induced Cartilage Matrix Degradation in the Chondrocytic Cells

To assess whether 3′-SL can prevent the production of MMPs, which are catabolic factors in OA pathogenesis, the expression of MMPs was examined in IL-1β-treated chondrocytic cells. 3′-SL significantly suppressed IL-1β-induced expression of MMP1, MMP3, and MMP13 (**Figure 4**, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Collagen II and aggrecan are the two major components of the matrix, and they are considered as ECM synthesis genes. To explore whether 3′-SL mitigates IL-1β-induced ECM degradation in the cell model of OA, the expression of ECM synthesis genes was examined. As shown in **Figure 4**, IL-1β decreased the expression levels of collagen II and aggrecan, while 3′-SL treatment significantly suppressed IL-1β-induced cartilage degradation ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Taken together, these results indicate that 3′-SL could inhibit cartilage matrix degradation by alleviating ECM degradation in IL-1β-induced chondrocytic cells.

3′-Sialyllactose Suppressed InterleukinL-1β-Induced Activation of Mitogen-Activated Protein Kinases and PI3K/AKT/NF-kB Signaling Pathways in the Chondrocytic Cells

Several studies have reported that the activation of MAPKs and phosphatidylinositol-3-kinase (PI3K)/AKT/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways plays an important role in OA progression (Hu et al., 2017; Zhang et al., 2019). Therefore, to explore whether 3′-SL can exert antioxidant protective effects, the two signaling cascades were examined. The MAPK signaling pathway was significantly activated after treatment with IL-1β in the chondrocytic cells (**Figure 5**, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). However, treatment with 3′-SL significantly suppressed the IL-1β-induced phosphorylation of P38, ERK, and JNK in chondrocytic cells (**Figure 5**, $^{*}p < 0.05$, $^{**}p < 0.01$,

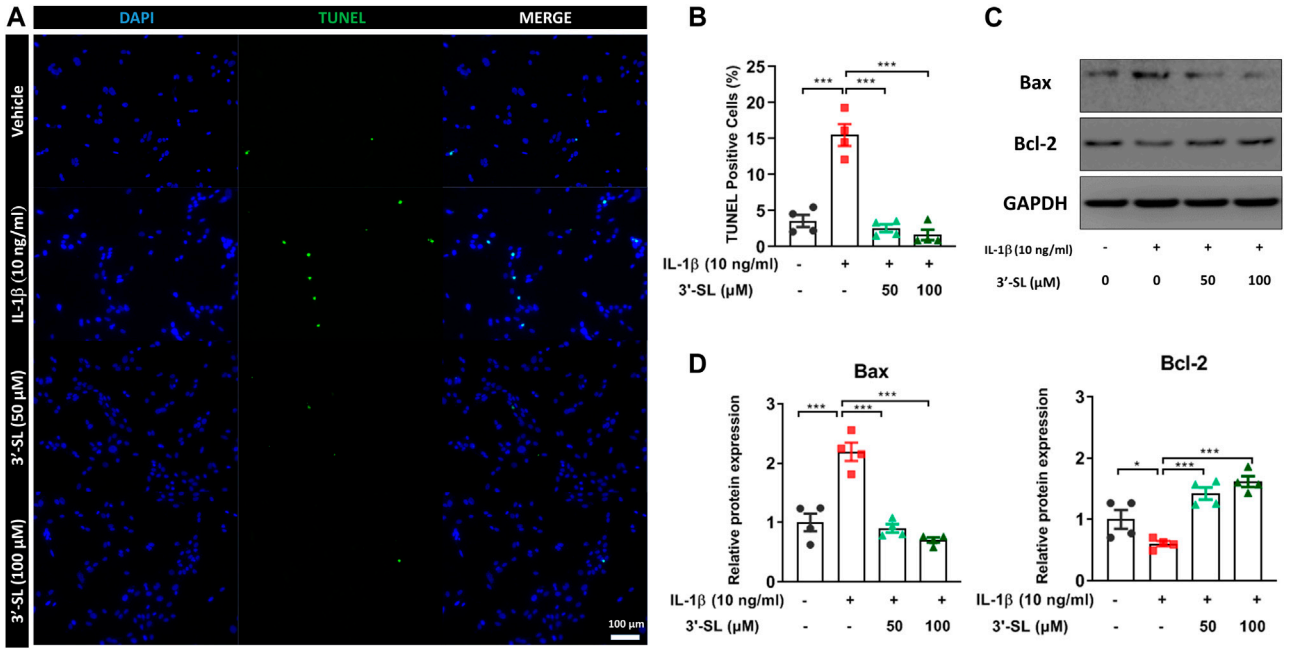


FIGURE 3 | Effect of 3′-SL on IL-1β-induced apoptosis in the chondrocytic cells. Chondrocytic cells were stimulated with or without IL-1β (10 ng/ml) for 24 h and then treated with different concentrations of 3′-SL (50 and 100 μM) for 24 h. **(A)** Cell death was evaluated by Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and representative images were shown. Scale bar: 100 μM. **(B)** The quantification of the number of TUNEL$^+$ cells. **(C)** The protein expression levels of Bax and Bcl-2 were determined by western blot and **(D)** quantification analysis. The values are the mean ± standard error of the mean. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. 3′-SL, 3′-Sialyllactose; IL-1β; interleukin-1β.

and $^{***}p < 0.001$). Moreover, 3′-SL significantly reversed the increased expression of PI3K, AKT, P65, and IκBα induced by IL-1β (**Figures 6A–D**, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). To further investigate the functional roles of 3′-SL, the cells were pretreated with Bay 11-7082, which significantly reduced the IL-1β-induced activation of NF-κB cascades (**Figures 6E,F**, $^{**}p < 0.01$, and $^{***}p < 0.001$). Taken together, these findings demonstrate the suppressive effects of 3′-SL on the activated MAPK and PI3K/AKT/NF-κB signaling in IL-1β-treated chondrocytic cells, which was presumably attributed to the suppression of oxidative stress and inflammation.

DISCUSSION

In the present study, we evaluated the underlying mechanisms of the therapeutic effects of 3′-SL using IL-1β-treated chondrocytic cells. Our data revealed that 3′-SL efficiently protects the cells from oxidative stress, inflammation, apoptosis, and cartilage matrix degradation by suppressing the activated MAPK and PI3K/AKT/NF-κB signaling pathways (**Figure 7**). These results provide novel insights into the therapeutic mechanisms of action of 3′-SL as a treatment for OA.

Oxidative stress is closely associated with cartilage destruction and OA progression (Sokolove and Lepus, 2013; Hu et al., 2017). The elevated production of ROS can make chondrocytes more vulnerable to oxidant-mediated cell death and lead to defective antioxidant mechanisms (Del Carlo and Loeser, 2003). Antioxidants, including SOD, catalase, and thioredoxin, are dysregulated and insufficient to detoxify ROS in OA patients (Altindag et al., 2007; Erturk et al., 2012; Gu et al., 2019; Zhong et al., 2019). Therefore, increasing the levels of these antioxidants can be used as a promising approach to prevent OA. Herein, our data revealed that 3′-SL significantly attenuated ROS production and changed intracellular redox status in the cell model of OA, as evidenced by decreased intracellular ROS production and increased oxidative stress defense system. These results demonstrated that 3′-SL can attenuate oxidative stress in IL-1β-treated chondrocytic cells.

Oxidative stress induces synovial inflammation, chondrocyte apoptosis, cartilage matrix synthesis, and intracellular signaling in OA progression (Lepetsos and Papavassiliou, 2016). Since a previous study showed that NLR family pyrin domain containing 3 (NLRP3) inflammasome activation in humans and mice is not involved in stressed-induced OA (Bougault et al., 2012), this study mainly focused on oxidative stress-related inflammation. It has been reported that nitric oxide catalyzed by iNOS and PGE2, produced from COX-2, could expedite the development of OA through ECM degradation (Sasaki et al., 1998). Moreover, IL-1β could induce an increase in iNOS and COX-2 (Daheshia and Yao, 2008; Sheu et al., 2015). Herein, the expression of iNOS, COX-2, NO, and PGE2 was usually enhanced in the cell model of OA. We found that the additional treatment with 3′-SL significantly downregulated the levels of iNOS, COX-2, NO, and PGE2. The synovial fluid and serum levels of IL-1β and IL-6 in OA patients are higher than in healthy normal subjects (Wojdasiewicz et al., 2014). In addition, it was observed that 3′-SL significantly suppressed the expression of IL-1 and IL-6 in the cell model of OA. These results demonstrated that 3′-SL can attenuate oxidative stress-derived inflammatory responses in IL-1β-treated chondrocytic cells.

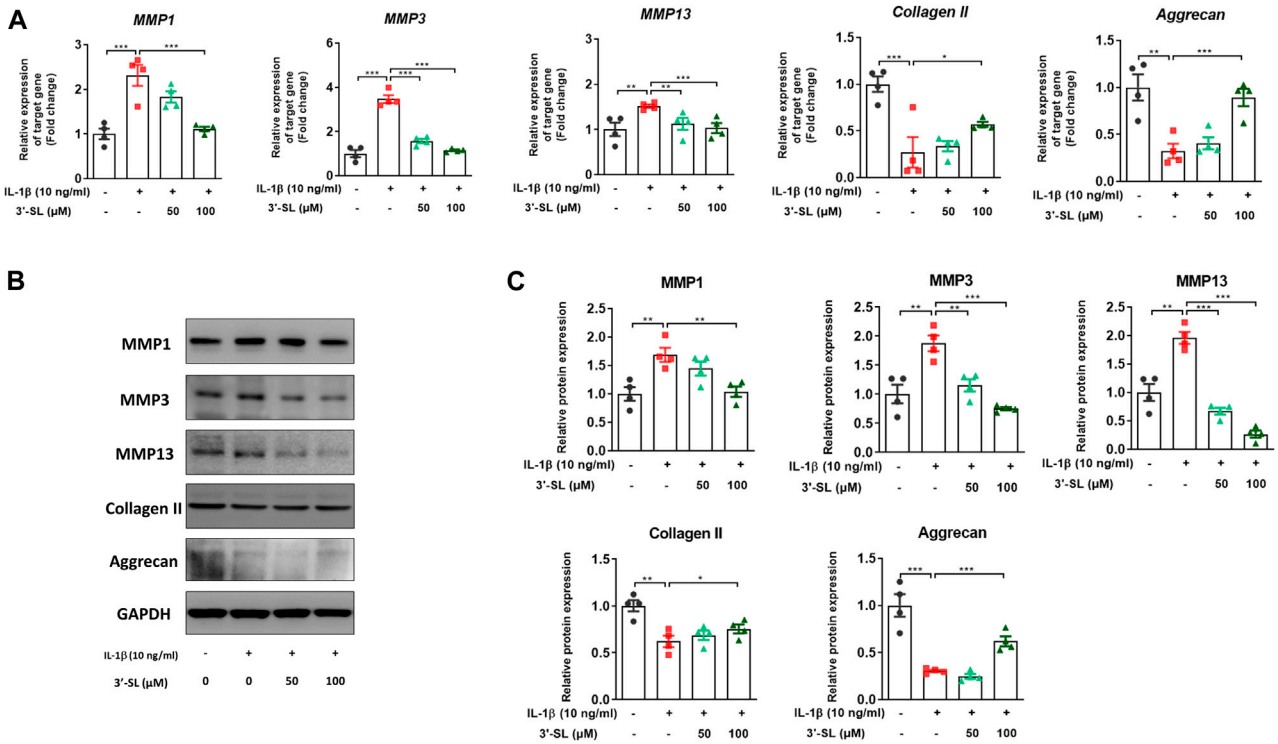


FIGURE 4 | Effect of 3′-SL on IL-1β-induced cartilage destruction in the chondrocytic cells. Chondrocytic cells were stimulated with or without IL-1β (10 ng/ml) for 24 h and then treated with different concentrations of 3′-SL (50 and 100 μM) for 24 h. **(A)** The mRNA expression levels of MMP-1, MMP-3, MMP-13, collagen II, and aggrecan were assayed by qRT-PCR. **(B)** The protein expression levels of MMP-1, MMP-3, MMP-13, collagen II, and aggrecan were determined by western blot and **(C)** quantification analysis. The values are the mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. 3′-SL, 3′-Sialyllactose; IL-1β; interleukin-1β; MMP, matrix metalloprotein.

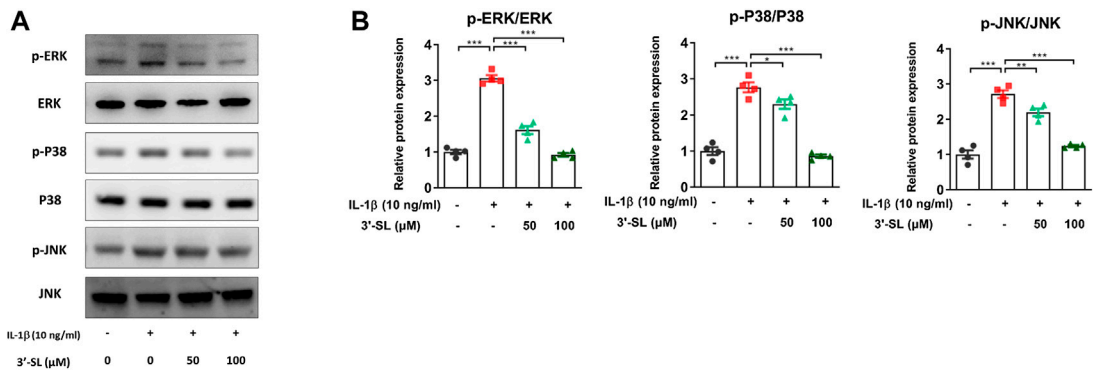


FIGURE 5 | Effect of 3′-SL on IL-1β-induced MAPK signaling pathway in the chondrocytic cells. Chondrocytic cells were stimulated with or without IL-1β (10 ng/ml) for 24 h and then treated with the different concentrations of 3′-SL (50 and 100 μM) for 24 h. **(A)** The protein expression levels of p-ERK, ERK, p-P38, P38, p-JNK, and JNK were determined by western blot and **(B)** quantification analysis. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. 3′-SL, 3′-Sialyllactose; IL-1β; interleukin-1β; MAPK, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase.

Oxidative stress can cause mitochondrial apoptosis through the increased expression of the pro-apoptotic protein Bax and the decreased expression of the anti-apoptotic protein Bcl-2 (Pena-Blanco and Garcia-Saez, 2018). Our data revealed that 3′-SL significantly attenuated these changes in the expression of apoptotic proteins in IL-1β-treated chondrocytic cells. Likewise, the number of TUNEL-positive cells in the chondrocytic cells was significantly increased after IL-1β treatment. However, 3′-SL significantly decreased the number of TUNEL-positive cells. These results demonstrated that 3′-SL could reduce oxidative stress-derived apoptosis in IL-1β-treated chondrocytic cells.

Furthermore, the release of excess cartilage matrix degrading enzymes, such as MMP1, MMP3, and MMP13, is implicated in OA

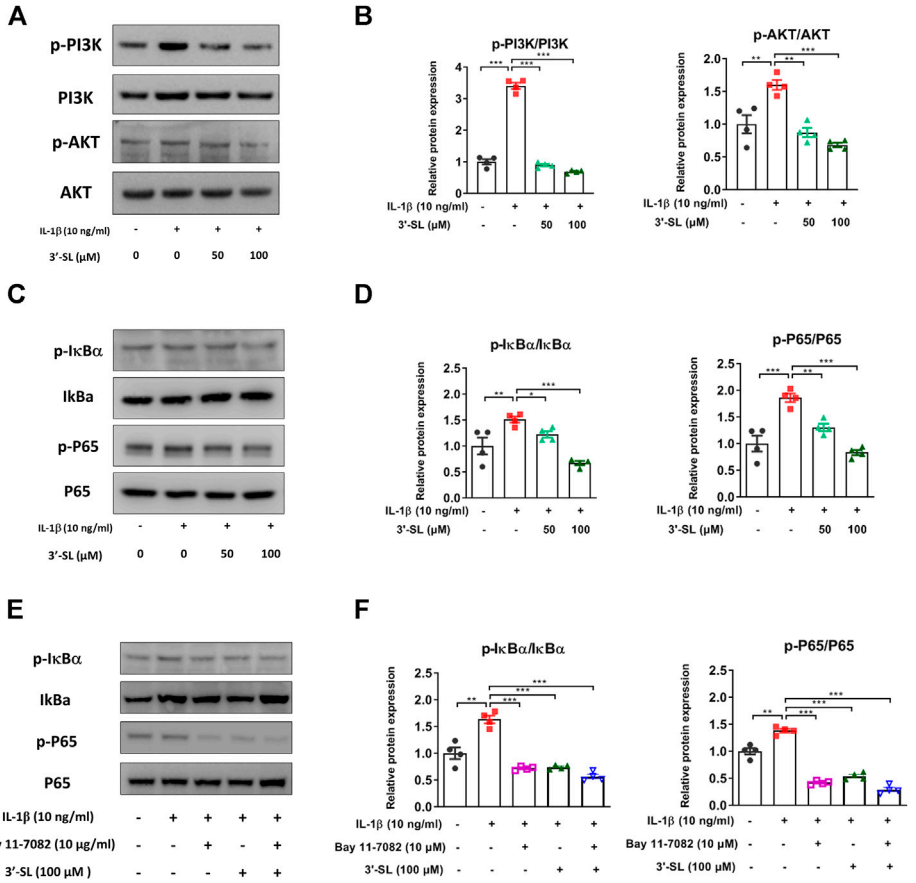


FIGURE 6 | Effect of 3′-SL on IL-1β-induced PI3K/AKT/NF-κB signaling pathway in the cell model of OA. Chondrocytic cells were stimulated with or without IL-1β (10 ng/ml) for 24 h and then treated with the different concentrations of 3′-SL (50 and 100 μM) for 24 h. **(A)** The protein expression levels of p-PI3K, PI3K, p-AKT, and AKT were determined by western blot and **(B)** quantification analysis. **(C)** The protein expression levels of p-IκBα, IκBα, p-P65, and P65 were determined by western blot and **(D)** quantification analysis. **(E)** Chondrocytic cells were pretreated with Bay 11–7,082 (10 μM) for 2 h, stimulated by IL-1β (10 ng/ml) for 24 h, and then treated with the different concentrations of 3′-SL (50 and 100 μM) for 24 h. The protein expression levels of p-IκBα, IκBα, p-P65, and P65 were determined by western blot and **(F)** quantification analysis. The values are mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. PI3K, Phosphatidylinositol 3-kinase; AKT, protein kinase B; NF-κB, nuclear factor kappa light chain enhancer of activated B cells.

progression (Ahmed et al., 2005; Wang et al., 2011; Maldonado and Nam, 2013). MMPs are a family of 23 enzymes with a specific function of inhibiting the synthesis of collagen II and aggrecan, which are critical for the synthesis of matrix-related proteins in cartilage (Dahlberg et al., 2000; Yamamoto et al., 2016). Proteolysis and pathological cartilage breakdown in OA are followed by abnormal expression of MMP members (Murphy et al., 2002). Among these MMP members, MMP1, MMP3, and MMP13 are responsible for the degradation of ECM in OA articular cartilage (Yoshihara et al., 2000). In this study, IL-1β increased the expression of MMP1, MMP3, and MMP13, while the expression of matrix related proteins, such as collagen II and aggrecan, was significantly downregulated in IL-1β-treated chondrocytic cells. However, all these changes could be restored by treatment with 3′-SL. These results demonstrate that 3′-SL could attenuate oxidative stress-derived cartilage matrix degradation in IL-1β-treated chondrocytic cells.

MAPK and PI3K/AKT/NF-κB signaling pathways are key mediators of cartilage degradation and OA progression (Sun et al., 2017; Zhang et al., 2019). MAPK signaling, which consists of ERK 1/2, P38, and JNK, can transduce extracellular stimuli into the nucleus (Keshet and Seger, 2010; Sugiura et al., 2011). In addition, MAPK activation is involved in the disruption of ECM (Sondergaard et al., 2010). Subsequently, the PI3K/AKT/NF-κB signaling cascade induces increased expression of catabolic factors and can contribute to cartilage degradation (Rigoglou and Papavassiliou, 2013; Jenei-Lanzl et al., 2019). Our data showed that the phosphorylation levels of P38, ERK, JNK, PI3K, and AKT, P65, and IκBα were significantly increased in the cell model of OA. However, these activated pathways were reversed by treatment with 3′-SL. The upstream regulators of NF-κB involve the MAPK and PI3K/AKT signaling pathways (Schulze-Osthoff et al., 1997; Yum et al., 2001). Thus, we evaluated the functional kinase activities using BAY 11–7082 in IL-1β-treated chondrocytic cells. In our study, the phosphorylation of IκBα and P65 was attenuated by pre-treatment with Bay 11–7082 in IL-1β-induced chondrocytic cells. Furthermore, the inhibition of NF-kB activation by 3′-

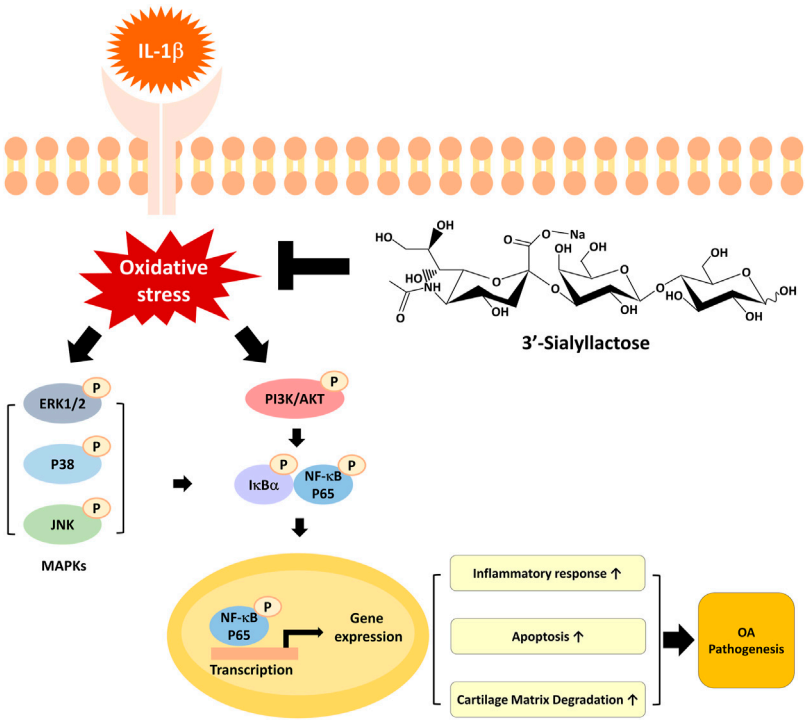


FIGURE 7 | A graphical representation of the mechanism for 3′-SL in chondrocytic cells stimulated with IL-1β. 3′-SL can attenuate oxidative stress and inflammation, which induces apoptosis and cartilage degradation via the suppression of activated MAPK and PI3K/AKT/NF-κB signaling pathways in chondrocytic cells stimulated with IL-1β.

SL and Bay 11–7082 was observed in IL-1β-induced chondrocytic cells. These observations highlight the importance and need to further investigate the detailed mechanisms for the activity of other kinases. Taken together, these results reveal that the regulation of MAPK and PI3K/AKT/NF-κB signaling pathways plays a vital role in preserving the structural integrity of the matrix in the cell model of OA.

It should be note that although the chondrosarcoma cell line SW1353 is widely used as a substitute for primary adult articular chondrocytes, our results cannot be totally translated to primary OA chondrocytes considering the difference in gene expression between SW1353 and primary OA chondrocytes after treatment with IL-1β (Gebauer et al., 2005). This was the limitation of this study.

Sialyllactose is a representative human milk oligosaccharide in human breast milk. It can regulate immune homeostasis through receptor-mediated endocytosis and phagocytosis (Kim et al., 2019). Considering this and the multiple inhibitory effects of 3′-SL on IL-1β-mediated effects observed in this study, 3′-SL may mediate the receptor-mediated mechanism and could be used as a therapeutic agent for OA treatment. Further prospective studies are warranted to determine the accurate target of 3′-SL on IL-1β-induced oxidative stress and inflammation.

In conclusion, results from this study demonstrated that 3′-SL can counteract oxidative stress and inflammation via the suppression of activated MAPK and PI3K/AKT/NF-κB signaling pathways in IL-1β treated chondrocytic cells. Based on these findings, 3′-SL may be potentially used to protect against OA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Conceptualization, AB; Data curation, AB and SJ; Formal analysis, AB and SJ; Funding acquisition, SK; Investigation, AB; Methodology, AB, LK, and EL.; Project administration, AB, SK, and S-RC; Supervision, SK and S-RC; Writing—original draft, AB and SJ; Writing—review and editing, SP, SK, and SJ. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was supported by the Ministry of Trade, Industry and Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for The Industries of Economic Cooperation Region (P0004751).

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.com) for English language editing.

REFERENCES

Ahmed, S., Wang, N., Hafeez, B. B., Cheruvu, V. K., and Haqqi, T. M. (2005). Punica granatum L. Extract inhibits IL-1β-induced expression of matrix metalloproteinases by inhibiting the activation of MAP kinases and NF-κB in human chondrocytes *in vitro*. *J. Nutr.* 135, 2096–2102. doi:10.1093/jn/135.9.2096

Altay, M. A., Ertürk, C., Bilge, A., Yaptı, M., Levent, A., and Aksoy, N. (2015). Evaluation of prolidase activity and oxidative status in patients with knee osteoarthritis: relationships with radiographic severity and clinical parameters. *Rheumatol. Int.* 35, 1725–1731. doi:10.1007/s00296-015-3290-5

Altindag, O., Erel, O., Aksoy, N., Selek, S., Celik, H., and Karaoglanoglu, M. (2007). Increased oxidative stress and its relation with collagen metabolism in knee osteoarthritis. *Rheumatol. Int.* 27, 339–344. doi:10.1007/s00296-006-0247-8

Ansari, M. Y., Ahmad, N., and Haqqi, T. M. (2020). Oxidative stress and inflammation in osteoarthritis pathogenesis: role of polyphenols. *Biomed. Pharmacother.* 129, 110452. doi:10.1016/j.biopha.2020.110452

Appleton, C. T. (2018). Osteoarthritis year in review 2017: biology. *Osteoarthr. Cartil.* 26, 296–303. doi:10.1016/j.joca.2017.10.008

Baek, A., Kim, M., Kim, S. H., Cho, S.-R., and Kim, H. J. (2018a). Anti-inflammatory effect of DNA polymeric molecules in a cell model of osteoarthritis. *J. Inflamm.* 41, 677–688. doi:10.1007/s10753-017-0722-2

Baek, A., Kim, Y., Lee, J. W., Lee, S. C., and Cho, S. R. (2018b). Effect of polydeoxyribonucleotide on angiogenesis and wound healing in an in vitro model of osteoarthritis. *Cell Transpl.* 27, 1623–1633. doi:10.1177/0963689718804130

Bao, G., Xu, L., Xu, X., Zhai, L., Duan, C., and Xu, D. (2016). SGTB promotes the caspase-dependent apoptosis in chondrocytes of osteoarthritis. *J. Inflamm.* 39, 601–610. doi:10.1007/s10753-015-0285-z

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism* 49, 3–8. doi:10.1016/s0026-0495(00)80077-3

Bolduc, J. A., Collins, J. A., and Loeser, R. F. (2019). Reactive oxygen species, aging and articular cartilage homeostasis. *Free Radic. Biol. Med.* 132, 73–82. doi:10.1016/j.freeradbiomed.2018.08.038

Bougault, C., Gosset, M., Houard, X., Salvat, C., Godmann, L., and Pap, T. (2012). Stress-induced cartilage degradation does not depend on the NLRP3 inflammasome in human osteoarthritis and mouse models. *Arthritis Rheum.* 64, 3972–3981. doi:10.1002/art.34678

Collins, J. A., Diekman, B. O., and Loeser, R. F. (2018). Targeting aging for disease modification in osteoarthritis. *Curr. Opin. Rheumatol.* 30, 101–107. doi:10.1097/bor.0000000000000456

Daheshia, M., and Yao, J. Q. (2008). The interleukin 1β pathway in the pathogenesis of osteoarthritis. *J. Rheumatol.* 35, 2306–2312. doi:10.3899/jrheum.080346

Dahlberg, L., Billinghurst, R. C., Manner, P., Nelson, F., Webb, G., and Ionescu, M. (2000). Selective enhancement of collagenase-mediated cleavage of resident type II collagen in cultured osteoarthritic cartilage and arrest with a synthetic inhibitor that spares collagenase 1 (matrix metalloproteinase 1). *Arthritis. Rheum.* 43, 673–682. doi:10.1002/1529-0131(200003)43:3<673::aid-anr25>3.0.co;2-8

Del Carlo, M., and Loeser, R. F. (2003). Increased oxidative stress with aging reduces chondrocyte survival: correlation with intracellular glutathione levels. *Arthritis. Rheum.* 48, 3419–3430. doi:10.1002/art.11338

Donovan, S. M., and Comstock, S. S. (2016). Human milk oligosaccharides influence neonatal mucosal and systemic immunity. *Ann. Nutr. Metab.* 69 (Suppl. 2), 42–51. doi:10.1159/000452818

Erturk, C., Altay, M. A., Selek, Ş., and Koçyiğit, A. (2012). Paraoxonase-1 activity and oxidative status in patients with knee osteoarthritis and their relationship with radiological and clinical parameters. *Scand. J. Clin. Lab. Invest.* 72, 433–439. doi:10.3109/00365513.2012.687116

Gebauer, M., Saas, J., Sohler, F., Haag, J., Söder, S., and Pieper, M. (2005). Comparison of the chondrosarcoma cell line SW1353 with primary human adult articular chondrocytes with regard to their gene expression profile and reactivity to IL-1β. *Osteoarthr. Cartil.* 13, 697–708. doi:10.1016/j.joca.2005.04.004

Gila-Diaz, A., Arribas, S. M., Algara, A., Martin-Cabrejas, M. A., Lopez De Pablo, A. L., and Saenz De Pipaon, M. (2019). A review of bioactive factors in human breastmilk: a focus on prematurity. *Nutrients* 11, 1307. doi:10.3390/nu11061307

Gu, Y. Q., Ren, K. W., Jiang, C. Z., Wang, L. M., and Yao, Q. Q. (2019). Regulation of cartilage damage caused by lack of Klotho with thioredoxin/peroxiredoxin (Trx/Prx) system and succedent NLRP3 activation in osteoarthritis mice. *Am. J. Translational Res.* 11, 7338–7350. doi:10.18632/aging.102603

Hu, J., Yan, Q., Shi, C., Tian, Y., Cao, P., and Yuan, W. (2017). BMSC paracrine activity attenuates interleukin-1β-induced inflammation and apoptosis in rat AF cells via inhibiting relative NF-κB signaling and the mitochondrial pathway. *Am. J. Transl Res.* 9, 79–89.

Huang, X. J., Xi, Y., Mao, Z. K., Chu, X. Y., Zhang, R., and Ma, X. H. (2019). Vanillic acid attenuates cartilage degeneration by regulating the MAPK and PI3K/AKT/NF-kappa B pathways. *Eur. J. Pharmacol.* 859, 172481. doi:10.1016/j.ejphar.2019.172481

Jenei-Lanzl, Z., Meurer, A., and Zaucke, F. (2019). Interleukin-1β signaling in osteoarthritis–chondrocytes in focus. *Cell Signal.* 53, 212–223. doi:10.1016/j.cellsig.2018.10.005

Jeon, J., Kang, L. J., Lee, K. M., Cho, C., Song, E. K., and Kim, W. (2018). 3′-Sialyllactose protects against osteoarthritic development by facilitating cartilage homeostasis. *J. Cel. Mol. Med.* 22, 57–66. doi:10.1111/jcmm.13292

Jeon, O. H., Kim, C., Laberge, R.-M., Demaria, M., Rathod, S., and Vasserot, A. P. (2017). Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. *Nat. Med.* 23, 775–781. doi:10.1038/nm.4324

Jia, P., Chen, G., Zhou, G., Zhong, Y., and Li, R. (2013). Fuyuan Decoction inhibits nitric oxide production via inactivation of nuclear factor-κB in SW1353 chondrosarcoma cells. *J. Ethnopharmacol* 146, 853–858. doi:10.1016/j.jep.2013.02.014

Jones, D. P. (2008). Radical-free biology of oxidative stress. *Am. J. Physiology Cell Physiol.* 295, C849–C868. doi:10.1152/ajpcell.00283.2008

Kang, L. J., Kwon, E. S., Lee, K. M., Cho, C., Lee, J. I., and Ryu, Y. B. (2018). 3′-Sialyllactose as an inhibitor of p65 phosphorylation ameliorates the progression of experimental rheumatoid arthritis. *Br. J. Pharmacol.* 175, 4295–4309. doi:10.1111/bph.14486

Kapoor, M., Martel-Pelletier, J., Lajeunesse, D., Pelletier, J. P., and Fahmi, H. (2011). Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat. Rev. Rheumatol.* 7, 33–42. doi:10.1038/nrrheum.2010.196

Keshet, Y., and Seger, R. (2010). The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Mol. Biol.* 661, 3–38. doi:10.1007/978-1-60761-795-2_1

Kim, J., Kim, Y. J., and Kim, J. W. (2019). Bacterial clearance is enhanced by alpha 2,3-and alpha 2,6-sialyllactose via receptor-mediated endocytosis and phagocytosis. *Infect. Immun.* 87, e00694-18. doi:10.1128/IAI.00694-18

Lepetsos, P., and Papavassiliou, A. G. (2016). ROS/oxidative stress signaling in osteoarthritis. *Biochim. Biophys. Acta* 1862, 576–591. doi:10.1016/j.bbadis.2016.01.003

Loeser, R. F. (2009). Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. *Osteoarthritis Cartilage* 17, 971–979. doi:10.1016/j.joca.2009.03.002

Lu, C., Li, Y. Q., Hu, S. Y., Cai, Y. Z., Yang, Z., and Peng, K. (2018). Scoparone prevents IL-1β-induced inflammatory response in human osteoarthritis chondrocytes through the PI3K/Akt/NF-κB pathway. *Biomed. Pharmacother.* 106, 1169–1174. doi:10.1016/j.biopha.2018.07.062

Luo, J., Nordvang, R. T., Morthensen, S. T., Zeuner, B., Meyer, A. S., and Mikkelsen, J. D. (2014). An integrated membrane system for the biocatalytic production of 3′-sialyllactose from dairy by-products. *Bioresour. Technol.* 166, 9–16. doi:10.1016/j.biortech.2014.05.003

Maldonado, M., and Nam, J. (2013). The role of changes in extracellular matrix of cartilage in the presence of inflammation on the pathology of osteoarthritis. *Biomed. Res. Int.* 2013, 284873. doi:10.1155/2013/284873

Murphy, G., Knäuper, V., Atkinson, S., Butler, G., English, W., and Hutton, M. (2002). Matrix metalloproteinases in arthritic disease. *Arthritis Res.* 4 (Suppl. 3), S39–S49. doi:10.1186/ar572

Pena-Blanco, A., and Garcia-Saez, A. J. (2018). Bax, Bak and beyond - mitochondrial performance in apoptosis. *FEBS J.* 285, 416–431. doi:10.1111/febs.14186

Rigoglou, S., and Papavassiliou, A. G. (2013). The NF-κB signalling pathway in osteoarthritis. *Int. J. Biochem. Cell Biol* 45, 2580–2584. doi:10.1016/j.biocel.2013.08.018

Sanchez, C., Deberg, M. A., Piccardi, N., Msika, P., Reginster, J.-Y. L., and Henrotin, Y. E. (2005). Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes. *Osteoarthr. Cartil.* 13, 988–997. doi:10.1016/j.joca.2005.07.012

Sanchez, C., Deberg, M. A., Bellahcène, A., Castronovo, V., Msika, P., and Delcour, J. P. (2008). Phenotypic characterization of osteoblasts from the sclerotic zones of osteoarthritic subchondral bone. *Arthritis Rheum.* 58, 442–455. doi:10.1002/art.23159

Sasaki, K., Hattori, T., Fujisawa, T., Takahashi, K., Inoue, H., and Takigawa, M. (1998). Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. *J. Biochem.* 123, 431–439. doi:10.1093/oxfordjournals.jbchem.a021955

Schulze-Osthoff, K., Ferrari, D., Riehemann, K., and Wesselborg, S. (1997). Regulation of NF-κB activation by MAP kinase cascades. *Immunobiology* 198, 35–49. doi:10.1016/s0171-2985(97)80025-3

Sheu, S. Y., Ho, S. R., Sun, J. S., Chen, C. Y., and Ke, C. J. (2015). Arthropod steroid hormone (20-Hydroxyecdysone) suppresses IL-1beta-induced catabolic gene expression in cartilage. *BMC Complement. Altern. Med.* 15, 1. doi:10.1186/s12906-015-0520-z

Sokolove, J., and Lepus, C. M. (2013). Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Ther. Adv. Musculoskelet. Dis.* 5, 77–94. doi:10.1177/1759720x12467868

Sondergaard, B. C., Schultz, N., Madsen, S. H., Bay-Jensen, A. C., Kassem, M., and Karsdal, M. A. (2010). MAPKs are essential upstream signaling pathways in proteolytic cartilage degradation - divergence in pathways leading to aggrecanase and MMP-mediated articular cartilage degradation. *Osteoarthr. Cartil.* 18, 279–288. doi:10.1016/j.joca.2009.11.005

Sugiura, R., Satoh, R., Ishiwata, S., Umeda, N., and Kita, A. (2011). Role of RNA-binding proteins in MAPK signal transduction pathway. *J. Signal. Transduct* 2011, 109746. doi:10.1155/2011/109746

Sun, H. Y., Hu, K. Z., and Yin, Z. S. (2017). Inhibition of the p38-MAPK signaling pathway suppresses the apoptosis and expression of proinflammatory cytokines in human osteoarthritis chondrocytes. *Cytokine* 90, 135–143. doi:10.1016/j.cyto.2016.11.002

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J. Physiol.* 552, 335–344. doi:10.1113/jphysiol.2003.049478

Wang, M., Shen, J., Jin, H., Im, H. J., Sandy, J., and Chen, D. (2011). Recent progress in understanding molecular mechanisms of cartilage degeneration during osteoarthritis. *Ann. N. Y Acad. Sci.* 1240, 61–69. doi:10.1111/j.1749-6632.2011.06258.x

Wojdasiewicz, P., Poniatowski, L. A., and Szukiewicz, D. (2014). The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm.* 2014, 561459. doi:10.1155/2014/561459

Xie, J., Lin, J., Wei, M., Teng, Y., He, Q., Yang, G., et al. (2019). Sustained Akt signaling in articular chondrocytes causes osteoarthritis via oxidative stress-induced senescence in mice. *Bone Res.* 7, 23. doi:10.1038/s41413-019-0062-y

Yamamoto, K., Okano, H., Miyagawa, W., Visse, R., Shitomi, Y., and Santamaria, S. (2016). MMP-13 is constitutively produced in human chondrocytes and co-endocytosed with ADAMTS-5 and TIMP-3 by the endocytic receptor LRP1. *Matrix Biol.* 56, 57–73. doi:10.1016/j.matbio.2016.03.007

Yoshihara, Y., Nakamura, H., Obata, K., Yamada, H., Hayakawa, T., and Fujikawa, K. (2000). Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Ann. Rheum. Dis.* 59, 455–461. doi:10.1136/ard.59.6.455

Yum, H. K., Arcaroli, J., Kupfner, J., Shenkar, R., Penninger, J. M., and Sasaki, T. (2001). Involvement of phosphoinositide 3-kinases in neutrophil activation and the development of acute lung injury. *J. Immunol.* 167, 6601–6608. doi:10.4049/jimmunol.167.11.6601

Zenhom, M., Hyder, A., De Vrese, M., Heller, K. J., Roeder, T., and Schrezenmeir, J. (2011). Prebiotic oligosaccharides reduce proinflammatory cytokines in intestinal caco-2 cells via activation of PPARγ and peptidoglycan recognition protein 3. *J. Nutr.* 141, 971–977. doi:10.3945/jn.110.136176

Zhang, H., Ji, L., Yang, Y., Wei, Y. L., Zhang, X. N., and Gang, Y. (2019). The therapeutic effects of treadmill exercise on osteoarthritis in rats by inhibiting the HDAC3/NF-KappaB pathway *in vivo* and *in vitro*. *Front. Physiol.* 10, 1060. doi:10.3389/fphys.2019.01060

Zhong, G., Yang, X. Y., Jiang, X. F., Kumar, A., Long, H. P., and Xie, J. (2019). Correction: dopamine-melanin nanoparticles scavenge reactive oxygen and nitrogen species and activate autophagy for osteoarthritis therapy. *Nanoscale* 11, 23504–23505. doi:10.1039/c9nr90272d

Conflict of Interest: Author LK was employed by the company GeneChem Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 13 April 2021
doi: 10.3389/fphar.2021.637904

New Trends in Injection-Based Therapy for Thumb-Base Osteoarthritis: Where Are We and where Are We Going?

Sara Tenti[1], *Sara Cheleschi*[1]*, *Nicola Mondanelli*[2], *Stefano Giannotti*[2] and *Antonella Fioravanti*[1]

[1]Department of Medicine, Surgery and Neuroscience, Rheumatology Unit, Clinic for the Diagnosis and Management of Hand Osteoarthritis, Azienda Ospedaliera Universitaria Senese, Siena, Italy, [2]Department of Medicine, Surgery and Neuroscience, Orthopedics and Traumatology Unit, University of Siena, Siena, Italy

Thumb-base osteoarthritis (TBOA) is a common condition, mostly affecting post-menopausal women, often inducing a significant impact on quality of life and hand functionality. Despite its high prevalence and disability, the therapeutic options in TBOA are still limited and few have been investigated. Among the pharmacological strategies for TBOA management, it would be worthwhile to mention the injection-based therapy. Unfortunately, its efficacy is still the subject of debate. Indeed, the 2018 update of the European League Against Rheumatism (EULAR) recommendations for the management of hand osteoarthritis (OA) stated that intra-articular (IA) injections of glucocorticoids should not generally be used, but may be considered in patients with painful interphalangeal joints, without any specific mention to the TBOA localization and to other widely used injections agents, such as hyaluronic acid (HA) and platelet-rich plasma (PRP). Even American College of Rheumatology (ACR) experts conditionally recommended against IA HA injections in patients with TBOA, while they conditionally encouraged IA glucocorticoids. However, the recommendations from international scientific societies don't often reflect the clinical practice of physicians who routinely take care of TBOA patients; indeed, corticosteroid injections are a mainstay of therapy in OA, especially for patients with pain refractory to oral treatments and HA is considered as a safe and effective treatment. The discrepancy with the literature data is due to the great heterogeneity of the clinical trials published in this field: indeed, the studies differ for methodology and protocol design, outcome measures, treatment (different formulations of HA, steroids, PRP, and schedules) and times of follow-up. For these reasons, the current review will provide deep insight into the injection-based therapy for TBOA, with particular attention to the different employed agents, the variety of the schedule treatments, the most common injection techniques, and the obtained results in terms of efficacy and safety. In depth, we will discuss the available literature on corticosteroids and HA injections for TBOA and the emerging role of PRP and other injection agents for this condition. We will consider in our analysis not only randomized controlled trials (RCTs) but also recent pilot or retrospective studies trying to step forward to identify satisfactory management strategies for TBOA.

OPEN ACCESS

Edited by:
Annalisa Bruno,
University of Studies G. d'Annunzio Chieti and Pescara, Italy

Reviewed by:
Piotr Bełdowski,
University of Technology and Life Sciences in Bydgoszcz, Poland
Seyed M. Rayegani,
Shahid Beheshti University, Iran

***Correspondence:**
Sara Cheleschi
saracheleschi@hotmail.com

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *04 December 2020*
Accepted: *23 February 2021*
Published: *13 April 2021*

Citation:
Tenti S, Cheleschi S, Mondanelli N, Giannotti S and Fioravanti A (2021) New Trends in Injection-Based Therapy for Thumb-Base Osteoarthritis: Where Are We and where Are We Going?. Front. Pharmacol. 12:637904. doi: 10.3389/fphar.2021.637904

Keywords: thumb-base osteoarthritis, trapezio-metacarpal osteoarthritis, first carpo-metacarpal osteoarthritis, rizoartrhosis, intra-articular injection, hyaluronic acid, corticosteroids, platelet-rich plasma

INTRODUCTION

Thumb-base osteoarthritis (TBOA) is a highly prevalent condition affecting middle-aged and older people; the condition increases with age, is more common in women—particularly post-menopausal—and it is often bilateral (Dahaghin et al., 2005; Haugen et al., 2011; Kloppenburg et al., 2017).

The prevalence of symptomatic TBOA among people aged >50 years was estimated from 5 to 7%, while the prevalence of radiographic TBOA is higher, ranging from 45 to 60% (Sodha et al., 2005; Sonne-Holm and Jacobsen, 2006).

The main symptoms of TBOA are pain, localized to the base of the thumb, stiffness, tenderness and loss of range of motion. The impairment function reduces the ability to perform activities of daily living, such as writing, opening a jar, turning a car key, and turning a door or handling small objects. In the more advanced stages, thenar muscle wasting combined with subluxation and adduction of the thumb metacarpal can induce a characteristic "squaring" joint deformity. Furthermore, patients with concomitant osteoarthritis (OA) of the interphalangeal (IP) joints and TBOA complain of more pain, functional disability, and reduced quality of life (Bijsterbosch et al., 2010; Tenti et al., 2020).

Despite its high prevalence and disability, the therapeutic options for TBOA are still limited and few investigated; its management usually requires a combination of non-pharmacological, pharmacological, and surgical strategies with a multidisciplinary approach (Kloppenburg et al., 2017).

Among the pharmacological strategies, it would be worthwhile to mention the use of intra-articular (IA) injection-based therapy with corticosteroid or hyaluronic acid (HA). Unfortunately, its efficacy is still the subject of debate and not universally shared by the current guidelines for the management of hand OA.

The 2007 European League Against Rheumatism (EULAR) recommendations for hand OA support the use of IA long-acting corticosteroids for painful flares of OA, especially for TBOA (Zhang et al., 2007).

Conversely, the 2018 update of EULAR recommendations state that IA injections of steroids should not generally be used, but may be considered in patients with painful IP joints, without any specific mention to the TBOA localization and to other widely used IA agents, as HA and platelet-rich plasma (PRP) (Kloppenburg et al., 2019). Even American College of Rheumatology (ACR) experts conditionally recommend against IA HA injections in patients with TBOA, while they conditionally encourage IA glucocorticoids (Kolasinski et al., 2020).

However, the recommendations from international scientific societies do not often reflect the clinical practice of all physicians who routinely take care of TBOA patients; indeed, corticosteroid injections are a mainstay of therapy in OA, especially for patients with pain refractory to oral treatments, and HA is considered as a safe and effective therapeutic option.

Considering the high prevalence of a disabling disease, such as TBOA, we aimed to perform a narrative review analyzing the current evidence on the efficacy and safety of the intra-articular therapy. For this purpose, we grouped the literature evidence for different used IA drugs (corticosteroids, hyaluronate, PRP, or other medications), adding a discussion to find the gaps in this area and to identify where additional research is needed.

METHODS

Data Sources and Searches

We created a comprehensive search strategy aimed to capture all relevant papers concerning injection-based therapy for TBOA. The search strategy was applied to the following bibliographic databases: Cochrane Library, PubMed, MEDLINE, EMBASE, Web of Science, and Scopus, using the terms "thumb-base joint osteoarthritis," "trapezio-metacarpal joint osteoarthritis," "first carpo-metacarpal joint osteoarthritis," "rizoartrhosis" in combination with "intra-articular injections," "injection-based therapy," "steroid injections," "hyaluronic acid injections," "platelet-rich plasma injections," and "prolotherapy." Additional articles were identified by searching bibliographies of each paper. Furthermore, we searched www.clinicaltrials.gov for active and/or recently completed clinical trials testing agents for IA therapy of TBOA.

We conducted the search of the literature in October 2020.

Inclusion/Exclusion Criteria

In this narrative review, we included all studies analyzing an injection-based intervention for patients suffering from TBOA. In particular, articles were considered eligible if they met the following criteria: 1) diagnosis of TBOA of the study population, according to the ACR criteria for hand OA (Altman et al., 1990); 2) any study design, including not only randomized controlled trials (RCTs), but even prospective open label or retrospective studies; 3) any studies presenting at least an evaluation of the efficacy, in terms of both pain and function, and tolerability of injection-based therapy; 4) any type of pharmacological agents or medical devices injected; 5) any injection approach included (with any or no image guidance); 6) studies published from 2000 to October 2020, totally written in English language. Studies were excluded if they did not evaluate the effects of injection therapy on both pain and function; review articles, studies not published as a full article (conference abstracts) and papers not totally written in the English language were also not considered.

Selection of Studies

Initially, duplicates were removed and relevant trials were independently screened by checking titles, keywords, and abstracts by two authors (T. S., M. N.). The references of

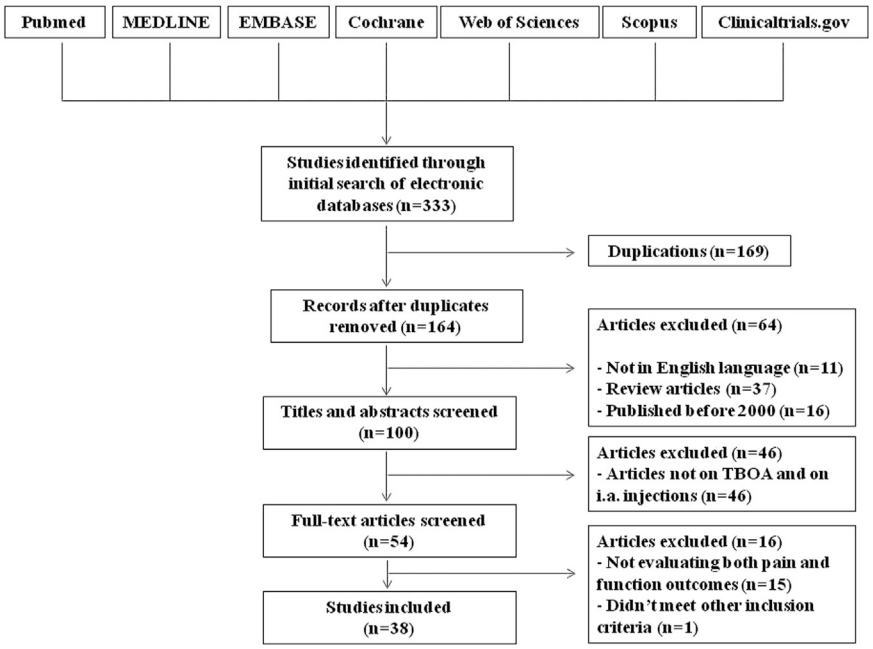


FIGURE 1 | Study flow diagram. TBOA, thumb-base osteoarthritis.

the selected articles and all significant reviews on the topic were also checked to identify other potential papers. Then, a full-text evaluation of the selected studies was performed by the same authors (T. S, M. N.) to determine whether the trials met the inclusion criteria regarding design, study population, outcomes, and interventions. Disagreement between the two reviewers was solved by involving a third author (F. A.).

Data Extraction

Data were independently extracted and aggregated into a Microsoft Excel®spreadsheet database by two authors (C. S. and G. S.). In particular, the data extraction sheet was designed to collect data about the study design, participants, details on the interventions undertaken, types of outcome measures evaluated, duration of follow-up, loss to follow-up, and results. Any inconsistencies between the two authors were solved by consensus discussion or by involving a third reviewer (F. A.) in case of persistent disagreement.

Outcomes and Data Analysis

Patient-reported pain and function were considered the main outcomes of interest; possible side effects related to the injection-based therapy were also recorded. A priori we defined as short-term follow-up, a follow-up period ranging from one week to 3 months, medium-term follow-up a period ranging from 3 to 6 months, and long-term follow-up above 6 months. Descriptive analysis was performed for all demographic data, interventions, and outcome parameters to facilitate narrative interpretation and comparison among the studies.

RESULTS

Literature Search Results and Trials Characteristics

In total, 164 potential eligible studies were found; no additional papers were obtained by hand searching of references. Of these, 11 studies were excluded because they were written in a language other than English, 37 because they were review articles and 16 because were published before 2000. Based on the title and the abstract content, 46 of these articles were not included in our review. The full texts of the remaining 54 studies were read, and a further 16 studies were excluded because they did not meet other inclusion criteria (**Figure 1**). We identified 38 assessable studies, six analyzing the effect of IA injections of corticosteroids, 20 evaluating the effects of IA hyaluronic acid, of whom seven in comparison to steroids, five dealing with IA injections of PRP and the remaining seven exploring new emerging IA therapy. Additionally, we identified two study protocols for trials planned for the coming years.

Corticosteroid Injections

Intra-articular corticosteroids have been used for decades in the management of symptomatic OA and remain a common practice given their potent anti-inflammatory properties and the favorable cost/effectiveness profile. Steroid injection is typically reserved to patients not responding to systemically delivered drugs or who do not tolerate pharmacological treatments (Jüni et al., 2015). The choice of the drug depends on the experience and preference of the physician, but generally includes triamcinolone, methylprednisolone, and betamethasone.

TABLE 1 | Summary of studies investigating intra-articular injections of corticosteroids for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Inclusion criteria[a]	Intervention	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Results
Jahangiri et al. (2014)	Double-blind RCT	60	-Age >40 years -Duration of pain ≥3 months -Pain intensity >30/ 100 mm VAS -Eaton grade >1	Group I: 2 monthly injections of 0.9% saline/ 1 ml followed by methylprednisolone acetate 40 mg/0.5 ml mixed with 2% lidocaine/0.5 ml after 1 month Group II: 3 monthly injections of 20% dextrose/ 0.5 ml mixed with 2% lidocaine/0.5 ml	24	None	VAS pain (0–100); tenderness intensity; HAQ-DI (0–3); pinch grip strength (lb)	The results on pain were better for steroid group at 1 month, and for dextrose group at 6 months; more effectiveness on functionality measures was observed for dextrose after 6 months
NCT00685880	Double-blind RCT	2	-Age > 45 years -Eaton grade 2–3 -Pain >3/10 on VAS -Symptoms duration >6 months	Group I: One injection of 10% dextrose solution Group II: one injection of betamethasone 3 mg/ 0.25–0.5 ml (CELESTONE® SOLUSPAN®)	24	None	VAS pain (0–10); analgesic use; grip strength; functional assessment of upper extremities	Early termination due to low enrollment; no subject data was analyzed
Day et al. (2004)	Open prospective study	30	-Isolated pain at TB -Tenderness over the TMCJ -Positive grind test	One injection of methylprednisolone acetate 40 mg/1 ml, mixed with 1% lidocaine/0.5 ml, 0.5% bupivacaine hydrochloride/ 0.5 ml and bicarbonate 0.5 ml followed by immobilization in a thumb spica splint for 3 weeks	72	None	Subjective pain relief (0–10); DASH (0–100)	Steroid injection with splinting provided long-term (until 18 months) benefit in early stage of the disease (eaton stage 1)
Joshi (2005)	Open prospective study	25	NR	One injection of methylprednisolone acetate 10 mg/0.25 ml	48	None	VAS pain (0–10); HAQ (0–3)	A significant long-term benefit wasn't observed; only a significant improvement of pain was reported after 1 month
Khan et al. (2009)	Open prospective study	40	Not reported	One injection of kenalog 10 mg/0.5 ml and a local anesthetic solution (not better specified)	24	None	VAS pain (0–10); DASH (0–100)	All patients reported a significant improvement in pain and hand function ($p < 0.05$), regardless of the disease stage. Additionally, a marked difference in the duration of improvement in hand function between early and late stages of the disease ($p = 0.0046$) was described
Rocchi et al. (2018)	Prospective comparative study	50	-Eaton stage 1–2 -Isolated pain at TB and tenderness over the TMCJ -Positive grind test	Group I: One injection of methylprednisolone acetate 40 mg/1 ml and lidocaine 10 mg Group II: 10 physical therapy sessions (including both physical agent application both exercise) with a hand therapist for 5 days a week for two consecutive weeks	48	None	TMC pain and restriction of activities (degrees); DASH (0–100); treatment satisfaction (1–10 scale); pinch strength (kg)	Group I reported a rapid decrease of pain and an increase of the functional performances, but this beneficial effect was short-lived. Group II experienced a more gradual improvement that lasted longer

[a]*All studies included patients with diagnosis of TBOA according to the ACR criteria (Altman et al., 1990).*

DASH, disabilities of the arm and shoulder; HAQ, health assessment questionnaire; HAQ-DI, health assessment questionnaire disability index; NR, not reported; pts, patients; TB, thumb-base; TMC, trapezio-metacarpal; TMCJ, trapezio-metacarpal joint; RCT, randomized controlled trial; VAS, visual analogue scale.

Intra-articular injection of steroid is mostly used and studied for inflammatory and degenerative disease of large joints, such as the knee, while the scientific evidence for TBOA is limited and conflicting. The characteristics of the few studies found by our literature research are summarized in **Table 1**.

A double-blind RCT compared the efficacy of IA steroids (methylprednisolone acetate 40 mg) with a 20% dextrose solution (prolotherapy treatment), both mixed with 0.5 ml of 2% lidocaine (Jahangiri et al., 2014). In this study, sixty patients with TBOA beyond stage one of the Eaton classification (Eaton and Glickel, 1987) were selected and randomly assigned to corticosteroids or prolotherapy. One group received two monthly placebo injections with a 0.9% saline solution and in the third month the steroid, the other one was treated with three monthly IA dextrose solution. The efficacy of the treatment was evaluated at 1, 2, and 6 months after the third injection. Methylprednisolone appeared more effective in the short-term, but at the sixth month the results showed a remarkable difference in favor of dextrose. No severe side effects were reported for prolotherapy.

Another randomized double-blind trial comparing IA 10% dextrose solution to betamethasone injection for the treatment of symptomatic TBOA was performed by the Mayo Hand Clinic (Clinicaltrials.gov, NCT00685880). The study started in 2008, but it appears to have been discontinued because of the small number of patients recruited.

A number of non-RCTs investigated the effectiveness and tolerability of IA steroid for TBOA. Thirty patients with TBOA were included in a long-term prospective open study and treated with a single injection of methylprednisolone acetate (40 mg) and 0.5 ml of 1% lidocaine followed by the use of a thumb spica splint for three weeks (Day et al., 2004). The clinical evaluation provided long-term (until 18 months) benefit in early stage of the disease, while in the severe form of disease (Eaton stage 4) the treatment appeared ineffective. On the contrary, Joshi R (Joshi, 2005) in a prospective case series of 25 patients treated with a single injection of 10 mg of methylprednisolone acetate showed a significant improvement of pain after 1 month, but not in the following observations at 3, 6, and 12 months. The Author did not report any information about the stage of the disease or about the concomitant use of other pharmacological or non-pharmacological treatments during the study period.

Khan et al. (Khan et al., 2009) conducted a prospective open study in 40 patients with TBOA to evaluate the improvement in pain and function of the hand after a single IA corticosteroid injection (triamcinolone acetonide 10 mg) and a local anesthetic solution. The symptomatic effect was evident in all patients in the short-term evaluation (2–4 weeks), but the duration of this benefit was different according to the stage of the disease.

Rocchi et al. (Rocchi et al., 2018) compared, prospectively, the effect of 10 sessions of physiotherapy to a single IA injection of methylprednisolone acetate (40 mg) and lidocaine in 50 patients with TBOA at early stages. The patients receiving IA therapy reported a rapid decrease of pain and an increase of the functional performances, but this beneficial effect was not maintained in the long-term follow-up (12 months). The group treated with physiotherapy (heat application, passive and active mobilization, massage, and stretching) experienced a more gradual improvement that lasted longer.

We did not report in this analysis the trials by Meenagh et al. (Meenagh et al., 2004) and by Swindells et al. (Swindells et al., 2010), because they evaluated only the effects of IA steroids on pain and not on functionality, as determined by our inclusion criteria.

Hyaluronic Acid Injections

HA represents another well-known IA treatment for OA; its use is based on its ability to restore the rheological properties of the synovial fluid and thus to decrease pain and improve functionality. For these reasons, it can represent a valid and safe alternative to IA corticosteroids in OA patients not responding to non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics. The role of viscosupplementation with HA is nowadays worldwide recognized for the treatment of knee OA, but its usefulness has been recently suggested also for other joints, such as hip, ankle, shoulder, temporomandibular joint, and thumb (Henrotin et al., 2015). However, as demonstrated in recent systematic reviews and meta-analysis, the scientific evidence on the efficacy of the IA therapy with HA in TBOA is still subject of debate, and often limited by the great heterogeneity of the trials performed in this field (Trellu et al., 2015; Kroon et al., 2016; Riley et al., 2019). The main sources of heterogeneity are represented by different HA formulations employed with variable injection schedules and IA techniques, different periods of follow-up and a great variety of assessed outcomes.

We identified a total of 20 papers, including nine RCTs, two retrospective comparative studies and nine open label trials evaluating the effects of the IA therapy with HA in TBOA patients. In the controlled studies, the comparator treatment was represented by IA corticosteroids (7 papers), IA saline solution (one paper) and extracorporeal shock wave therapy (ESWT) (one paper). The remaining controlled trials evaluated different schedules of IA HA in one case, and assessed a combination therapy with IA HA and IA ketorolac vs. IA HA alone in another one.

Hyaluronic Acid Versus Corticosteroids Injections

The individual characteristics of each study (6 RCTs and one retrospective comparative study) are reported in **Table 2**. A direct comparison among these trials is not possible, considering the great heterogeneity of the studies for a variety of parameters. Four research papers evaluated, as HA formulation, sodium hyaluronate from different commercial brands, two studies analyzed hylan and another one considered a hybrid

TABLE 2 | Summary of studies investigating intra-articular injections of hyaluronic acid for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Inclusion criteria[a]	Intervention	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Main results on pain and function	N° of reported adverse events
Stahl et al. (2005)	RCT	52	NR	Group I: One injection of methylprednisolone acetate (depomedrol®) 40 mg/1 ml Group II: one injection of sodium hyaluronate (orthovisc®) 15 mg/1 ml	24	None	VAS pain (0–10); grip and pinch strength (kg); PPT	A reduction of pain was observed in both groups after 1 month. Grip strength improved significantly in both groups at 6 months; patients treated with HA showed an improvement of pinch strength and PPT at 3 months, too	0
Fuchs et al. (2006)	Single-blind RCT	56	-VAS pain ≥40 mm for at least 6 months -Good general condition and compliance	Group I: 3 weekly injections of 1% sodium hyaluronate 10 mg/1 ml (ostenil® mini), average MW 1.2 milion dalton Group II: 3 weekly injections of triamcinolone acetonide (volon® A10) 10 mg/1 ml	26	None	VAS pain (0–100); lateral pinch grip (kg); pulp pinch grip (kg); radial and palmar ab/adduction and opposition (degrees)	VAS pain improved in a more significantly manner in group II at 2–3 weeks and in group I at 26 weeks. At the end of follow-up, a superiority of HA was found for the improvement of lateral pinch strength, pulp pinch strength and for radial abduction/adduction and opposition	0
Heyworth et al. (2008)	Double-blind RCT	60	NR	Group I: 2 weekly injections of 1 ml of hylan G-F 20 (synvisc®) Group II: one injection of 1 ml placebo of normal saline (0.9% sodium chloride), followed by one week by an injection of 1 ml of sodium betamethasone sodium phosphate–betamethasone acetate (celestone soluspan®) Group III: 2 weekly injections of 1 ml placebo of normal saline (0.9% sodium chloride)	26	None	VAS pain (0–10); DASH (0–100); ROM (degrees); grip and pinch strength (lbs)	There were no statistically significant differences among the three studied groups for most of the outcome measures at any of the follow-up time points	0

(Continued on following page)

TABLE 2 | (*Continued*) Summary of studies investigating intra-articular injections of hyaluronic acid for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Inclusion criteria[a]	Intervention	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Main results on pain and function	N° of reported adverse events
Monfort et al. (2015)	Single-blind RCT	88	-Age ≥ 18 years -Clinical symptoms for at least 90 days requiring analgesics or NSAIDs treatment	Group I: 3 weekly injections of 500–1,000 kDa HA (suplasyn®) 5 mg/0.5 cm^3, MW 500–1,000 kDa Group II: 3 weekly injections of 0.5 cm^3 of betamethasone disodium phosphate 1.5 mg and betamethasone acetate 1.5 mg	24	Yes, US-guidance	VAS pain (0–10); FIHOA (0–30); SF-36 PCS and MCS (0–100)	VAS and FIHOA significantly improved trough follow-up without significant differences between groups. A sub-analysis of patients with FIHOA ≥ 5 and VAS ≥ 3 at baseline showed a significantly major improvement of FIHOA score in the HA group vs steroid group at 12 and 24 weeks	Group I: 5 Group II: 5
Tenti et al. (2017)	Retrospective comparative study	100	-Age between 45 and 75 years -Clinical symptoms for at least 3 months -VAS pain >30 mm and FIHOA ≥ 6	Group I: 2 injections performed 15 days apart of a 3.2% hybrid formulation of HA (sinovial H-L®) 16 mg + 16 mg/1 ml; combination of 1,100–1,400 kDa MW and 80–100 kDa MW Group II: 2 injections performed 15 days apart of triamcinolone acetonide (kenacort®) 20 mg/0.5 ml	24	None	VAS pain (0–100); FIHOA (0–30); HAQ (0–3); duration of morning stiffness (minutes); SF-36 PCS and MCS (0–100)	Both therapies provided effective pain relief and functional improvement, but the benefits achieved were significantly superior in group I vs group II, after 1 month and persisted until 6 months. HA was also associated to a significant improvement of morning stiffness, HAQ and SF-36 PCS	Group I: 2 Group II: 4
Bahadir et al. (2009)	Single-blind RCT	40	-Eaton stage 2 or 3	Group I: 3 weekly injections of sodium hyaluronate (ostenil®) 5 mg/0.5 ml Group II: one injection of triamcinolone acetonide (kenacort®) 20 mg/0.5 ml	48	None	VAS pain (0–10); pinch strength (pound); grip strength (pound); DHI (0–90)	VAS pain decreased significantly vs baseline over 12 months in group II and over 6 months in group I. Pinch strength didn't improve in any group, while grip strength increased significantly in both. DHI improved significantly only in group II	0
NCT00398866	Three arms RCT	200	-Unacceptable pain despite modification of activity and NSAIDs -Failure/intolerance of conservative therapy with NSAIDs and/or COX-2 inhibitors	Group I: 2 weekly injections of 1 ml of hylan GF-20 (synvisc®) Group II: one injection of triamcinolone (kenalog®) 40 mg/1 ml, followed by a placebo injection of 1 ml 0.5% bupivacaine after 1 week Group III: Two weekly injections of 1 ml of bupivicaine 0.5%	26	None	VAS pain (0–100); DASH (0–100)	Only partial results reported	Group I: 0 Group II:0 Group III: 1

(Continued on following page)

TABLE 2 | (*Continued*) Summary of studies investigating intra-articular injections of hyaluronic acid for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Inclusion criteria[a]	Intervention	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Main results on pain and function	N° of reported adverse events
Roux et al. (2007)	Three arms RCT	42	-VAS > 40 mm -Failure of other therapies -Kellgren grade II-IV	Group I: One injection of 1 ml of sodium hyaluronate (sinovial®) Group II: 2 weekly injections of 1 ml of sodium hyaluronate (sinovial®) Group III: 3 weekly injections of 1 ml of sodium hyaluronate (sinovial®)	12	Yes (radioscopic control)	VAS pain (0–100); FIHOA (0–30)	No significant differences were found among the groups over the study for VAS and FIHOA. Intra-groups analyses showed significant improvement in VAS and FIHOA in group II and III, but not in group I. Efficacy was evident after 1 month and persisted at 3 months	NR
Figen Ayhan and Ustün (2009)	RCT	66 joints of 33 pts	-VAS > 40 mm -Eaton grade 1–4	Group I: One injection of 1 ml of hylan G-F 20 (synvisc®) Group II: one injection of 1 ml of saline solution	24	None	VAS pain (0–100); FIHOA (0–30); pinch strength (lbs)	Statistically significant improvements of VAS, FIHOA and pinch strength were observed in group I at 24 weeks, while only VAS decreased temporarily in group II at 6 weeks	NR
Ioppolo et al. (2018)	RCT	58	-Pain duration ≥6 months -Age >40 years -VAS >4 mm -Eaton grade 2 or 3	Group I: 3 weekly injections of 0.5 cm^3 HA (sinovial mini®) Group II: 3 weekly sessions of ESWT (2,400 pulses for each session with a frequency of 4 Hz and an EFD of 0.09 mJ/mm^2)	24	Yes (US guidance)	VAS pain (0–10); DHI (0–90); grip and pinch strength (kg)	A significant improvement of VAS and DHI was observed in both groups over time, but a greater average improvement was detected in group II at 24 weeks. A significant increase in strength was reported in both groups, but it was superior in group II vs group I starting immediately after the treatment	0
Koh et al. (2019)	Retrospective comparative study	74	-Age > 40 years -Failure to other conservative treatments -Eaton grade 2 or 3 -Pain duration ≥3 months	Group I: One injection of 0.5 ml of sodium hyaluronate mixed with 0.5 ml of ketorolac 30 mg/ml Group II: one injection of 0.5 ml of sodium hyaluronate mixed with 0.5 ml of saline	24	Yes (US-guidance)	DASH (0–100); VNS for pain (0–10)	The DASH and VNS scores improved at 1, 3 and 6 months in both groups, but the onset of pain relief was more rapid/ at 1 month) in group I vs group II	Group I: 5 Group II: 0

(Continued on following page)

TABLE 2 | (*Continued*) Summary of studies investigating intra-articular injections of hyaluronic acid for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Inclusion criteria[a]	Intervention	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Main results on pain and function	N° of reported adverse events
Schumacher et al. (2004)	Open-label study	16	-Pain and/or tenderness at TMCJ	5 weekly injections of sodium hyaluronate (hyalgan®) 10 mg/ml, MW 500–730 kDa	24	None	VAS pain (0–10); tenderness (0–3); crepitus (0–3); 5-question non validated hand function survey; pinch strength (kg)	Mean pain score at rest decreased of 46% and pain on use of 27% at 6 months vs baseline. No other significant improvement in the evaluated parameters were reported	2
Frizziero et al. (2014)	Open-label retrospective study	58	Not reported	3 weekly injections of 0.8 ml of HA 10 mg/ml, MW 500–730 kDa	24	None	VAS pain (0–10) at rest and on voluntary and passive movements; lateral pinch strength; morning stiffness; NSAIDs consumption (pills/days/month)	At 1, 3 and 6 months from baseline, VAS pain at rest and on movements significantly improved, as well as the duration of morning stiffness and NSAIDs consumption	15
Coaccioli et al. (2006)	Open-label study	43 (56 TMCJ in total)	-VAS spontaneous pain >40 mm -Provoked pain under pressure >60 mm	3 weekly injections of 0.5 ml of HA	7	None	VAS spontaneous pain (0–100); VAS provoked pain (0–100); grip strength (mmHg); FIHOA (0–30); NSAIDs/ analgesics consumption (%)	Pain and FIHOA significantly decreased at the end of the study. A reduction of symptomatic drugs consumption was also observed	0
Salini et al. (2009)	Open-label study	18	-Kellgren grade II-III -Symptoms duration > 1 month	One injection of 1 ml of 0.8% HA, MW 0.8–1.2 million dalton	4	Yes (US guidance)	VAS pain at rest (0–10); VAS pain during common activities (0–10); NSAIDs consumption (nr pts and tablets/week); FIHOA (0–30); grip strength (kg); lateral and pulp pinch strength (kg)	Pain at rest and during activities significantly reduced after 1 month, as well as FIHOA. A significant decrease of NSAIDs consumption was also reported	2
Mandl et al. (2009)	Open-label study	32	-Kellgren grade II-IV	3 weekly injections of 1 ml of hylan G-F 20 (synvisc®)	26	None	VAS pain (0–100); DASH (0–100); opposition grip strength (lbs); overall pts satisfaction	VAS pain and DASH significantly improved at 26 weeks, while grip strength didn't significantly change. VAS pain correlated with patient satisfaction at 26 weeks	4

(Continued on following page)

TABLE 2 | (*Continued*) Summary of studies investigating intra-articular injections of hyaluronic acid for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Inclusion criteria[a]	Intervention	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Main results on pain and function	N° of reported adverse events
Ingegnoli et al. (2011)	Open-label study	16 (32 TMCJ in total)	-VAS pain ≥40 mm -Failure of prior treatments (NSAIDs, physical therapy, splinting)	3 weekly injections of 0.5 ml of high MW HA (hyalubrix®)	24	Yes (US guidance)	VAS pain (0–100); FIHOA (0–30); synovial hypertrophy and PDS (0–3) assessed by US	VAS pain and FIHOA score significantly decreased after 2 weeks and are maintained at week 24. PDS significantly decrease after 2 weeks, but it was not maintained at week 24. No significant reduction of synovial hypertrophy was reported during the follow-up	0
Di Sante et al. (2011)	Open-label study	31	-VAS pain ≥4 cm -DHI ≥ 24	3 weekly injections of 1 ml of HA	24	Yes (US guidance)	VAS pain (0–10); DHI (0–90)	A significant decrease of VAS pain was detected after 1 and 3 months, but not at 6-months follow-up. No significant differences were found for DHI at 1, 3 and 6 months	0
Velasco et al. (2017)	Open-label study	35	-Age between 18 and 75 years -Eaton grade 2 or 3 -Pain duration at TMCJ >6 months -VAS pain ≥4 cm in the target hand and <4 in the controlateral hand	One injection of 0.7–1 ml of NASHA 20 mg/ml (durolane®)	24	Yes (fluoroscopy guidance)	VAS pain (0–10); Q-DASH (0–100); kapandji thumb opposition test (0–10); radial abduction (degrees); MCP joint flexion (degrees); strength of fist and clamp (kg); crepitus (%); morning stiffness (%); mobility difficulties (%)	Mean VAS pain decreased of 27.8% after 6 months vs baseline and a reduction >25% was already present after 1 month. All other evaluated parameters, excepted for strength of fist significantly improved at 6 months vs baseline	5
Bartoloni et al. (2019)	Open-label study	12	-VAS pain ≥40 mm	Two injections, 15 days apart, of 1 ml of hybrid HA (sinovial H-L®)	24	Yes (US guidance)	VAS pain (0–100); DASH (0–100)	VAS pain significantly decreased after 3 and 6 months- a significant improvement of DASH was reported at any evaluation times (1, 3 and 6 months)	0

[a]*All studies included patients with diagnosis of TBOA according to the ACR criteria (Altman et al., 1990).*

COX-2, cycloxigenase-2; DASH, disabilities of the arm and shoulder; DHI, Duruöz hand index; EFD, energy flux density; ESWT, extracorporeal shock wave therapy; FIHOA, functional index for hand osteoarthritis; HA, hyaluronic acid; HAQ, health assessment questionnaire; MCP, metacarpophalangeal; MW, molecular weight; NASHA, nonanimal hyaluronic acid; NR, not reported; NSAIDs, non steroidal anti-inflammatory drugs; PDS, power doppler signal; PPT, Purdue Pegboard test; pts, patients; Q-DASH, quick-disabilities of the arm and shoulder; RCT, randomized controlled trial; ROM, range of motion; SF-36 PCS, short form-36 physical component summary; SF-36 MCS, short form-36 mental component summary; TMCJ, trapezio-metacarpal joint; US, ultrasound; VAS, visual analogue scale; VNS, verbal numeric scale.

formulation of HA. As corticosteroid comparator, the Authors chose triamcinolone acetonide in four cases, although with different dosages, betamethasone disodium phosphate in two works and methylprednisolone in the remaining one. Injections courses ranged from a single injection to three weekly injections. The length of follow-up was of 6 months for all trials, except from one in which the follow-up lasted until 12 months. Image guidance was employed in only one study. The only outcome parameter evaluated in all studies was pain by a visual analogue scale (VAS). Functionality was assessed by a variety of different tests.

Concerning the efficacy of the results, the RCTs by Stahl et al. (Stahl et al., 2005) and Fuchs et al. (Fuchs et al., 2006) showed a significant effect of both IA steroid and IA HA on pain relief (VAS) and function improvement (assessed by grip strength in the former study and by pinch grip and pulp pinch grip in the latter). However, Stahl et al. (Stahl et al., 2005) observed a significant improvement of the functional Purdue Pegboard Test (PPT), which measures the fine hand function, only in the HA group. Consistent with these results, Fuchs et al. (Fuchs et al., 2006) found a superiority of HA over steroids in all assessed parameters (VAS pain, grip power, and range of motion) in the medium-term. The more recent 6-months, single-blind, RCT by Heyworth et al. (Heyworth et al., 2008) reported no statistically significant differences among the three studied groups, of whom one was treated with two IA injections of hylan, one with a single injection of normal saline (0.9% sodium chloride) followed, after a week, by IA betamethasone, and another one with two IA injections of normal saline; however, a positive trend in hand function, assessed by Disabilities of the Arm, Shoulder, and Hand (DASH) scores, was observed in patients treated with HA. A positive trend in hand function, measured by Functional Index for Hand Osteoarthritis (FIHOA) score, was observed in patients treated with IA HA [3 weekly injections of a formulation of HA with molecular weight (MW) 500–1,000 kDa] also by Monfort et al. (Monfort et al., 2015) in a 6-months single-blinded randomized trial vs. betamethasone. These findings became particularly evident and reached statistical significance when patients with more severe symptoms (FIHOA score of at least five and VAS score of 50 or more) were considered for analysis.

These encouraging data on the HA therapy in patients with TBOA were recently confirmed by a 6-months retrospective comparative study which assessed the efficacy of a new hybrid formulation of HA vs. triamcinolone acetonide in 100 patients (Tenti et al., 2017). The Authors found both IA therapies effective in controlling pain (by VAS) and improving joint functionality (by FIHOA), but the benefits achieved were significantly superior in the HA group than in the steroid group after 1 month and until the end of follow-up. Furthermore, the HA formulation studied also resulted in an association with a significant decrease in the duration of morning stiffness and with a significant improvement of Health Assessment Questionnaire (HAQ) and physical component summary (PCS)-SF-36.

Contrasting results were reported by Bahadir et al. (Bahadir et al., 2009) in an RCT evaluating in the long-term 20 patients treated with a single injection of 20 mg triamcinolone acetonide and 20 patients who received three weekly injections of 5 mg sodium hyaluronate. Pain levels were significantly decreased in both groups, but the beneficial effect persisted until 12 months only in the steroid group; similarly, the improvement in hand functionality, assessed by the Duruoz Hand Index (DHI), reached statistical significance only in patients treated with triamcinolone.

Interestingly, the protocol of a new randomized multicenter study, the RHIZ'ART trial, aimed to analyze, for the first time, the possible synergistic effect of corticosteroids associated with HA, compared to steroid alone, in TBOA patients, was published last year (Cormier et al., 2019). The Authors would like to compare VAS pain, Cochin score, grip strength and opposition force, 3 months after a single injection of 0.5 ml of corticosteroid and 0.5 ml of physiological saline or 0.5 ml of corticosteroid and 0.5 ml of HA and would like to continue the follow-up until 12 months.

A phase three triple-blind (participants, care provider, investigator) RCT comparing the safety and effectiveness of hyaluronan (Hylan G-F20 injected once a week for two consecutive weeks) to corticosteroids (triamcinolone, 40 mg injected the first week, followed by a placebo injection of 1 ml 0.5% bupivacaine the second week) and local anesthetic (Bupivicaine 0.5% 1 ml injected once a week for 2 weeks) in relieving symptoms of TBOA has recently been completed (Clinicaltrials.gov, NCT00398866). Unfortunately, only partial results have been reported.

Hyaluronic Acid Versus Other IA Treatment Comparators

Considering the lack of guidelines for the IA HA treatment schedule, in 2007 Roux et al. (Roux et al., 2007) compared the efficacy on pain and function of one, two, or three IA injections of 1 ml sodium hyaluronate, performed weekly under radioscopic control in the carpometacarpal joint of 44 patients. No significant differences were found among the three groups over the study period (3 months) for VAS pain and FIHOA, while intra-groups differences between baseline and the end of follow-up were significant only for patients treated with two or three injections.

In a 6-months Turkish RCT conducted in 2009, IA HA was compared to IA saline injection in 33 women with bilateral TBOA; in particular, hands of the same patient were divided to hylan G-F 20 injection and saline injection, randomly. The Authors found a significant improvement of VAS pain, FIHOA, and pinch strength at the 24th week only in the hylan group, while a short-term (at the sixth week) placebo analgesic effect was described for the control group (Figen Ayhan and Ustün, 2009).

In another RCT on 58 TBOA patients, three weekly IA injections of 0.5 cm^3 HA were compared to ESWT performed once a week for three consecutive weeks. Although a significant improvement in VAS pain, DHI score and grip and pinch strength was observed in both groups at 3 and 6 months, a greater benefit was reported in the ESWT group for all the assessed parameters (Ioppolo et al., 2018).

Finally, very recently, in a retrospective comparative study, Koh et al. (Koh et al., 2019) treated 74 TBOA patients with ultrasound-guided IA injection of 0.5 ml of sodium hyaluronate

and 0.5 ml of ketorolac or 0.5 ml of sodium hyaluronate and 0.5 ml of saline. The DASH and verbal numeric scale (VNS) pain scores improved at 1, 3, and 6 months post-injection in both groups, but the pain reduction was significantly more rapid (at 1 month) after the injection of HA plus ketorolac compared to HA alone, suggesting a possible role of this combined IA therapy for a fast onset of analgesia.

Hyaluronic Acid in Open Label Trials

In the last two decades, a variety of papers investigating the potential efficacy of different formulations of HA have been published (**Table 2**).

In 2004 the open-label study by Schumacher et al. (Schumacher et al., 2004) provided preliminary evidence that a cycle of five weekly injections of low MW (500–730 kDa) HA into the trapezio-metacarpal joint of 16 TBOA patients, was effective in reducing pain at 6 months follow-up, although a significant effect on pinch strength could not be observed. The beneficial effects of the same HA formulation have been subsequently confirmed by a retrospective open study conducted by Frizziero et al. (Frizziero et al., 2014). The Authors demonstrated that 58 patients treated with three weekly IA injections of low MW HA (500–730 kDa) presented a significant reduction of pain at rest and on voluntary or passive movements of flexion, extension, abduction, and rotation (on a 0–10 mm VAS scale), of morning stiffness duration and of NSAIDs consumption at any evaluation time (1, 3, and 6 months); furthermore, a significant improvement of radial and palmar ab-/adduction was registered at each follow-up visit.

The use of IA HA for TBOA was encouraged also in two different studies by Coaccioli et al. (Coaccioli et al., 2006) and Salini et al. (Salini et al., 2009); however, both trials were limited by a very short-term follow-up (1 month). In the former trial, 43 TBOA patients for a total of 56 trapezio-metacarpal joints were treated with three weekly injections of 0.5 ml HA and experienced a significant reduction of VAS pain, FIHOA score and NSAIDs/analgesic consumption, other than a significant improvement of grip strength after 1 month from the first injection (Coaccioli et al., 2006). In the latter study, a small group of TBOA patients (n = 18) received a single ultrasound-guided injection of a formulation of HA with a MW of 0.8–1.2 million Dalton; a significant decrease of pain at rest and during activities, as well as of FIHOA score were reported at the end of 1 month follow-up, together with a significant reduction of NSAIDs intake (Salini et al., 2009).

Other HA formulations also resulted to be beneficial for patients with TBOA in open label pilot trials. In an American study on 32 patients, a cycle of three weekly injections of hylan G-F 20 determined a significant improvement of VAS pain and DASH score (Mandl et al., 2009). In 2011, Ingegnoli et al. (Ingegnoli et al., 2011) evaluated the effects of three ultrasound-guided IA injections, performed 1 week apart, with high MW HA in 32 TB joints of 16 patients. The Authors reported a significant clinical improvement, characterized by VAS pain and FIHOA score decrease, 2 weeks after the injections and this effect persisted until 6 months. At the same time, a significant reduction of power doppler signal was observed at 2 weeks, suggesting a potential role of HA in reducing local inflammation, although this result was not maintained at week 24. In the same year, an Italian trial assessed the efficacy of an ultrasound-guided procedure for the treatment of TBOA with HA. Thirty-one patients received three weekly injections of 1 ml HA and experienced a statistically significant VAS reduction at 1 and 3 months after the end of the IA therapy, but not a 6-months follow-up; no significant differences were described for DHI at any evaluation times (Di Sante et al., 2011).

More recently, a 6-months, prospective, open-label study investigated the effects of a single IA injection of nonanimal hyaluronic acid (NASHA) into the trapezio-metacarpal joint of 35 TBOA patients. This HA formulation differs from the others above mentioned for the presence of synthetic cross-linking which creates a three-dimension gel network, responsible for an increased viscosity and half-life. The Authors reported a significant mean change from baseline in VAS pain score at any evaluation times (month 1, 3, and 6) with a reduction of 27.8% at 6 months. Further, a significant improvement of quickDASH, Kapandji thumb opposition test, radial abduction, metacarpal flexion, and strength of clamp scores were observed at the end of follow-up (Velasco et al., 2017). Finally, an open study on a small sample of patients (n = 12) confirmed the positive results of the above-mentioned study by Tenti et al. (Tenti et al., 2017) on the use of an hybrid formulation of HA. Indeed, the Authors reported a statistically significant reduction of VAS pain after 3 and 6 months and a significant improvement of DASH score at 1, 3, and 6 months (Bartoloni et al., 2019).

We did not report in this analysis the trial by Dauvissat et al. (Dauvissat et al., 2018) on a single injection of mannitol-modified cross-linked HA in patients with TBOA, because it evaluated only the effects on pain and not on functionality, as determined by our inclusion criteria.

Platelet-Rich Plasma Injections

PRP is an autologous blood product derived by centrifugation of the whole blood and characterized by a high concentration of platelets above the normal levels (Marx, 2001). Many protocols for preparing PRP exist; one possibility is to include the leukocyte-containing buffy coat obtaining the so-called leukocyte-rich PRP, while another one is to exclude leukocytes resulting in the so-called leukocyte-poor PRP which is the standard PRP preparation for OA (Evans et al., 2020). Its use for the treatment of OA of large joints, particularly knee and hip OA, has emerged since the first decade of twenty-first century. The rationale of efficacy of this IA treatment lies on its ability to reverse pro-inflammatory processes and to modify the microenvironment inside the joint, restoring the articular homeostasis (Ornetti et al., 2016). In depth, after PRP injection, a subset of cytokines and growth factors, as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), interleukin-1 receptor antagonist (IL-1RA), soluble receptor of tumor necrosis factor-alpha (TNF-alpha) transforming growth factor-beta (TGF-beta), and many others, are released into the joint, through the degranulation of the

TABLE 3 | Summary of studies investigating intra-articular injections of platelet-rich plasma for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Intervention	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Results	No of reported adverse events
Loibl et al. (2016)	Open label study	10	2 injections of 1–2 ml of PRP with a platelet concentrations of 2.4 higher vs baseline, performed 4 weeks apart	24	Yes (fluoroscopic guidance)	VAS pain (0–10); DASH (0–100); mayo wrist score (0–100); grip and pinch strength (kg)	VAS significantly improved at 6 months vs baseline, as well as mayo wrist score. DASH and grip strength were unaffected. Pinch strength significantly declined at 6 months	1
Malahias et al. (2018)	RCT	33	Group I: 2 injections of 2 ml of PRP with a platelet concentrations of 2.6 higher vs baseline, performed 15 days apart Group II: 2 injections of 125 mg/2 ml methylprednisolone sodium succinate (solu medrol®) and lidocaine hydrochloride 2%, performed 15 days apart	48	Yes (US guidance)	VAS pain (0–100); Q-DASH (0–100); patient satisfaction (yes/no)	After 12 months' follow-up, PRP treatment yielded significantly better results vs steroid in terms of VAS pain, Q-DASH and patients' satisfaction	NR
Medina-Porqueres et al. (2019)	Case report	1	3 weekly injections of 3 ml of PRP and 10% calcium chloride	48	None	VAS pain (0–10), grip and pinch strength (kg); kapandji opposition score; Q-DASH (0–100)	After 6 months, the patient reported an improvement of pain and functional disability. At 12 months, no recurrences or complications were observed	0
NCT03196310, ongoing	Three arms single-blind RCT	150 (estimated)	Group I: PRP injection Group II: corticosteroid (kenalog) injection Group III: Normal saline injection	48	NR	VAS pain; DASH; pinch strength	No results posted	No results posted
NCT04218591, ongoing	Double-blinded RCT	90 (estimated)	Group I: PRP injection Group II: normal saline injection	24	NR	VAS pain (0–10); nelson thumb score (0–100); EQ-5D (0–1); PRWHE (0–100); DASH (0–100); HADS (0–21); PCS (0–52); ROM (degrees); strength (kg)	No results posted	No results posted

DASH, disabilities of the arm and shoulder; EQ-5D, EuroQoL-5D; NR, not reported; HADS, hospital anxiety and depression score; PCS, pain catastrophizing score; PRP, platelet-rich plasma; PRWHE, patient-rated wrist and hand evaluation; pts, patients; Q-DASH, quick-disabilities of the arm and shoulder; RCT, randomized controlled trial; ROM, range of motion; US, ultrasound; VAS, visual analogue scale.

platelets α-granules. Globally, these mediators exert an anti-catabolic and anti-inflammatory action, modulate the metabolic functions of chondrocytes and subchondral bone and stimulate fibroblasts to synthesize HA (Moussa et al., 2017).

Actually, only a very limited number of papers, often with a very small sample size and a not controlled design, investigating the possible efficacy of PRP in TBOA are published (**Table 3**).

The first one dates back to 2016 and analyzed the effect of two IA injections of 1–2 ml of PRP, administered 4 weeks apart to a small number of patients (n = 10). After 6 months of follow-up, the Authors reported a significant improvement of VAS pain and Mayo wrist score, while no differences vs. baseline were observed for DASH score and grip strength (Loibl et al., 2016). These results are supported by a RCT published in 2018 and assessed the efficacy of two ultrasound-guided IA PRP injections, performed 2 weeks apart, in 16 patients compared to two ultrasound-guided IA methylprednisolone and lidocaine injections at a 2-weeks interval in 17 patients. The Authors demonstrated a significant efficacy of PRP in improving pain (measured by VAS) and function (assessed by quick-DASH) both in the mid-

TABLE 4 | Summary of studies investigating intra-articular injections of mesenchymal-derived stem cell populations for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Intervention	Source of MSCs	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Main results on pain and functionality	No of reported adverse events
Centeno and Freeman (2014)	Case series	10	Group I: One injection of 0.3–1 ml of MSCs formulation in addition to a platelet product Group II: pts interested in the procedure, but not treated	Bone marrow (from iliac crest)	48	Yes (fluoroscopy)	VAS pain (0–10); strength (kg); ROM (degrees)	Positive outcomes were observed in pts treated with MSCs, compared with a reported worsening among the controls	0
Bohr et al. (2015)	Case report	1	One injection of 1 ml cell-enriched lipoaspirate	Adipose tissue (from abdomen)	48	Yes (X-ray control)	Pain; DASH (0–100)	The patient reported to be free of pain after 5 weeks and reported a reduction of DASH score at 12 months	NR
Herold et al. (2017)	Open label study	50	One injection of 1 cc of lipoaspirate	Adipose tissue (from abdomen and tights)	48	Yes (radiographic control)	VAS pain (0–10); pinch strength (bar); kapandji test; DASH (0–100)	All the evaluated parameters significantly improved at all evaluation times until 48 weeks, but in patients with higher degrees of OA (eaton grade 3 or 4) the benefit was lower than in patients with eaton grade 2	5
Erne et al. (2018)	Retrospective comparative study	21	Group I: One injection of 1.3 ± 0.2 ml of autologous fat Group II: Lundborg resection arthroplasty	Adipose tissue (from low abdomen)	72	None	VAS pain (0–10); DASH (0–100); grip strength (kg); pinch strength (kg); patient satisfaction (0–10)	Both treatments resulted effective in improving VAS pain and DASH without any significant differences between groups at one year follow-up; however, the time until complete symptoms resolution was significantly shorter for group I	Group I: 1 Group II: 1
Haas et al. (2020)	Open label trial	89 (99 TMCJ)	One injection of 1–2 ml of autologous fat	Adipose tissue (from abdomen)	48	None	VAS pain; pinch and grip strength (kg); MHQ (0–100)	VAS pain and MHQ significantly improved from 2 to 6 weeks, respectively and continued to improve over 12 months	2

(Continued on following page)

TABLE 4 | (*Continued*) Summary of studies investigating intra-articular injections of mesenchymal-derived stem cell populations for the treatment of thumb-base osteoarthritis.

Authors, publication year	Study design	Sample size (pts)	Intervention	Source of MSCs	Follow-up duration (weeks)	Injection guidance	Outcomes evaluated	Main results on pain and functionality	No of reported adverse events
NCT03166410, ongoing	Open label study	500 (estimated)	Injection of autologous adipose-derived stromal vascular cellular fraction	NR	96	NR	Pain, function and stiffness	No results posted	No results posted
NCT04455763, ongoing	RCT	60 (estimated)	Group I: Injection of autologous adipose-derived stromal vascular cellular fraction in association with splinting Group II: splinting alone	NR	24	NR	VAS pain (0–100); PRWE (0–10); global improvement; grip and pinch strength (kg); MHQ	No results posted	No results posted

DASH, disabilities of the arm and shoulder; MHQ, Michigan hand outcomes questionnaire; MSCs, mesenchymal stem cells; NR, not reported; OA, osteoarthritis; PRWE, patient-rated wrist evaluation; pts, patients; RCT, randomized controlled trial; ROM, range of motion; TMCJ, trapezio-metacarpal joint; VAS, visual analogue scale.

(3 months) and long-term (12 months) with a superior effect of PRP compared to steroids at 12 months of follow-up (Malahias et al., 2018). The beneficial effect of PRP in TBOA was supported also by the case report by Medina-Porqueres et al. (Medina-Porqueres et al., 2019). The Authors reported the clinical history of a pianist affected by TBOA and treated with three weekly IA PRP injections who experienced a significant improvement of VAS pain, grip and pinch strength, and quick-DASH score after 6 months; at 12 months follow-up no recurrences or complications were identified.

There are two ongoing clinical trials with IA PRP for TBOA registered in ClinicalTrials.gov. A single-blind (patients) study with IA injections of leukocyte depleted PRP vs. triamcinolone acetonide and vs. placebo (normal saline) for TBOA started in United States in September 2018 (NCT03196310); no results have been reported yet.

Finally, a double-blind randomized trial is currently ongoing in Sweden in patients with radiological Eaton class 1–3 of TBOA comparing the efficacy of PRP vs. placebo (saline solution) (NCT04218591).

New Emerging Intra-articular Therapies

New data are emerging about the possible use of IA injections based on mesenchymal-derived stem cell populations for the treatment of OA, due to their properties of providing mechanical support into the joint and stimulating cartilage repair and regeneration (Bosetti et al., 2016); however, the evidence for TBOA is still very limited. In **Table 4** is reported the summary of the studies investigating such kind of IA treatment in TBOA.

A case series on a small study population investigated the efficacy of fluoroscopy-guided IA injections of autologous mesenchymal stem cells, derived from bone marrow aspirate of iliac crest, administered to six patients, and compared to four participants who remained untreated. The Authors reported positive encouraging results for both pain and function after one year of follow-up, although they claimed caution for the several limitations of the study (Centeno and Freeman, 2014). Subsequently, Bohr et al. (Bohr et al., 2015) described the case of a 62-year old man, affected by TBOA, treated with cell-enriched lipoaspirate arhroplasty, after abdominal liposuction, who experienced pain relief after five weeks and a significant improvement vs. baseline of DASH score after one year. Herold et al. (Herold et al., 2017) confirmed the positive results in their prospective open study, which included 50 TBOA patients treated with IA injection of processed autologous fat. This therapy resulted beneficial in terms of VAS score, DASH score, grip, and pinch strength at 12 months follow-up. However, a sub-groups analysis showed significantly better outcomes in patients at Eaton stage 2, while only partial or no improvement in stage 3 or 4.

More recently, Erne et al. (Erne et al., 2018) performed a retrospective study aimed to compare the results of a surgical technique of trapeziectomy with autologous fat injections. Twelve patients underwent the Lundborg resection arthroplasty, while nine patients received autologous fat injection, harvested from their own abdomen. Both treatments resulted effective in improving pain and function (measured by VAS and DASH questionnaires, respectively) without any significant differences between groups at one-year follow-up; however, autologous fat injections seemed to determine a shorter time until symptoms resolution and shorter operative time compared with Lundborg arthroplasty.

Data on a wider cohort of TBOA patients ($n = 99$) treated with autologous fat injection was derived from the most recent study by Haas et al. (Haas et al., 2020). They reported that pain during activities at 2 and 6 weeks as well as 3, 6, and 12 months was significantly lower than at baseline. Furthermore, Michigan Hand

Outcome Questionnaire (MHQ) scores were significantly higher at 6 weeks, 3, 6, and 12 months.

Two open label studies are currently undergoing to evaluate the safety and efficacy of injection therapy with autologous stromal vascular fraction (SVF) derived from adipose tissue (Clinicaltrials.gov, NCT03166410; NCT04455763). The SVF exerts anti-inflammatory, immunosuppressive and chondroprotective effects; due to its potential properties being tried in treating patients with different OA localizations (Pak et al., 2018).

Interestingly, still ongoing at the Cochin Hospital of Paris is an RCT aimed to evaluate the possible efficacy of IA injections of botulinum toxin A, associated with splinting, and compared to IA injection of saline associated with splinting. The rationale for use of botulinum toxin A in OA lies on its potential role in suppressing the release of some mediators involved in nociception (Gil et al., 2018).

Safety of Intra-articular Therapy

In general, IA therapy represents a valid and safe alternative in OA patients with multiple comorbidities, for whom pharmacological treatments often present a not favorable risk/benefit ratio or are contraindicated. However, IA therapy is not free of several side effects.

In particular, corticosteroids are known to be associated with both local reactions, as skin atrophy or hypopigmentation, acute corticosteroid-microcrystalline joint flare and hemarthrosis and both systemic effects, including facial flush, hyperglycemia, blood pressure increase, Tachon's syndrome, vagal reaction and hypersensitivity (Nguyen and Rannou, 2017). Furthermore, it is noteworthy to report the potential chondrotoxicity of IA steroids which still remains one of the more debated issues in this field. Indeed, some *in vitro* and animal studies demonstrated that corticosteroids can have an adverse effect on cartilage, especially at high doses, probably due to its ability to modulate cartilage proteins production and breakdown (Wernecke et al., 2015). From a clinical point of view, some trials showed a greater cartilage volume loss in patients treated with IA steroids compared to placebo (McAlindon et al., 2017; Zeng et al., 2019).

In the trials on TBOA patients summarized in this review, the adverse events related to IA steroids injections are not discussed in depth and rarely reported. The side effects occurred in a minority of patients and consisted mainly in temporary acute local pain starting 1–6 h after the injections and resolved spontaneously after one or two days. Only one patient reported mild skin atrophy and hypopigmentation (Day et al., 2004; Joshi, 2005; Khan et al., 2009; Jahangiri et al., 2014; Rocchi et al., 2018).

Intra-articular HA is usually recognized as a safe treatment for OA; the incidence of adverse events in RCTs, especially on knee OA, is rather low. The most frequent side effects consist of mild transient local reactions, such as pain, swelling, flares, and effusion at the site injection, while systemic events are seldom reported. Furthermore, rare cases of acute pseudoseptic reactions are observed in association with avian high MW cross-linked HA (Nguyen and Rannou, 2017). Actually, there is no evidence of a direct influence of the number of joint injections on the occurrence of side effects, while high MW and cross-linked formulations of HA were more frequently associated to local reactions and post-injection flares in comparison with intermediate or low MW (Reichenbach et al., 2007; Nguyen and Rannou, 2017).

The analysis of the literature papers on IA HA therapy for TBOA patients, confirmed what had already been demonstrated for HA treatment safety in OA in general. Indeed, several trials did not report any side effects after HA injections and others documented only minor local adverse reactions consisting of pain and/or swelling at the site injection, usually lasting a few hours and were spontaneously resolved (Schumacher et al., 2004; Stahl et al., 2005; Coaccioli et al., 2006; Fuchs et al., 2006; Roux et al., 2007; Heyworth et al., 2008; Bahadir et al., 2009; Figen Ayhan and Ustün, 2009; Salini et al., 2009; Di Sante et al., 2011; Ingegnoli et al., 2011; Frizziero et al., 2014; Monfort et al., 2015; Tenti et al., 2017; Ioppolo et al., 2018; Bartoloni et al., 2019; Koh et al., 2019). Only in two different studies evaluating high MW and cross-linked HA formulations, local adverse events of moderate intensity and needing ice, NSAIDs and/or selective cyclooxygenase-2 inhibitors (COXIBs) for resolution were recorded (Mandl et al., 2009; Velasco et al., 2017).

In 2007 Karalezli et al. (Karalezli et al., 2007) conducted a prospective study on 16 TBOA patients to analyze pain and tolerability of viscosupplementation therapy with HA. Patients underwent a cycle of three weekly injections of 0.3 cm^3 sodium hyaluronate: eight patients under fluoroscopy control (group A) and the others without fluoroscopy control (group B). The results confirmed the tolerability of IA HA therapy, but pain and discomfort are frequent during the injection procedure with a major degree of pain experienced by subjects from group B.

Furthermore, the analysis of an American database containing data of patients with TBOA, the Truven MarketScan® Databases, revealed that both steroid both HA injections were associated with early post-operative complications after surgical treatment of TBOA. In particular, infectious complications were associated with corticosteroids injections, while wound-healing complications were found to be related mainly to IA HA therapy (Giladi et al., 2018).

The current evidence suggests a comparable safety profile of PRP to IA HA with self-limited post-injection pain and swelling representing the most frequent reported adverse events (Nguyen and Rannou, 2017). Unfortunately, there are no data available about the tolerability of PRP injections for TBOA.

The few studies on the IA therapy with mesenchymal-derived stem cell populations did not show severe complications and consisted mainly in persisting pain after the procedure injection. In particular, Herold et al. (Herold et al., 2017) observed a transient paraesthesia of branches of the superficial radial nerve that completely resolved after 2 months in two patients, while three patients underwent additional surgical treatment for insufficient pain relief induced by the injection therapy. Also, Haas et al. (Haas et al., 2020) reported that in 2% of the cases, further operation was needed for persisting pain. Similarly, Erne et al. (Erne et al., 2018) found one patient who needed revision surgery because of persisting pain.

DISCUSSION

The present narrative review provides an updated and comprehensive overview of the efficacy and safety of different IA injection-based therapies currently employed for the management of TBOA. Concerning IA steroids and HA, it seems that IA HA may be useful in TBOA, especially in improving functional capacity and IA corticosteroids in reducing painful symptomatology (Trellu et al., 2015; Kroon et al., 2016; Riley et al., 2019), but the current evidence remains equivocal and inconclusive. Indeed, in agreement of what has already been reported by some systematic reviews and meta-analysis with a robust methodological quality, the great heterogeneity among the trials published until now does not deserve a definite conclusion about the efficacy of these treatments and whether an injection-based therapy is more effective than another one (Trellu et al., 2015; Kroon et al., 2016; Riley et al., 2019). First of all, the studies differed for the design, with only few RCTs or retrospective comparative studies; in almost all cases, they were small single-center studies with a very limited number of patients. The population analyzed was heterogeneous, particularly for the severity of the radiological grade, evaluated according to different criteria (Kellgren-Lawrence or Eaton grade). Only twelve studies included a specific symptom threshold for inclusion (e.g., VAS $\geq$ 30 mm, VAS $\geq$ 40 mm, and FIHOA $\geq$ 6) (Coaccioli et al., 2006; Fuchs et al., 2006; Roux et al., 2007; Figen Ayhan and Ustün, 2009; Di Sante et al., 2011; Ingegnoli et al., 2011; Jahangiri et al., 2014; Tenti et al., 2017; Velasco et al., 2017; Ioppolo et al., 2018; Bartoloni et al., 2019). This factor is a potential source of bias in interpreting the trials' results, considering that including participants with relatively low levels of symptoms could make less likely that a clinical meaningful difference in outcomes could be obtained. For these reasons, both Osteoarthritis Research Society International (OARSI) and European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases (ESCEO) recommendations for the conduct of pharmacological clinical trials in hand OA recommend a minimum cut-off for inclusion in terms of pain and function (Kloppenburg et al., 2015a; Reginster et al., 2018).

Another important source of heterogeneity is represented by different formulations of IA corticosteroids and HA tested with different injected volumes. Among steroids, triamcinolone acetonide, methylprednisolone and betamethasone are the most frequent used; there are no evidence supporting the superiority of a formulation over another one in TBOA, although in large joints OA, triamcinolone acetonide seems to have a greater effectiveness (Cushman et al., 2018). The HA preparations explored in the above discussed trials included HA of different MW (low, intermediate, and high), hylan, cross-linked HA and hybrid formulations. Unfortunately, no data are available about a possible difference in efficacy according to MW and viscosity in TBOA. The number of injections was variable ranging from one to three injections both for steroids and HA, as well as the technique of IA injections. In this sense, particularly debated was the accuracy of TBOA injections with and without imaging guidance, nowadays represented essentially by ultrasound. Indeed, the consensus statement on viscosupplementation (Henrotin et al., 2015) suggested to inject the trapezio-metacarpal joint under fluoroscopy or ultrasonography guidance and a recent United States cadaveric study showed a 25% higher accuracy when thumb-base joint was injected with ultrasound guidance compared to no imaging control (To et al., 2017). Conversely, other studies demonstrated success rates comparable with those obtained under ultrasound-control when the injections were performed by an experienced physician based on palpation of landmarks (Helm et al., 2003; Mandl et al., 2006).

Furthermore, another important element of heterogeneity is represented by a great variety of analyzed outcomes. Outcome Measures in Rheumatology (OMERACT) consensus recommended to evaluate in hand OA clinical trials pain, functional capacity, joint activity, and patient global assessment (Kloppenburg et al., 2015b). Few studies followed these suggestions, and for hand functionality different scores were often used, sometimes evaluating not only the hand, but the arm in its globality; few papers investigated FIHOA, validated in hand OA and considered a reliable measure of hand functionality (Kloppenburg et al., 2015b).

Also, the times of follow-up are extremely variable, ranging from 1 to 12 months, contributing to make difficult the comparison across the studies.

Another important point often poorly explored is represented by the description of the concomitant pharmacological and non-pharmacological therapy for TBOA. Indeed, in real-world application, TBOA is managed not only with injections, but with a multidisciplinary approach, so we think that more detailed information, particularly on the concomitant use of NSAIDs/analgesics and splint, can provide useful clinical implications.

Concerning PRP and mesenchymal-derived stem cell populations injections, the data are encouraging, but still too limited for any kind of conclusion. In particular, the small sample size of the analyzed studies makes it very difficult to extrapolate the results to a large scale population. Furthermore, a better understanding of the mechanism of actions of PRP and mesenchymal-derived stem cell populations and a standardized preparation method are needed to achieve a higher level of evidence in this field. It is possible that in the future both therapies can obtain a place in the management of TBOA, mainly thanks to their properties of promoting healing cartilage defects, stem cell proliferation and preventing chondrocytes and extra-cellular matrix degradation (Bonetti et al., 2020).

The tolerability of all the discussed IA therapies were found to be quite good. Local side effects are the most frequently reported and consisted mainly of painful, moderate, local inflammatory reactions at the injection site. Corticosteroids injections have the disadvantages to potentially determine skin and/or ligaments alterations, particularly in the case of repeated injections and in diabetic subjects. However, the most serious risk for IA injections remains septic arthritis which has not been described in any of the above-presented studies.

The current review of the scientific literature allowed us to find out some important points which, in our opinion, deserve further investigation. First of all, the discrepancy between the clinical experience of several physicians with expertize in this field, and the published recommendations from international scientific societies has become more evident throughout the last few years. This gap deriving from the literature evidence, which is methodologically very poor, is likely to determine negative implications, restricting patients' access to this valuable treatment option and accelerating the referrals to the surgery, a more expensive strategy and without minor risks. In our opinion, the only way to solve this discrepancy is to realize well-designed and well-conducted controlled trials, preferably double-blind RCTs or real-life studies on a large sample size of patients. Further, there is a need for homogenous trials which can follow the OARSI and ESCEO criteria for the conduct of clinical studies in hand OA, not only in selecting patients, but also in defining the most reliable pain and function outcomes (Kloppenburg et al., 2015a; Reginster et al., 2018). The follow-up should be performed in the long-term with results at 1 year. The injection procedure should be standardized, as well as the schedule of the injected agent. At this regard, we think that studies of comparisons between the different IA therapies and placebo, between different agents within the same class and between different IA treatment belonging to various pharmaceutical categories should be encouraged. Also comparing the injection-based therapies with other conservative strategies including oral pharmacological drugs, exercise, splint, different kinds of physical therapies, as laser therapy or extracorporeal shockwave therapy, should be very interesting. Finally, to understand if some disease characteristics (e.g., radiological grade) could be useful in helping clinicians in the choice of the IA therapy, should be desirable.

The main limitation of this review lies in its narrative nature with all the limitations inherent to a non-rigorous systematic review. In particular, this paper did not identify the quality and the strength of the discussed trials, and has not been built on a robust methodology structure. Further limitations are those intrinsic to the included papers which presented several consistent methodological flaws, as the not randomized controlled design.

CONCLUSION

The intra-articular injection of therapeutic agents is an attractive strategy for the local treatment of TBOA, which takes a place within the multidisciplinary approach for the management of hand OA. However, the current evidence remains equivocal. The main reason behind this is related to the poor methodology of the available scientific studies, which makes the results quite inconclusive. Some data supported the clinical usefulness of IA HA, especially in improving functional capacity and of IA corticosteroids in reducing painful symptomatology; new emerging and encouraging results derived from PRP and mesenchymal-derived stem cell populations, but they are still preliminary. At this regard, we auspicate a growing development of the scientific evidence in the field of regenerative medicine until now poorly explored in TBOA. For an exhaustive understanding of all therapeutic possibilities related to the different intra-articular agents in TBOA patients there is a need for large, independent, methodologically robust RCTs with long-term follow-up.

RESEARCH AGENDA

- To publish well-conducted double-blind RCTs on a large TBOA population and with a long-term follow-up
- To use standardized selection criteria and standardized efficacy outcomes to make the different studies uniform and comparable
- To uniform the injection technique and the therapeutic regimens (dosage, number of injections, kind of formulation of steroid and HA)
- To compare the IA agents with each other, with placebo, and with other conservative therapeutic options
- To find out if a corticosteroid or HA formulation is superior to another one in TBOA
- To study the additional symptomatic effect of the different IA therapies, combined with other therapeutic options such as pharmacological management, physiotherapy and splinting
- To identify patients and disease characteristics useful to guide the choice of the IA agent

AUTHOR CONTRIBUTIONS

AF and ST conceived the topic and the design of work. ST and NM contributed to the literature search, while SC and SG organized the database and contributed to the analysis and interpretation of the literature data. AF solved disagreement between the two Authors. AF and ST wrote the first draft of the manuscript. SC, NM, and SG revised the paper critically for important intellectual content and provided approval for publication of the content.

REFERENCES

Altman, R., Alarcón, G., Appelrouth, D., Bloch, D., Borenstein, D., Brandt, K., et al. (1990). The American college of rheumatology criteria for the classification and reporting of osteoarthritis of the hand. *Arthritis. Rheum.* 33, 601–610. doi:10.1002/art.1780331101

Bahadir, C., Onal, B., Dayan, V. Y., and Gürer, N. (2009). Comparison of therapeutic effects of sodium hyaluronate and corticosteroid injections on trapeziometacarpal joint osteoarthritis. *Clin. Rheumatol.* 28, 529–533. doi:10.1007/s10067-008-1079-6

Bartoloni, E., Luccioli, F., La Paglia, G. M. C., Cafaro, G., Marcucci, E., and Gerli, R. (2019). Effect of Sinovial High-Low® injections in trapeziometacarpal osteoarthritis. *Clin. Exp. Rheumatol.* 37, 166.

Bijsterbosch, J., Visser, W., Kroon, H. M., Stamm, T., Meulenbelt, I., Huizinga, T. W., et al. (2010). Thumb base involvement in symptomatic hand osteoarthritis is associated with more pain and functional disability. *Ann. Rheum. Dis.* 69, 585–587. doi:10.1136/ard.2009.104562

Bohr, S., Rennekampff, H. O., and Pallua, N. (2015). Cell-enriched lipoaspirate arthroplasty: a novel approach to first carpometacarpal joint arthritis. *Hand Surg.* 20, 479–481. doi:10.1142/S0218810415720259

Bonetti, M. A., Rovere, G., Fulchignoni, C., De Santis, V., Ziranu, A., Maccauro, G., et al. (2020). Autologous fat transplantation for the treatment of trapeziometacarpal joint osteoarthritis. *Orthop. Rev. (Pavia)* 12, 8666. doi:10.4081/or.2020.8666

Bosetti, M., Borrone, A., Follenzi, A., Messaggio, F., Tremolada, C., and Cannas, M. (2016). Human lipoaspirate as autologous injectable Active scaffold for one-step repair of cartilage defects. *Cel Transpl.* 25, 1043–1056. doi:10.3727/096368915X689514

Centeno, C. J., and Freeman, M. D. (2014). Percutaneous injection of autologous, culture-expanded mesenchymal stem cells into carpometacarpal hand joints: a case series with an untreated comparison group. *Wien Med. Wochenschr.* 164, 83–87. doi:10.1007/s10354-013-0222-4

Coaccioli, S., Pinoca, F., and Puxeddu, A. (2006). Short term efficacy of intra-articular injection of hyaluronic acid in osteoarthritis of the first carpometacarpal joint in a preliminary open pilot study. *Clin. Ter.* 157, 321–325.

Cormier, G., Le Goff, B., Denis, A., Varin, S., Auzanneau, L., Dimet, J., et al. (2019). Corticosteroids injections versus corticosteroids with hyaluronic acid injections in rhizarthrosis: the randomised multicentre RHIZ'ART trial study protocol. *BMJ Open* 9, e022553. doi:10.1136/bmjopen-2018-022553

Cushman, D. M., Bruno, B., Christiansen, J., Schultz, A., and McCormick, Z. L. (2018). Efficacy of injected corticosteroid type, dose, and volume for pain in large joints: a narrative review. *PM R.* 10, 748–757. doi:10.1016/j.pmrj.2018.01.002

Dahaghin, S., Bierma-Zeinstra, S. M. A., Ginai, A. Z., Pols, H. A. P., Hazes, J. M. W., and Koes, B. W. (2005). Prevalence and pattern of radiographic hand osteoarthritis and association with pain and disability (the Rotterdam study). *Ann. Rheum. Dis.* 64, 682–687. doi:10.1136/ard.2003.017087

Dauvissat, J., Rizzo, C., Lellouche, H., Porterie, J., Melac-Ducamp, S., Locquet, V., et al. (2018). Safety and predictive factors of short-term efficacy of a single injection of mannitol-modified cross-linked hyaluronic acid in patients with trapeziometacarpal osteoarthritis. Results of a multicentre prospective open-label pilot study (INSTINCT trial). *Clin. Med. Insights Arthritis Musculoskelet. Disord.* 11, 1179544118782901. doi:10.1177/1179544118782901

Day, C. S., Gelberman, R., Patel, A. A., Vogt, M. T., Ditsios, K., and Boyer, M. I. (2004). Basal joint osteoarthritis of the thumb: a prospective trial of steroid injection and splinting. *J. Hand Surg. Am.* 29, 247–251. doi:10.1016/j.jhsa.2003.12.002

Di Sante, L., Cacchio, A., Scettri, P., Paoloni, M., Ioppolo, F., and Santilli, V. (2011). Ultrasound-guided procedure for the treatment of trapeziometacarpal osteoarthritis. *Clin. Rheumatol.* 30, 1195–1200. doi:10.1007/s10067-011-1730-5

Eaton, R. G., and Glickel, S. Z. (1987). Trapeziometacarpal osteoarthritis, staging as a rationale for treatment. *Hand Clin.* 3, 455–471.

Erne, H. C., Cerny, M. K., Ehrl, D., Bauer, A. T., Schmauss, V., Moog, P., et al. (2018). Autologous fat injection versus Lundborg resection arthroplasty for the treatment of trapeziometacarpal joint osteoarthritis. *Plast. Reconstr. Surg.* 141, 119–124. doi:10.1097/PRS.0000000000003913

Evans, A., Ibrahim, M., Pope, R., Mwangi, J., Botros, M., Johnson, S. P., et al. (2020). Treating hand and foot osteoarthritis using a patient's own blood: a systematic review and meta-analysis of platelet-rich plasma. *J. Orthop.* 18, 226–236. doi:10.1016/j.jor.2020.01.037

Figen Ayhan, F., and Ustün, N. (2009). The evaluation of efficacy and tolerability of Hylan G-F 20 in bilateral thumb base osteoarthritis: 6 months follow-up. *Clin. Rheumatol.* 28, 535–541. doi:10.1007/s10067-008-1080-0

Frizziero, A., Maffulli, N., Masiero, S., and Frizziero, L. (2014). Six-months pain relief and functional recovery after intra-articular injections with hyaluronic acid (mw 500–730 kDa) in trapeziometacarpal osteoarthritis. *Muscles Ligaments Tendons J.* 4, 256–261.

Fuchs, S., Mönikes, R., Wohlmeiner, A., and Heyse, T. (2006). Intra-articular hyaluronic acid compared with corticoid injections for the treatment of rhizarthrosis. *Osteoarthritis Cartilage* 14, 82–88. doi:10.1016/j.joca.2005.07.016

Gil, C., Abdoul, H., Campagna, R., Guerini, H., Ieong, E., Chagny, F., et al. (2018). Intra-articular botulinum toxin A for base-of-thumb osteoarthritis: protocol for a randomised trial (RHIBOT). *BMJ Open* 8, e022337. doi:10.1136/bmjopen-2018-022337

Giladi, A. M., Rahgozar, P., Zhong, L., and Chung, K. C. (2018). Corticosteroid or hyaluronic acid injections to the carpometacarpal joint of the thumb joint are associated with early complications after subsequent surgery. *J. Hand Surg. Eur.* 43, 1106–1110. doi:10.1177/1753193418805391

Haas, E. M., Eisele, A., Arnoldi, A., Paolini, M., Ehrl, D., Volkmer, E., et al. (2020). One-year outcomes of intraarticular fat transplantation for thumb carpometacarpal joint osteoarthritis: case review of 99 joints. *Plast. Reconstr. Surg.* 145, 151–159. doi:10.1097/PRS.0000000000006378

Haugen, I. K., Englund, M., Aliabadi, P., Niu, J., Clancy, M., Kvien, T. K., et al. (2011). Prevalence, incidence and progression of hand osteoarthritis in the general population: the Framingham Osteoarthritis Study. *Ann. Rheum. Dis.* 70, 1581–1586. doi:10.1136/ard.2011.150078

Helm, A. T., Higgins, G., Rajkumar, P., and Redfern, D. R. (2003). Accuracy of intra-articular injections for osteoarthritis of the trapeziometacarpal joint. *Int. J. Clin. Pract.* 57, 265–266.

Henrotin, Y., Raman, R., Richette, P., Bard, H., Jerosch, J., Conrozier, T., et al. (2015). Consensus statement on viscosupplementation with hyaluronic acid for the management of osteoarthritis. *Semin. Arthritis Rheum.* 45, 140–149. doi:10.1016/j.semarthrit.2015.04.011

Herold, C., Rennekampff, H. O., Groddeck, R., and Allert, S. (2017). Autologous fat transfer for thumb carpometacarpal joint osteoarthritis: a prospective study. *Plast. Reconstr. Surg.* 140, 327–335. doi:10.1097/PRS.0000000000003510

Heyworth, B. E., Lee, J. H., Kim, P. D., Lipton, C. B., Strauch, R. J., and Rosenwasser, M. P. (2008). Hylan versus corticosteroid versus placebo for treatment of basal joint arthritis: a prospective, randomized, double-blinded clinical trial. *J. Hand Surg. Am.* 33, 40–48. doi:10.1016/j.jhsa.2007.10.009

Ingegnoli, F., Soldi, A., and Meroni, P. L. (2011). Power Doppler sonography and clinical monitoring for hyaluronic Acid treatment of rhizarthrosis: a pilot study. *J. Hand Microsurg.* 3, 51–54. doi:10.1007/s12593-011-0037-8

Ioppolo, F., Saracino, F., Rizzo, R. S., Monacelli, G., Lanni, D., Di Sante, L., et al. (2018). Comparison between extracorporeal shock wave therapy and intra-articular hyaluronic acid injections in the treatment of first carpometacarpal joint osteoarthritis. *Ann. Rehabil. Med.* 42, 92–100. doi:10.5535/arm.2018.42.1.92

Jahangiri, A., Moghaddam, F. R., and Najafi, S. (2014). Hypertonic dextrose versus corticosteroid local injection for the treatment of osteoarthritis in the first carpometacarpal joint: a double-blind randomized clinical trial. *J. Orthop. Sci.* 19, 737–743. doi:10.1007/s00776-014-0587-2

Joshi, R. (2005). Intraarticular corticosteroid injection for first carpometacarpal osteoarthritis. *J. Rheumatol.* 32, 1305–1306.

Jüni, P., Hari, R., Rutjes, A. W., Fischer, R., Silletta, M. G., Reichenbach, S., et al. (2015). Intra-articular corticosteroid for knee osteoarthritis. *Cochrane Database Syst. Rev. Cd005328*. doi:10.1002/14651858.CD005328.pub3

Karalezli, N., Ogun, T. C., Kartal, S., Saracgil, S. N., Yel, M., and Tuncay, I. (2007). The pain associated with intraarticular hyaluronic acid injections for trapeziometacarpal osteoarthritis. *Clin. Rheumatol.* 26, 569–571. doi:10.1007/s10067-006-0354-7

Khan, M., Waseem, M., Raza, A., and Derham, D. (2009). Quantitative assessment of improvement with single corticosteroid injection in thumb CMC joint osteoarthritis?. *Open Orthop. J.* 3, 48–51. doi:10.2174/1874325000903010048

Kloppenburg, M., Bøyesen, P., Visser, A. W., Haugen, I. K., Boers, M., Boonen, A., et al. (2015b). Report from the OMERACT hand osteoarthritis working group: set of core domains and preliminary set of instruments for use in clinical trials and observational studies. *J. Rheumatol.* 42, 2190–2197. doi:10.3899/jrheum.141017

Kloppenburg, M., Kroon, F. P., Blanco, F. J., Doherty, M., Dziedzic, K. S., Greibrokk, E., et al. (2019). 2018 update of the EULAR recommendations for the management of hand osteoarthritis. *Ann. Rheum. Dis.* 78, 16–24. doi:10.1136/annrheumdis-2018-213826

Kloppenburg, M., Maheu, E., Kraus, V. B., Cicuttini, F., Doherty, M., Dreiser, R. L., et al. (2015a). OARSI hand clinical trial recommendations work GroupOARSI clinical trials recommendations: design and conduct of clinical trials for hand

osteoarthritis. *Osteoarthritis Cartilage* 23, 772–786. doi:10.1016/j.joca.2015.03.007

Kloppenburg, M., van Beest, S., and Kroon, F. P. B. (2017). Thumb base osteoarthritis: a hand osteoarthritis subset requiring a distinct approach. *Best Pract. Res. Clin. Rheumatol.* 31, 649–660. doi:10.1016/j.berh.2018.08.007

Koh, S. H., Lee, S. C., Lee, W. Y., Kim, J., and Park, Y. (2019). Ultrasound-guided intra-articular injection of hyaluronic acid and ketorolac for osteoarthritis of the carpometacarpal joint of the thumb: a retrospective comparative study. *Medicine (Baltimore)* 98, e15506. doi:10.1097/MD.0000000000015506

Kolasinski, S. L., Neogi, T., Hochberg, M. C., Oatis, C., Guyatt, G., and Block, J. (2020). 2019 American College of rheumatology/arthritis foundation guideline for the management of osteoarthritis of the hand, hip, and knee. *Arthritis Rheumatol.* 72, 220–233. doi:10.1002/art.41142

Kroon, F. P., Rubio, R., Schoones, J. W., and Kloppenburg, M. (2016). Intra-articular therapies in the treatment of hand osteoarthritis: a systematic literature review. *Drugs Aging* 33, 119–133. doi:10.1007/s40266-015-0330-5

Loibl, M., Lang, S., Dendl, L. M., Nerlich, M., Angele, P., Gehmert, S., et al. (2016). Leukocyte-reduced platelet-rich plasma treatment of basal thumb arthritis: a pilot study. *Biomed. Res. Int.* 2016, 9262909. doi:10.1155/2016/9262909

Malahias, M. A., Roumeliotis, L., Nikolaou, V. S., Chronopoulos, E., Sourlas, I., and Babis, G. C. (2018). Platelet-rich plasma versus corticosteroid intra-articular injections for the treatment of trapeziometacarpal arthritis: a prospective randomized controlled clinical trial. *CARTILAGE.* 12(1):51-61. doi:10.1177/1947603518805230

Mandl, L. A., Hotchkiss, R. N., Adler, R. S., Ariola, L. A., and Katz, J. N. (2006). Can the carpometacarpal joint be injected accurately in the office setting? Implications for therapy. *J. Rheumatol.* 33, 1137–1139.

Mandl, L. A., Hotchkiss, R. N., Adler, R. S., Lyman, S., Daluiski, A., Wolfe, S. W., et al. (2009). Injectable hyaluronan for the treatment of carpometacarpal osteoarthritis: open label pilot trial. *Curr. Med. Res. Opin.* 25, 2103–2108. doi:10.1185/03007990903084016

Marx, R. E. (2001). Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant. *Dent* 10, 225–228. doi:10.1097/00008505-200110000-00002

McAlindon, T. E., LaValley, M. P., Harvey, W. F., Price, L. L., Driban, J. B., Zhang, M., et al. (2017). Ward rj. Effect of intra-articular triamcinolone vs saline on knee cartilage volume and pain in patients with knee osteoarthritis: a randomized clinical trial. *JAMA* 317, 1967–1975. doi:10.1001/jama.2017.5283

Medina-Porqueres, I., Martin-Garcia, P., Sanz-De Diego, S., Reyes-Eldblom, M., and Cantero-Tellez, R. (2019). Platelet-rich plasma for thumb carpometacarpal joint osteoarthritis in a professional pianist: case-based review. *Rheumatol. Int.* 39, 2167–2175. doi:10.1007/s00296-019-04454-x

Meenagh, G. K., Patton, J., Kynes, C., and Wright, G. D. (2004). A randomised controlled trial of intra-articular corticosteroid injection of the carpometacarpal joint of the thumb in osteoarthritis. *Ann. Rheum. Dis.* 63, 1260–1263. doi:10.1136/ard.2003.015438

Monfort, J., Rotés-Sala, D., Segalés, N., Montañes, F. J., Orellana, C., Llorente-Onaindia, J., et al. (2015). Comparative efficacy of intra-articular hyaluronic acid and corticoid injections in osteoarthritis of the first carpometacarpal joint: results of a 6-month single-masked randomized study. *Jt. Bone Spine* 82, 116–121. doi:10.1016/j.jbspin.2014.08.008

Moussa, M., Lajeunesse, D., Hilal, G., El Atat, O., Haykal, G., Serhal, R., et al. (2017). Platelet rich plasma (PRP) induces chondroprotection via increasing autophagy, anti-inflammatory markers, and decreasing apoptosis in human osteoarthritic cartilage. *Exp. Cel Res.* 352, 146–156. doi:10.1016/j.yexcr.2017.02.012

Nguyen, C., and Rannou, F. (2017). The safety of intra-articular injections for the treatment of knee osteoarthritis: a critical narrative review. *Expert Opin. Drug Saf.* 16, 897–902. doi:10.1080/14740338.2017.1344211

Ornetti, P., Nourissat, G., Berenbaum, F., Sellam, J., Richette, P., and Chevalier, X. (2016). Under the aegis of the Osteoarthritis Section of the French Society for Rheumatology (Société Française de Rhumatologie, SFR).Does platelet-rich plasma have a role in the treatment of osteoarthritis?. *Jt. Bone Spine* 83, 31–36. doi:10.1016/j.jbspin.2015.05.002

Pak, J., Lee, J. H., Pak, N., Pak, Y., Park, K. S., Jeon, J. H., et al. (2018). Cartilage regeneration in humans with adipose tissue-derived stem cells and adipose stromal vascular fraction cells: updated status. *Int. J. Mol. Sci.* 19, 2146. doi:10.3390/ijms19072146

Reginster, J. L., Arden, N. K., Haugen, I. K., Rannou, F., Cavalier, E., Bruyère, O., et al. (2018). Guidelines for the conduct of pharmacological clinical trials in hand osteoarthritis: consensus of a working group of the European society on clinical and economic Aspects of Osteoporosis, osteoarthritis and musculoskeletal diseases (ESCEO). *Semin. Arthritis Rheum.* 48, 1–8. doi:10.1016/j.semarthrit.2017.12.003

Reichenbach, S., Blank, S., Rutjes, A. W., Shang, A., King, E. A., Dieppe, P. A., et al. (2007). Hylan versus hyaluronic acid for osteoarthritis of the knee: a systematic review and meta-analysis. *Arthritis Rheum.* 57, 1410–1418. doi:10.1002/art.23103

Riley, N., Vella-Baldacchino, M., Thurley, N., Hopewell, S., Carr, A. J., and Dean, B. J. F. (2019). Injection therapy for base of thumb osteoarthritis: a systematic review and meta-analysis. *BMJ Open* 9, e027507. doi:10.1136/bmjopen-2018-027507

Rocchi, L., Merolli, A., Giordani, L., Albensi, C., and Foti, C. (2018). Trapeziometacarpal joint osteoarthritis: a prospective trial on two widespread conservative therapies. *Muscles Ligaments Tendons J.* 7, 603–610. doi:10.11138/mltj/2017.7.4.603

Roux, C., Fontas, E., Breuil, V., Brocq, O., Albert, C., and Euller-Ziegler, L. (2007). Injection of intra-articular sodium hyaluronidate (Sinovial) into the carpometacarpal joint of the thumb (CMC1) in osteoarthritis. A prospective evaluation of efficacy. *Jt. Bone Spine* 74, 368–372. doi:10.1016/j.jbspin.2006.08.008

Salini, V., De Amicis, D., Abate, M., Natale, M. A., and Di Iorio, A. (2009). Ultrasound-guided hyaluronic acid injection in carpometacarpal osteoarthritis: short-term results. *Int. J. Immunopathol. Pharmacol.* 22, 455–460. doi:10.1177/039463200902200222

Schumacher, H. R., Meador, R., Sieck, M., and Mohammed, Y. (2004). Pilot investigation of hyaluronate injections for first metacarpal-carpal (MC-C) osteoarthritis. *J. Clin. Rheumatol.* 10, 59–62. doi:10.1097/01.rhu.0000120894.49180.99

Sodha, S., Ring, D., Zurakowski, D., and Jupiter, J. B. (2005). Prevalence of osteoarthrosis of the trapeziometacarpal joint. *J. Bone Jt. Surg. Am.* 87, 2614–2618. doi:10.2106/JBJS.E.00104

Sonne-Holm, S., and Jacobsen, S. (2006). Osteoarthritis of the first carpometacarpal joint: a study of radiology and clinical epidemiology. Results from the Copenhagen Osteoarthritis Study. *Osteoarthritis Cartilage* 14, 496–500. doi:10.1016/j.joca.2005.12.001

Stahl, S., Karsh-Zafrir, I., Ratzon, N., and Rosenberg, N. (2005). Comparison of intraarticular injection of depot corticosteroid and hyaluronic acid for treatment of degenerative trapeziometacarpal joints. *J. Clin. Rheumatol.* 11, 299–302. doi:10.1097/01.rhu.0000191194.39926.c9

Swindells, M. G., Logan, A. J., Armstrong, D. J., Chan, P., Burke, F. D., and Lindau, T. R. (2010). The benefit of radiologically-guided steroid injections for trapeziometacarpal osteoarthritis. *Ann. R. Coll. Surg. Engl.* 92, 680–684. doi:10.1308/003588410X12699663905078

Tenti, S., Ferretti, F., Gusinu, R., Gallo, I., Giannotti, S., Pozza, A., et al. (2020). Impact of thumb osteoarthritis on pain, function, and quality of life: a comparative study between erosive and non-erosive hand osteoarthritis. *Clin. Rheumatol.* 39, 2195–2206. doi:10.1007/s10067-020-04982-z

Tenti, S., Pascarelli, N. A., Giannotti, S., Galeazzi, M., Giordano, N., and Fioravanti, A. (2017). Can hybrid hyaluronic acid represent a valid approach to treat rizoarthrosis? A retrospective comparative study. *BMC Musculoskelet. Disord.* 18, 444. doi:10.1186/s12891-017-1809-5

To, P., McClary, K. N., Sinclair, M. K., Stout, B. A., Foad, M., Hiratzka, S., et al. (2017). The accuracy of common hand injections with and without ultrasound: an anatomical study. *Hand (N Y)* 12, 591–596. doi:10.1177/1558944717692086

Trellu, S., Dadoun, S., Berenbaum, F., Fautrel, B., and Gossec, L. (2015). Intra-articular injections in thumb osteoarthritis: a systematic review and meta-analysis of randomized controlled trials. *Jt. Bone Spine* 82, 315–319. doi:10.1016/j.jbspin.2015.02.002

Velasco, E., Ribera, M. V., and Pi, J. (2017). Single-arm open-label study of Durolane (NASHA nonanimal hyaluronic acid) for the treatment of osteoarthritis of the thumb. *Open Access Rheumatol.* 9, 61–66. doi:10.2147/OARRR.S128675

Wernecke, C., Braun, H. J., and Dragoo, J. L. (2015). The effect of intra-articular corticosteroids on articular cartilage: a systematic review. *Orthop. J. Sports Med.* 3, 2325967115581163. doi:10.1177/2325967115581163

Zeng, C., Lane, N. E., Hunter, D. J., Wei, J., Choi, H. K., McAlindon, T. E., et al. (2019). Intra-articular corticosteroids and the risk of knee osteoarthritis progression: results from the Osteoarthritis Initiative. *Osteoarthritis Cartilage* 27, 855–862. doi:10.1016/j.joca.2019.01.007

Zhang, W., Doherty, M., Leeb, B. F., Alekseeva, L., Arden, N. K., Bijlsma, J. W., et al. (2007). EULAR evidence based recommendations for the management of hand osteoarthritis: report of a task force of the EULAR standing committee for international clinical studies including therapeutics (ESCISIT). *Ann. Rheum. Dis.* 66, 377–388. doi:10.1136/ard.2006.062091

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 15 April 2021
doi: 10.3389/fphar.2021.645842

New Trends in Pharmacological Treatments for Osteoarthritis

Xiaoyan Cai[1†], Shiwen Yuan[1†], Yanting Zeng[1], Cuicui Wang[1], Na Yu[1] and Changhai Ding[1,2,3]**

[1]Department of Rheumatology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, Guangzhou, China, [2]Clinical Research Centre, Zhujiang Hospital, Southern Medical University, Guangzhou, China, [3]Menzies Institute for Medical Research, University of Tasmania, Hobart, TAS, Australia

Osteoarthritis (OA) is the leading cause of function loss and disability among the elderly, with significant burden on the individual and society. It is a severe disease for its high disability rates, morbidity, costs, and increased mortality. Multifactorial etiologies contribute to the occurrence and development of OA. The heterogeneous condition poses a challenge for the development of effective treatment for OA; however, emerging treatments are promising to bring benefits for OA management in the future. This narrative review will discuss recent developments of agents for the treatment of OA, including potential disease-modifying osteoarthritis drugs (DMOADs) and novel therapeutics for pain relief. This review will focus more on drugs that have been in clinical trials, as well as attractive drugs with potential applications in preclinical research. In the past few years, it has been realized that a complex interaction of multifactorial mechanisms is involved in the pathophysiology of OA. The authors believe there is no miracle therapeutic strategy fitting for all patients. OA phenotyping would be helpful for therapy selection. A variety of potential therapeutics targeting inflammation mechanisms, cellular senescence, cartilage metabolism, subchondral bone remodeling, and the peripheral nociceptive pathways are expected to reshape the landscape of OA treatment over the next few years. Precise randomized controlled trials (RCTs) are expected to identify the safety and efficacy of novel therapies targeting specific mechanisms in OA patients with specific phenotypes.

Keywords: osteoarthritis, novel therapeutics, DMOADs, therapy selection, clinical prospect

OPEN ACCESS

Edited by:
Per-Johan Jakobsson, Karolinska Institutet (KI), Sweden

Reviewed by:
Martijn H. Van Den Bosch, Radboud University Nijmegen Medical Centre, Netherlands
Giustino Orlando, University of Studies G. d'Annunzio Chieti and Pescara, Italy

****Correspondence:***
Na Yu
546046726@qq.com
Changhai Ding
changhai.ding@utas.edu.au

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *24 December 2020*
Accepted: *16 March 2021*
Published: *15 April 2021*

Citation:
Cai X, Yuan S, Zeng Y, Wang C, Yu N and Ding C (2021) New Trends in Pharmacological Treatments for Osteoarthritis. Front. Pharmacol. 12:645842. doi: 10.3389/fphar.2021.645842

INTRODUCTION

Osteoarthritis (OA) can be viewed as the structural and functional failure of the synovial joint organ (Loeser et al., 2012). All tissues of the joint can be involved, including articular cartilage, subchondral bone, and synovium (Felson, 2006). OA is the leading cause of function loss and disability among elderly, which makes these patients suffer from chronic pain (Hunter and Bierma-Zeinstra, 2019). Traditionally the management of OA has been constrained to symptom relieving (Arden et al., 2020); the non-steroidal anti-inflammatory drugs (NSAIDs) or analgesics are most commonly applied to OA for relieving pain, however, their side-effects often restrict their use (Bally et al., 2017; da Costa et al., 2017; Fuggle et al., 2019; Leopoldino et al., 2019). In recent years, there has been substantial progress made in understanding the pathogenesis of OA.

OA is a very complicated pathophysiologic process and is a result of interacting action of multiple mechanisms. Mechanical overload, genetic alterations, sex hormone deficiency, aging, metabolic imbalance and low-grade chronic inflammation all may contribute to the imbalance between catabolism and anabolism of joint tissues, and lead to eventual joint damage in OA. The

TABLE 1 | Major emerging drugs to control structural damage and relieve pain in OA clinical trials.

Type of drug	Route of administration	Major findings	Stage of development	Clinical trials. gov identifier
Targeting inflammatory mechanisms				
IL-1 inhibitors				
Anakinra	Intra-articular	Anakinra did not significantly improve symptoms in patients with knee OA.	Phase II (knee OA)	NCT00110916
AMG 108	Subcutaneous/Intra-articular	AMG 108 showed statistically insignificant but numerically greater improvements in pain.	Phase II (knee OA)	NCT00110942
Canakinumab	Intra-articular	The clinical trial was completed, but the results have not been published.	Phase II (knee OA)	NCT01160822
Gevokizumab	Subcutaneous	The clinical trials were completed, but the results have not been published.	Phase II (erosive hand OA) Phase II (erosive hand OA)	NCT01683396 NCT01882491
Lutikizumab (ABT-981)	Subcutaneous	Lutikizumab was generally well tolerated in patients with knee OA and elicited an anti-inflammatory response.	Phase I (knee OA)	NCT01668511
		Lutikizumab did not improve pain or imaging outcomes in erosive hand OA compared with placebo.	Phase IIa (erosive hand OA)	NCT02384538
		Lutikizumab was not an effective analgesic/anti-inflammatory therapy in most patients with knee OA associated synovitis.	Phase IIa (knee OA)	NCT02087904 (ILL-USTRATE- K trail)
TNF-α inhibitors				
Etanercept	Subcutaneous	Subcutaneous injection of Etanercept for 24 weeks did not relieve pain effectively in patients with erosive hand OA compared with placebo.	—	NTR1192 (EHOA trail)
Infliximab	Intra-articular	Treatment with Infliximab can reduce the incidence of secondary OA in proximal interphalangeal joints in patients with active RA.	Exploratory observational longitudinal study	—
		Infliximab was safe, and significantly improved pain symptoms	Plot study (erosive hand OA)	—
Adalimumab	Subcutaneous	Adalimumab was not superior to placebo in relieving pain in patients with erosive hand OA.	Phase III (erosive hand OA)	NCT00597623
		Adalimumab did not affect synovitis or BMLs in patients with hand OA with MRI-detected synovitis.	—	ACTRN12612000791831 (HUMOR trial)
		Adalimumab significantly slowed the progression of joint aggressive lesions in a subpopulation with palpable tissue swelling of the interphalangeal joints.	—	EudraCT 2006–000925–71
DMARDs				
HCQ	Oral	HCQ did not relieve symptoms or delay structural damage.	—	ISRCTN91859104 (HERO trial)
MTX	Oral	MTX significantly reduced pain and improved synovitis in patients with symptomatic knee OA.	—	NCT01927484
		MTX added to usual care demonstrated significant reduction in knee OA pain at 6 months, and significant improvements in WOMAC stiffness and function. No effect on synovitis	Phase III (knee OA)	ISRCTN77854383 (PROMOTE trial)
		The clinical trial is ongoing	—	NCT03815448
Removing SnCs				
UBX0101	Intra-articular	The clinical trials were completed, but the results have not been published.	Phase I (knee OA) Phase I (knee OA) Phase II (knee OA)	NCT03513016 NCT04229225 NCT04129944
Curcuma longa extract	Oral	Curcuma longa extract was more effective than placebo for knee pain but did not affect knee effusion–synovitis or cartilage composition.	Phase II (knee OA)	ACTRN12618000080224
		The clinical trial is ongoing	Phase III (hip or knee pain)	NCT04500210
Targeting Cartilage Metabolism				
Wnt pathway inhibitors				
Lorecivivint (SM04690)	Intra-articular	Lorecivivint 0.07 mg was superior to the placebo in improving pain and function, and increased the JSW in patients with knee OA.	Phase I (knee OA)	NCT02095548
		Lorecivivint had no significant effects in knee OA patients, but significantly relieved pain, improved joint function, and increased JSW in a subgroup of patients (patients with unilateral symptomatic knee OA and unilateral symptomatic knee OA without extensive pain).	Phase IIa (knee OA)	NCT02536833
		The clinical trial is ongoing	Phase III (knee OA)	NCT03928184
Cathepsin-K inhibitors				
MIV-711	Oral	MIV-711 was not more effective than placebo for pain, but it significantly reduced bone and cartilage progression with a reassuring safety profile.	Phase IIa (knee OA)	NCT02705625

(Continued on following page)

TABLE 1 | (*Continued*) Major emerging drugs to control structural damage and relieve pain in OA clinical trials.

Type of drug	Route of administration	Major findings	Stage of development	Clinical trials. gov identifier
MMP/ADAMTS inhibitors				
AGG-523	Oral	The clinical trials were completed, but the results have not been published	Phase I (knee OA) Phase I (knee OA)	NCT00454298 NCT00427687
M6495	Subcutaneous	The clinical trial was completed, but the results have not been published.	Phase Ib (knee OA)	NCT03583346
Growth factors				
Sprifermin (rhFGF18)	Intra-articular	Sprifermin appeared safe and well-tolerated, and it showed a statistically significant dose-dependent effect in reducing the loss of total and lateral femorotibial cartilage thickness and loss of lateral radiographic JSW.	Phase I (knee OA)	NCT01033994
		Sprifermin had a limited effect on pain improvement, but had a statistically significant effect in reducing the loss of total femorotibial cartilage thickness.	Phase II (knee OA)	NCT01919164 (FO-RWARD trial)
GEC-TGF-β1	Intra-articular	GEC-TGF-β1 significantly improved pain function and physical ability.	Phase II (knee OA) Phase II (knee OA)	NCT01221441 NCT01671072
		GEC-TGF-β1 had beneficial effects on pain and functional improvement in patients with OA, but had limited effects on structural improvement.	Phase III (knee OA)	NCT02072070
Activating AMPK pathway				
Metformin	Oral	Metformin may have a beneficial effect on long-term knee joint outcomes in those with knee OA and obesity.	Prospective cohort study (knee OA)	—
Targeting the Subchondral Bone				
Bisphosphonate				
Zoledronic Acid	Intra-articular	Zoledronic acid did not significantly reduce cartilage volume loss, relieve pain, or improve BMLs.	Phase **III** (Knee OA)	ACTRN12613000039785
Calcitonin				
Salmon calcitonin	Oral	Salmon calcitonin did not improve pain symptoms and JSW in patients with symptomatic knee OA.	Phase **III** (Knee OA)	NCT00486434 NCT00704847
Strontium Ranelate	Oral	Strontium Ranelate significantly inhibited the narrowing of the medial femoral joint space, relieved pain, and improved physical function in patients with moderate to severe knee OA.	Phase **III** (Knee OA)	ISRCTN41323372 (SEKOIA trial
Teriparatide	Subcutaneous	The clinical trial is ongoing.	Phase **II** (knee OA)	NCT03072147
Vitamin D	Oral	Vitamin D supplementation, compared with placebo, did not result in significant differences in change in MRI-measured tibial cartilage volume or WOMAC knee pain score over 2 years, but might have beneficial effects on physical function, foot pain, depressive symptoms and effusion-synovitis.	Phase **III** (Knee OA)	NCT01176344
Investigational Drugs to relieve pain				
NGF inhibitors				
Tanezumab	Subcutaneous	Tanezumab was significantly better than the placebo in improving pain and physical function, and PGA-OA.	Phase III (hip or knee OA)	NCT02697773
		Tanezumab statistically significantly improved pain, physical function and PGA-OA in patients with moderate to severe OA who had not responded to or could not tolerate standard-of-care analgesics	Phase III (hip or knee OA)	NCT02709486
Fasinumab	Subcutaneous	Fasinumab significantly improved pain and function in patients with OA, even in those who obtained little benefit from previous analgesics	Phase IIb/III (hip or knee OA)	NCT02447276
		The clinical trials are ongoing	Phase III (hip or knee OA)	NCT02683239 NCT03285646 NCT03161093 NCT03304379
Triamcinolone acetonide sustained-release agent				
Zilretta (FX006)	Intra-articular	Zilretta significantly reduced ADP-intensity compared with saline-solution placebo. Zilretta significantly improved pain, stiffness, physical function, and the quality of life compared with both placebo and TAcs	Phase III (knee OA)	NCT02357459

OA: osteoarthritis; RA: rheumatoid arthritis; BMLs: bone marrow lesions; DMARDs: disease-modifying antirheumatic drugs; HCQ: hydroxychloroquine; MTX: methotrexate; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index; SnCs: senescent cells; JSW: joint space width; MMP: matrix metalloproteinase; ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs; rhFGF18: recombinant human fibroblast growth factor 18; NGF: nerve growth factor; PGA-OA: patient's Global assessment of OA; ADP: average-daily-pain; TAcs: triamcinolone acetonide crystal suspensions.

etiological heterogeneity causes a great difficulty on the development of an effective treatment for OA. The development of OA is a very complicated pathophysiologic process and is a result of interaction of multiple mechanisms. Mechanical overload, genetic alterations, sex hormone deficiency, aging, metabolic imbalance and low-grade chronic inflammation all may contribute to the imbalance between catabolism and anabolism of joint tissues and lead to eventual joint damage in OA (Chen D. et al., 2017; Oo et al., 2018). The etiological heterogeneity causes a great difficulty on the development of an effective treatment for OA. Epidemiological data support significant associations between structural changes and long-term outcome. However, the available therapeutic regimens of OA are merely symptom-relieving drugs unable to modify the progression of OA and to prevent long-term disability, and the symptom-structure discordance is well-recognized in clinical course of OA. Thus, the guidelines from the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) point out that the effective disease-modifying osteoarthritis drugs (DMOADs) should be developed (Reginster et al., 2015; Oo et al., 2018). A DMOAD is expected a drug that modifies the underlying OA pathophysiology, thereby inhibiting structural damage to prevent or reduce long-term disability and offer potential symptomatic relief (Latourte et al., 2020). Currently, there are no US FDA- or EMA-approved DMOADs. But emerging treatments targeting inflammation, cartilage metabolism, and subchondral bone remodeling, which may retard the structural progression and induce disease remission, are promising to bring benefits to OA management in the future.

This narrative review will discuss recent developments of agents for the treatment of OA, including potential DMOADs and novel therapeutics for pain relief (**Table 1**). This review will focus more on drugs that have been in clinical trials, as well as attractive drugs with potential applications in preclinical research, to provide clinicians with recent advances in OA pharmacological therapies.

INVESTIGATIONAL DRUGS TARGETING INFLAMMATORY MECHANISMS

The inflammatory mediators can be detected in both synovial fluid and serum in OA patients, indicating that inflammation does play a significant role in the pathogenesis of OA (LeGrand et al., 2001). OA is now seen as a low-grade inflammatory disease compared to rheumatoid arthritis (RA) (Scanzello and Loeser, 2015). Recently, studies have revealed that the low-grade, chronic, sterile inflammation associated with OA is closely related to dysregulation of the immune system as aging (Millerand et al., 2019). Anti-inflammatory therapeutics and treatment modalities targeting senescence processes may be promising approaches to attenuate disease progression of OA.

Interleukin (IL)-1 Inhibitors

IL-1 has an increased expression in cartilage, synovium, and synovial fluid in OA patients (Sohn et al., 2012). It is an important proinflammatory cytokine and pain mediator resulting in pain sensitization, bone resorption, and cartilage destruction. Thus, IL-1 inhibitors may protect against structural changes in OA (Miller et al., 2014; Schett et al., 2016). Cytokines of the IL-1 family members include IL-1α, IL-1β, and endogenous IL-1 receptor antagonist (IL-1Ra). The ideal treatment is to effectively inhibit IL-1α and IL-1β without interfering with IL-1Ra.

1) Drugs targeting IL-1 receptor include human IL-1 receptor antagonist Anakinra, and human IL-1 receptor type 1 (IL-1R1) monoclonal antibody AMG 108 produced by genetic recombination technology. In two randomized, double-blind, placebo-controlled studies, it was found that subcutaneous (SC) or intravenous (IV) of AMG 108 and a single intra-articular (IA) injection of Anakinra were well tolerated (Chevalier et al., 2009; Cohen et al., 2011). Patients in the study received SC or IV injection of AMG 108 every 4 weeks for 12 weeks, and the results showed that patients who received AMG 108 showed statistically insignificant but numerically greater improvements in pain compared to placebo (Cohen et al., 2011). Similarly, IA injection of Anakinra did not significantly improve symptoms in patients with knee OA (Chevalier et al., 2009). Neither of these studies evaluated the effects on the joint structure.
2) Drugs targeting IL-1β include the humanized monoclonal antibody Canakinumab and the IL-1β allosteric modulating antibody Gevokizumab, which inhibit IL-1β receptor activation by tightly binding IL-1β. Canakinumab is considered as a disease-modifying antirheumatic drug (DMARD), has been shown to improve symptoms of juvenile idiopathic arthritis and RA, and decrease cartilage destruction (Sota et al., 2018). A recent preclinical study demonstrated that Canakinumab had protective effects on human OA chondrocytes *in vitro* (Cheleschi et al., 2015). In the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS trial), it was observed that Canakinumab reduced not only cardiovascular events but also the incidence of total knee or hip replacement as a result of OA (Chevalier and Eymard, 2019). A phase II study (NCT01160822) on the safety, tolerability, pharmacokinetics, and pain effects of a single IA injection of Canakinumab in patients with knee OA was completed, but the results have not been published. Another phase II studies (NCT01683396; NCT01882491) to test the safety and biologic activity of Gevokizumab, and an open-label safety extension study of Gevokizumab (NCT02293564) in patients with hand OA were completed, but no published results are available.
3) Lutikizumab (formerly ABT-981) is a human dual variable domain immunoglobulin (DVD-Ig), simultaneously binding and inhibiting IL-1α and IL-1β (Lacy et al., 2015). In a randomized placebo-controlled phase I study, Lutikizumab was generally well tolerated in patients with mild to moderate knee OA, and

significantly reduced serum concentrations of matrix metalloproteinase (MMP)-1 and high-sensitivity C-reactive protein (hsCRP) (Wang S. X. et al., 2017). However, the results from two recent phase II clinical studies to assess the efficacy of Lutikizumab in patients with hand OA and knee OA were unsatisfactory (Fleischmann et al., 2019; Kloppenburg et al., 2019). In erosive hand OA, Lutikizumab was administrated subcutaneously every 2 weeks for 26 weeks, but there were no significant differences in pain score, and in changes of X-ray or magnetic resonance imaging (MRI) scores between Lutikizumab and placebo (Kloppenburg et al., 2019). In knee OA with evidence of synovitis (ILLUSTRATE-K trial), Lutikizumab was administrated subcutaneously with three different doses (25, 100, and 200 mg) every 2 weeks for 50 weeks, the results showed that only lutikizumab 100 mg was slightly superior to the placebo in pain improvement at week 16 (Fleischmann et al., 2019). Moreover, at weeks 26 and 52, there were no significant differences between the lutikizumab and placebo groups in MRI-detected synovitis, radiographic medial and lateral joint space narrowing (JSN), and cartilage thickness (Fleischmann et al., 2019). These results suggest that IL-1 inhibition is not effective in most patients with OA. Whether subgroups of OA patients might have symptomatic or disease-modifying benefits from IL-1 inhibition remains an open question.

Tumor Necrosis Factor-Alpha Inhibitors

TNF-α, a proinflammatory cytokine produced by synoviocytes and chondrocytes in OA, plays a central role in the induction of structural damage and pain modulation in OA. Besides, TNF-α enhances the production of a series of other proinflammatory cytokines (such as IL-6 and IL-8), stimulates the synthesis of MMP and cyclooxygenase (COX), and increases NO production (Orita et al., 2011). Preclinical studies suggested that anti-TNF-α therapy might exert a protective effect on articular cartilage by improving the structure of the subchondral bone and reducing cartilage matrix degradation (Ma et al., 2015). Thus, inhibitors of TNF-α might be considered as potential candidates for disease-modifying therapy in OA.

(1) Etanercept is a recombinant human tumor necrosis factor receptor type II antibody fusion protein. A study investigated the effect of IA injection of Etanercept for pain in moderate and severe knee OA. The results showed that compared with the hyaluronic acid group, direct injection of Etanercept into OA knee joints could effectively relieve the pain symptoms in OA patients (Ohtori et al., 2015). However, A recent randomized, double-blind, placebo-controlled trial (EHOA trial) found that the SC injection of Etanercept for 24 weeks did not relieve pain effectively in patients with erosive hand OA compared with placebo (Kloppenburg et al., 2018). In subgroup analysis, joints treated with Etanercept for 52 weeks showed more radiographic remodeling and less MRI bone marrow lesions (BMLs), which was more pronounced in actively inflamed joints at the baseline (Kloppenburg et al., 2018). In this study, Etanercept was observed to reduce serum levels of MMP-3, an important mediator of joint destruction (Kroon et al., 2020). Overall, this study did not provide evidence for the use of Etanercept to treat hand OA, but from a therapeutic strategy targeting inflammation, the authors believed that short-term treatment with TNF-α inhibitors during disease flares could be considered.

(2) Infliximab is a human/mouse chimeric monoclonal antibody of immunoglobulin G (IgG) 1/k subtype (composed of human IgG1 constant region and murine variable region). An exploratory observational longitudinal study found that treatment with Infliximab can reduce the incidence of secondary OA in proximal interphalangeal joints in patients with active RA (Guler-Yuksel et al., 2010). A pilot study investigated the efficacy and tolerability of IA injection of Infliximab in erosive hand OA (Fioravanti et al., 2009). The results showed that IA injection of Infliximab was safe, and significantly improved pain symptoms. Infliximab tended to reduce radiological scores of anatomical lesions in the hand, but the difference did not reach statistical significance. The study suggested a possible symptom- and disease-alleviating effect of Infliximab, but clinical trials are still needed to elucidate the true effect of Infliximab in OA.

(3) Adalimumab is the first bioengineered fully human monoclonal antibody that binds specifically to TNF and neutralizes the biological function of TNF by blocking its interaction with both Types 1 and 2 TNF receptors (TNF-R1 and -R2). A 12-month, double-blind, randomized controlled trial evaluated the efficacy and safety of Adalimumab in controlling structural damage in patients with erosive hand OA (Verbruggen et al., 2012). The tolerability and safety of Adalimumab in patients with erosive hand OA were similar to those in patients with other systemic rheumatic diseases. Compared with placebo, Adalimumab did not halt the progression of joint damage in overall patients, but it significantly slowed the progression of joint aggressive lesions in a subpopulation with palpable tissue swelling of the interphalangeal joints. However, in two randomized double-blind placebo-controlled trials, Adalimumab was not superior to placebo in relieving pain in patients with erosive hand OA (Chevalier et al., 2015; Aitken et al., 2018), and one study (HUMOR trial) also indicated that Adalimumab did not affect synovitis or BML in patients with hand OA with MRI-detected synovitis (Aitken et al., 2018).

DMARDs

With the increasing acceptance of the inflammatory phenotype of OA, traditional DMARDs may have the potential to reduce pain and slow structural degeneration in OA. Hydroxychloroquine (HCQ) has been successfully used in the treatment of mild RA

and other autoimmune diseases for many years (Ghouri and Conaghan, 2019). A randomized trial during 24 weeks showed that compared with placebo, HCQ was not effective in reducing the symptoms of hand OA (Lee et al., 2018). Recently, a randomized double-blind placebo-controlled trial (HERO trial) with 12-month follow-up evaluated the efficacy of HCQ in hand OA patients with moderate to severe pain, and the results showed that HCQ did not relieve symptoms or delay structural damage (Kingsbury et al., 2018).

Methotrexate (MTX) is a traditional DMARD for the treatment of some autoimmune diseases such as RA. The study (NCT01927484) reported that oral MTX significantly relieved pain and reversed features of synovitis in patients with symptomatic knee OA, which indicated MTX as an option for the treatment of knee OA (Abou-Raya et al., 2018). A pragmatic phase III RCT was completed (PROMOTE trial) to determine whether oral MTX reduced pain and synovitis associated with knee OA in 2019 (Kingsbury et al., 2015). The results presented at Osteoarthritis Research Society International (OARSI) Annual Congress showed that MTX significantly reduced knee OA pain, and significantly improved Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores for stiffness and function. However, MTX did not change the synovial volume assessed by MRI in this study. Meanwhile, a multicenter RCT study to investigate the effect of oral MTX on pain and synovitis in patients with mid-to late-stage knee OA (NCT03815448) is ongoing (Zhu et al., 2020), and further data are expected to come soon. Overall, more evidence is needed to clearly define the role of MTX in OA treatment.

Targeting Senescent Cells

The innate immune activation caused by the dysregulation of the immune system with aging is considered to play a crucial role in the chronic inflammation of OA (Jeon et al., 2018). Age-related mitochondrial dysfunction and associated oxidative stress might induce senescence in joint tissue cells (Coryell et al., 2020). The accumulation of SnCs in joints causes the secretion of pro-inflammatory and pro-catabolic factors (cytokines, chemokines, MMPs), which is called a "senescence-associated secretory phenotype" (SASP) (Childs et al., 2017; Millerand et al., 2019). Direct targeting the SnCs provides a potential opportunity to eliminate the source of OA disease (Childs et al., 2017; Jeon et al., 2017). UBX0101 is a small molecule lysosomal agent that can reduce the expression of SASP factors and improve overall joint function (Jeon et al., 2017). Currently, several randomized, placebo-controlled clinical trials of UBX0101 are all completed in 2020 to evaluate the efficacy, safety, and tolerability of IA injection of UBX0101 in knee OA patients (NCT03513016, NCT04229225, and NCT04129944), and the results will be released soon.

Curcuma Longa Extract

Curcuminoids, are the principal extracted from the CL root (Family Zingiberaceae), which comprise curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) (Cao et al., 2014). The curcumin is the main active and effective ingredient. Curcumin is known to suppress oxidative stress and inflammation by scavenging active oxygen and inhibiting nuclear factor-kappa β (NF-κβ) pathway (Shen and Ji, 2012; Wang J. et al., 2017). A systematic review and meta-analysis of RCT enrolled 797 patients with primarily knee OA demonstrated that Curcuminoids had some beneficial effects on knee pain and quality of life in patients with knee OA (Onakpoya et al., 2017). Recently, a single-center, randomized, placebo-controlled trial with 12-week follow-up evaluated the efficacy of CL in patients with symptomatic knee OA and effusion-synovitis, and the results showed that CL was superior to placebo in relieving knee pain but did not affect the effusion-synovitis volume or cartilage composition as assessed by MRI (Wang et al., 2020). However, the follow-up time was relatively short so that it might be insufficient to detect a change in the cartilage- and synovium-specific outcomes in this study. Another double-blind, randomized, parallel-group, phase III comparative study (NCT04500210) of CL and placebo to patients with mild to moderate OA of the knee and or hip is still recruiting. Further researches with larger sample sizes are needed to assess the clinical significance of CL in OA treatment.

INVESTIGATIONAL DRUGS TARGETING CARTILAGE METABOLISM

The characteristic sign of OA is cartilage destruction, so emerging drugs targeting the molecular mechanism of articular cartilage should be an attractive therapeutic strategy for OA. The research direction is mainly to delay cartilage destruction by anti-catabolic agents and stimulate cartilage development and repair by anabolic agents.

Wnt Signaling Pathway Inhibitors

The balance of Wnt pathway activity is integral for regulating the differentiation of progenitor cells in the joint and maintaining cartilage homeostasis (Lories et al., 2013; Thysen et al., 2015). In OA, aberrant Wnt pathway activity leads to the differentiation of progenitor cells into osteoblasts while chondrocyte development is blocked, as well as the increased secretion of catabolic enzymes and inflammation.

Preclinical studies demonstrated that Wnt pathway inhibitors could delay the development of OA in animal models; however, excessive inhibition, in turn, caused cartilage and bone destruction. Thus, targeting the Wnt pathway and controlling it within an optimal range is a potential therapeutic avenue (Usami et al., 2016; Deshmukh et al., 2018).

Lorecivivint (formerly SM04690) is a small-molecule Wnt pathway inhibitor and modulates the Wnt pathway by inhibiting two intranuclear targets, intranuclear kinases CDC-like kinase 2 (CLK2) and dual-specificity tyrosine phosphorylation-regulated kinase 1 A (DYRK1A) (Deshmukh et al., 2019). In a 24-week, randomized, placebo-controlled phase I study, a single IA injection of Lorecivivint (0.03, 0.07, or 0.23 mg) appeared safe and well-tolerated (Yazici et al., 2017). Lorecivivint 0.07 mg was superior to the placebo in improving WOMAC pain scores and function scores in patients with moderate to severe knee OA, while the 0.07 mg dose group

also showed an increase from baseline in radiographic joint space width (JSW) (Yazici et al., 2017).

Recently, the results of a 52-week multicenter, randomized, double-blind, placebo-controlled phase IIa study announced that Lorecivivint treatment was not superior to placebo for improving pain, joint function, and radiographic JSW in patients with moderate to severe knee OA (Deshmukh et al., 2019), but in subgroup patients with unilateral symptomatic knee OA or unilateral symptomatic knee OA without extensive pain, Lorecivivint 0.07 mg significantly relieved pain, improved joint function, and increased JSW compared with placebo (Deshmukh et al., 2019). The study suggested that Lorecivivint might be effective in OA patients with a certain phenotype.

Besides, a phase III clinical study (NCT03928184) has been initiated in 2019 to assess the long-term efficacy and safety of Lorecivivint in the treatment of knee OA, and Lorecivivint has the potential to be an effective treatment for OA.

Cathepsin-K Inhibitors

Cathepsin-K is the predominant cysteine cathepsin in the skeleton and it plays an important role in the resorption of cartilage and bone (Dejica et al., 2008). Several observations have demonstrated up-regulation of cathepsin K in OA cartilage and inflamed synovial tissue (Salminen-Mankonen et al., 2007). Cathepsin-K may be an attractive therapeutic target for diseases with excessive bone resorption such as osteoporosis and OA. Cathepsin K inhibitors have shown structural protection and analgesic effects in animal models of joint degeneration (Lindström et al., 2018a; Nwosu et al., 2018).

The results of phase II clinical study evaluating the efficacy and safety of the Cathepsin-K inhibitor Balicatib in OP and OA patients showed that it could improve bone mineral density in OP patients, but it failed to decrease cartilage volume loss (CVL) in patients with knee OA (Duong et al., 2016). Also, Balicatib could lead to dose-related adverse effects-Morphea-like skin reactions (Runger et al., 2012).

MIV-711 is a highly selective cathepsin K inhibitor that has been shown in preclinical animal models of OA to reduce cartilage lesions, reduce levels of biomarkers reflecting the degradation of bone and cartilage [carboxy-terminal collagen cross links (CTX)-I and CTX-II] and prevent subchondral bone loss (Lindström et al., 2018a; Lindström et al., 2018b). A recent randomized, double-blind, placebo-controlled phase IIa study to assess the efficacy and safety of MIV-711 in symptomatic patients with Kellgren-Lawrence (KL) grade 2 and 3 knee OA (Conaghan et al., 2020). The results showed that oral administration of MIV-711 (100 mg/d or 200 mg/d) for 26 weeks had a significant protective effect on both bone and cartilage structures, and significantly reduced the levels of CTX-I and CTX-II, but failed to meet the primary study endpoint of alleviating knee joint pain (Conaghan et al., 2020). MIV-711 has a good safety profile, but its clinical efficacy remains to be validated in longer-term and larger-scale clinical studies.

MMP/ADAMTS Inhibitors

Aggrecan and type II collagen are two main components of articular cartilage, which are essential for maintaining the function and integrity of cartilage (Malfait and Tortorella, 2019). Aggrecan provides the compressibility of cartilage, while collagen provides its elasticity. These macromolecules are decomposed by proteolysis. MMPs and aggrecanase (a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), mainly ADAMTS-4 and ADAMTS-5) are demonstrated to have critical roles in the degradation of type II collagen and aggrecan, respectively, and are considered as potential targets for OA treatment.

(1) In preclinical trials, highly selective MMP-13 inhibitors (such as ALS1-0635 and PF152) have shown advantages in slowing the progression of OA (Piecha et al., 2010; Schnute et al., 2010). However, the available data on the role of MMP-13 inhibitors in OA treatment is limited, and human clinical trials are still needed to observe the efficacy of MMP-13 inhibitors as DMOADs.

(2) At present, the investigational drugs targeting ADAMTS-5/ADAMTS-4 include a chimeric murine/human ADAMTS-5 monoclonal antibody-CRB0017, which was reported to slow OA disease progression after IA administration in animal models of OA (Chiusaroli et al., 2013), and a humanized ADAMTS-5-selective monoclonal antibody, GSK2394002, which was reported to have structural modification and analgesic effects in animal models of OA (Larkin et al., 2015; Miller et al., 2016). AGG-523, an orally small molecule inhibitor of ADAMTS-4 and ADAMTS-5, was the first to enter the human phase I study (NCT00454298 and NCT00427687), but these trials were discontinued for unknown reasons. M6495, a novel anti-ADAMTS-5 inhibiting Nanobody, showed dose-dependent protection against cartilage deterioration in *ex vivo* cartilage cultures (Siebuhr et al., 2020). A phase Ib (NCT03583346) clinical trial to assess safety, tolerability, immunogenicity, pharmacokinetics, and pharmacodynamics of SC injections of M6495 in knee OA patients was completed in 2019, but the results have not yet been published.

Growth Factors

Different from using anti-catabolic agents to delay the progression of cartilage destruction, an alternative approach is to stimulate the growth and repair of cartilage for the treatment of OA. Several growth factors have been shown to stimulate cartilage anabolism and promote cartilage repair *in vitro* and animal models of OA. Growth factors may have potential therapeutic effects on OA.

(1) Sprifermin is a recombinant human fibroblast growth factor 18 (rhFGF18) (Onuora, 2014), and preclinical data had shown that Sprifermin bound to and activated fibroblast growth factor receptor 3 (FGFR3) in cartilage to promote chondrogenesis, cartilage matrix formation, and cartilage repair *in vivo* and *in vitro* (Moore et al., 2005; Gigout et al., 2017; Reker et al., 2017; Sennett et al., 2018). A randomized, double-blind,

placebo-controlled phase I b proof-of-concept trial evaluated the efficacy and safety of IA injection of Sprifermin (10, 30, and 100 μg) in patients with symptomatic knee OA (Lohmander et al., 2014). The results showed that Sprifermin appeared safe and well-tolerated. Although Sprifermin was not superior to placebo in reducing the loss of central medial femorotibial compartment (cMFTC) cartilage thickness and improving pain, it showed a statistically significant dose-dependent effect in reducing the loss of total and lateral femorotibial cartilage thickness and loss of lateral radiographic JSW (Lohmander et al., 2014). Two post-hoc analyses of this study demonstrated that Sprifermin (100 μg) reduced cartilage loss, increased cartilage thickness, and improved BMLs (Eckstein et al., 2015; Roemer et al., 2016).

A 5-years, dose-finding, multicenter phase II clinical trial (FORWARD trial), published in 2019, showed that IA injection of 100 μg Sprifermin every 6 or 12 months significantly increased the total femorotibial joint cartilage thickness in patients with symptomatic knee OA after 2 years, and this effect was dose-dependent. Sprifermin had a limited effect on pain improvement in this study (Hochberg et al., 2019). Recently, two post-hoc exploratory analyses were carried out on this study, and the results showed that sprifermin treatment could significantly increase cartilage thickness and reduce cartilage loss, making cartilage loss in patients with knee OA similar to that of healthy subjects (Brett et al., 2020; Eckstein et al., 2020). The above studies supported the conclusions that sprifermin modified structural progression and could be a potential DMOAD.

(2) Transforming growth factor-β1 (TGF-β1) plays an important role in the development and maturation of articular cartilage and the phenotypic maintenance of chondrocytes (Yang et al., 2001; Crane et al., 2016). The expression of TGF-β1 in healthy cartilage is significantly higher than that in OA cartilage; however, it has been found that overexpression of TGF-β1 leads to OA-like changes in the knee joint of C57Bl/6 mice, including hyperplasia of the synovium and osteophyte formation (Bakker et al., 2001). Recently, Liu et al. demonstrated that TGF-β had different effects on human OA mesenchymal stromal cells (OA-MSC) and chondrocytes (OAC). While TGF-β stimulated chondrogenesis in OAC, it induced hypertrophy, mineralization, and MMP-13 in OA-MSC (Liu et al., 2020).

SB-505124 is a TGF-β type I receptor inhibitor, and it was found *in vitro* and in animal models of OA that TGF-β1 overexpression in osteoclasts was responsible for chondrocyte apoptosis and cartilage degeneration in OA, and SB-505124 could inhibit the degradation of articular cartilage (Zhang et al., 2018).

Tissue Gene-c (TG-C) is a cell-mediated gene therapy that delivers allogeneic chondrocytes expressing TGF-β1 directly to the damaged knee joint, consisting of irradiated allogeneic human chondrocytes that express TGF-β1 and normal allogeneic human chondrocytes in a 1:3 ratio (GEC-TGF-β1) (Ha et al., 2012). Two randomized, double-blind, placebo-controlled phase II studies to evaluate the safety and efficacy of IA injection of GEC-TGF-β1 in patients with knee OA (Cherian et al., 2015; Ha et al., 2015). The results showed that most of the adverse events were local reactions and did not require further treatment, and only a small number of patients had allergic reactions but recovered within 24 h. Moreover, compared with the placebo, GEC-TGF-β1 could significantly improve pain and physical function. However, neither of these studies evaluated the effect of GEC-TGF-β1 on cartilage regeneration and OA imaging changes. The results of a phase III trial (NCT02072070) suggested that GEC-TGF-β1 had beneficial effects on pain and functional improvement in patients with OA, but had limited effects on structural improvement (Kim et al., 2018).

Metformin

Metformin is a safe and well-tolerated oral biguanide that has been used as the first-line therapy for type 2 diabetes for more than 50 years. Preclinical studies had shown that Metformin could significantly attenuate articular cartilage degeneration and relieve pain in the OA mouse model (Li H. et al., 2020). Besides, it was found that the chondroprotective effect of metformin was mediated by activation of adenosine monophosphate-activated protein kinase (AMPK) signaling. Metformin could enhance AMPK expression and phosphorylation in chondrocytes, and increase the production of type II collagen and reduce the level of MMP-13 by activating AMPK pathway (Li J. et al., 2020). A nationwide, retrospective, matched-cohort study evaluated 968 patients with OA and type 2 diabetes mellitus (T2DM) during 10 years of follow-up and the results showed that OA patients with T2DM under combination COX-2 inhibitors and Metformin therapy were associated with lower joint replacement surgery rates than COX-2 inhibitors only (Lu et al., 2018). Recently, a prospective cohort study reported that metformin had a beneficial effect on long-term knee outcomes in obese knee OA patients, and metformin significantly reduced the loss of medial knee cartilage volume (Wang et al., 2019). Currently, randomized controlled trials are still needed to confirm these findings and to determine whether metformin can be considered as a potential disease-modifying drug for knee OA with or without obese phenotype.

INVESTIGATIONAL DRUGS TARGETING THE SUBCHONDRAL BONE

Increased subchondral bone resorption and bone turnover contribute to the pathogenesis of OA (Karsdal et al., 2014). Thus, the subchondral bone may be a potential target for OA therapy. However, currently available agents targeting the subchondral bone haven't been approved for the treatment of OA due to the inconsistent efficacy or safety considerations, including Zoledronic Acid, Calcitonin, and Strontium ranelate.

Bisphosphonate

One small randomized clinical trial stated that intravenous Zoledronic Acid was beneficial in improving pain and BMLs in knee OA patients at 6 months (Vaysbrot et al., 2018). BMLs detected by MRI represented areas of high bone turnover and active bone remodeling, and bisphosphonates might be beneficial for patients with high metabolic activity (Kuttapitiya et al., 2017). However, recently, a 24-month multicenter, double-blind placebo-controlled randomized clinical trial assessed the effects of twice-yearly intravenous Zoledronic Acid for 24 months on CVL in patients with symptomatic knee OA and BMLs (Cai et al., 2020). The results showed that Zoledronic Acid did not significantly reduce cartilage volume loss, relieve pain, or improve BMLs. These findings did not support intravenous Zoledronic Acid to treat knee OA. A randomized, double-blind, parallel-group, multicenter, placebo-controlled, dose-ranging study (EudraCT2018-002081-39) to assess the efficacy and safety of IA injection of clodronate for knee OA is currently ongoing, and no results are available.

Calcitonin

A combined reporting of two randomized, double-blind, multi-center, placebo-controlled trials (NCT00486434 and NCT00704847) that included 1176 and 1030 patients, respectively, showed that oral salmon calcitonin (sCT) for 24 months did not improve pain symptoms and joint space width (JSW) measured by X-ray in patients with symptomatic knee OA (Karsdal et al., 2015).

Strontium Ranelate

Strontium Ranelate is indicated for the treatment of postmenopausal osteoporosis (Han et al., 2017). Preclinical studies indicated that it reduced subchondral bone resorption and stimulated cartilage matrix formation *in vitro* and in rat OA model (Coulombe et al., 2004; Tat et al., 2011; Yu et al., 2013). A 3-year multicenter, randomized, double-blind, placebo-controlled Phase III clinical trial (SEKOIA trial) showed that Strontium Ranelate significantly inhibited the narrowing of the medial femoral joint space, relieved pain, and improved physical function in patients with moderate to severe knee OA compared with placebo (Reginster et al., 2013). A post hoc analysis of the SEKOIA trial found that Strontium Ranelate was also significantly associated with decreased MRI-assessed CVL and BMLs (Pelletier et al., 2015). However, although Strontium Ranelate has a significant protective effect on the joint structure and clinically relevant improvement of symptoms of knee OA, the use of Strontium Ranelate in OA is limited by its cardiovascular risk, particularly the side effects of thromboembolism.

Teriparatide

Teriparatide is a recombinant human parathyroid hormone (PTH), derived from the 1–34 amino acid fragment of human PTH (Oo and Hunter, 2019). It promotes the proliferation and survival of osteoblasts, which is a bone anabolic therapy for osteoporosis (Sampson et al., 2011). A preclinical study showed that Teriparatide could decelerate cartilage degeneration and induced cartilage matrix regeneration in mice administered a meniscal/ligamentous knee injury (Macica et al., 2011). Teriparatide may become a novel candidate therapy for injury-induced OA. A phase II study (NCT03072147) to assess the chondroregenerative efficacy and safety of Teriparatide for knee OA is still ongoing, and the estimated study completion date is in 2022.

Vitamin D

A prospective study determined that sunlight exposure and serum 25(OH)D levels were both positively associated with knee cartilage volume in older people, suggesting that vitamin D is an important hormonal contributor to cartilage homeostasis (Ding et al., 2009). Thus, Vitamin D supplementation potentially prevented the progression of OA. However, A 2-year RCT showed that Vitamin D supplementation at a dose sufficient to elevate serum levels of 25-hydroxyvitamin D to >36 ng/ml did not reduce knee pain or CVL in patients with symptomatic knee OA (McAlindon et al., 2013). A multicenter randomized, double-blind, placebo-controlled clinical trial (VIDEO trial) evaluated the effects of vitamin D supplementation in patients with symptomatic knee OA and low serum 25-hydroxyvitamin D levels (Jin et al., 2016). The results showed that Vitamin D supplementation did not prevent tibial cartilage loss or relieve knee pain over 2 years, but improved physical function (Jin et al., 2016) and reduced joint effusion synovitis (Wang X. et al., 2017). Three post-hoc exploratory analysis were carried out on the VIDEO trial. Vitamin D supplementation and maintaining vitamin D sufficiency (25-hydroxyvitamin D > 50 nmol/L at month 3 and 24) over 24 months might be benefificial for depressive symptoms (Zheng et al., 2019) and foot pain (assessed by manchester foot pain and disability index) (Tu et al., 2020) in patients with knee OA. Maintaining vitamin D sufficiency significantly reduced tibial cartilage volume loss and effusion-synovitis volume, and improved physical function compared with those who did not (Zheng et al., 2017).

INVESTIGATIONAL DRUGS TO RELIEVE PAIN

NSAIDs and opioid drugs are primary pharmacological treatments for pain palliation in OA. But these medications are unsuitable for long-term use because of side effects, and their roles in pain control are limited (McAlindon and Bannuru, 2010; Zhang et al., 2010). Patients with OA continue to suffer from inadequate pain relief. Thus, although the development of drugs that can reverse the structural progression of joint damage in OA is important, it is still necessary to consider the effect of drugs against pain (Karsdal et al., 2016; Miller et al., 2018). Besides, there is also an urgent need to develop new ideal therapies, which are safe, simple, long-acting, and convenient to treat the chronic pain associated with OA.

Monoclonal Antibodies Neutralizing Nerve Growth Factor

NGF is a neurotrophin that stimulates the growth of nociceptive nerve fibers and the expression of nociceptive cell surface receptors (Denk et al., 2017; Vincent, 2020). Almost all structures in the joint are innervated with nociceptive nerve fibers, and elevated NGF levels may be sources of refractory knee pain in OA (Malfait and Schnitzer, 2013; Denk et al., 2017). NGF is therefore an attractive target for novel analgesic agents. Tanezumab, Fulranumab, and Fasinumab are monoclonal antibodies that specifically target NGF and inhibit binding to its receptors (Ghouri and Conaghan, 2019). Tanezumab is the most widely studied and has completed pivotal phase III clinical trials, and Fasinumab is in the midst of phase III clinical trials (NCT02683239, NCT03285646, NCT03161093, and NCT03304379), while Janssen has discontinued the clinical development of Fulranumab, with no active trials being underway (Cao et al., 2020). The US FDA recently has granted fast-track certification (a process designed to facilitate the development and expedite the review of new therapies to treat serious conditions and fill unmet medical needs) for Tanezumab for the treatment of chronic pain in patients with OA or chronic low back pain, and Tanezumab is expected to be approved for clinical use soon.

A meta-analysis of 10 randomized controlled trials enrolled 7,665 patients demonstrated that Tanezumab was superior to placebo in pain relief and improvement in physical function and patient's global assessment (PGA) in knee and hip OA patients (Chen J. et al., 2017). A phase IIb/III clinical trial assessed the efficacy, tolerability, and joint safety of Fasinumab in patients with hip and/or knee OA (Dakin et al., 2019). The results showed that Fasinumab significantly improved pain and function in patients with OA, even in those who obtained little benefit from previous analgesics (Dakin et al., 2019). A phase III clinical trial evaluated 696 patients with hip and/or knee OA who had not responded to or were unable to receive standard analgesics (Schnitzer et al., 2019). Patients received by 2 SC injections of Tanezumab (2.5 mg administered at baseline and week 8 or 2.5 mg administered at baseline and 5 mg at week 8) or placebo at day 1 and week 8. The results showed that Tanezumab was significantly better than the placebo in improving scores assessing pain and physical function, and PGA-OA (Schnitzer et al., 2019). Recently, another phase III clinical trial evaluated 849 patients with hip and/or knee OA who had not responded to or could not tolerate standard-of-care analgesics. Patients received SC Tanezumab 2.5 mg or 5 mg or placebo every 8 weeks (Berenbaum et al., 2020). The results showed that Tanezumab 5 mg statistically significantly improved pain, physical function and PGA, and Tanezumab 2.5 mg significantly improved pain and physical function, but did not improve PGA (Berenbaum et al., 2020).

It should be noted that anti-NGF treatment may lead to treatment-related rapidly progressive OA (PROA) and osteonecrosis (Hochberg, 2015). These serious joint-related adverse events drove the FDA to place a partial clinical hold on NGF antibodies. By reviewing the adverse events reported in clinical trials, it was found a dose-response relationship between osteonecrosis and NGF antibodies, with the dose of Tanezumab ranging from 2.5 to 10 mg and the dose of Fasinumab ranging from 3 to 9 mg (Hochberg, 2015; Lane and Corr, 2017; Dakin et al., 2019). Therefore, the maximum dose of Tanezumab was reduced to 5 mg after resuming the clinical trials in 2015. Importantly, compared with Tanezumab monotherapy, Tanezumab combined with NSAIDs treatment appeared to increase the risk of RPOA (Hochberg et al., 2016). It seemed that more joint replacements had been observed in patients treated with Tanezumab, but most were personal choices and not associated with adverse events (Schnitzer et al., 2019).

The anti-NGF treatment undoubtedly provides great potential for improving the pain and function of patients with severely symptomatic OA, but it carries the risk of aggravating the structural progression of OA (Miller et al., 2017). Therefore, in addition to using the lowest effective dose to mitigate the risk, it is essential to identify the patient population most suitable for this therapeutic approach. Jayabalan and Schnitzer believed that individuals with preexisting joint abnormalities, such as subchondral insufficiency fractures, who were at increased risk for PROA when treated with anti-NGF, should not be considered for the anti-NGF treatment. On the other hand, anti-NGF may be a particularly useful drug for specific populations for whom NSAIDs are contraindicated and/or not recommended (Jayabalan and Schnitzer, 2017).

Intra-articular Corticosteroid

Triamcinolone Acetonide Sustained-Release Agent

Triamcinolone acetonide (TA) is an intra-articular corticosteroid to relieve pain, but its magnitude of benefit rapidly wanes post-injection for rapid systemic absorption (Kraus et al., 2018). Zilretta (formerly FX006) is a novel type of extended-release TA formulation in 75:25 poly microsphere, which is designed to prolong TA residence in the joint compared with standard TA crystal suspensions (TAcs) (Conaghan et al., 2018a). A phase III, multicenter, double-blind, randomized controlled trial compared FX006 (32 mg), TAcs (40 mg), and saline placebo in 484 patients with knee OA (Conaghan et al., 2018b). Although FX006 did not significantly reduce the average-daily-pain (ADP)-intensity of OA compared to TAcs at 12 weeks, it reached the primary endpoint of a significant improvement in ADP-intensity compared with placebo. In addition, FX006 significantly improved Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores for pain, stiffness, and physical function, and Knee Injury and Osteoarthritis Outcome Score Quality of Life (KOOS-QOL) scores for the quality of life at 12 weeks compared with both placebo and TAcs (Conaghan et al., 2018b). FX006 causes less blood glucose elevation compared to standard TAcs in type 2 diabetic patients. For this reason, FX006 has been licensed by the FDA in October 2017 for the treatment of OA-related knee pain.

IA Triamcinolone

A two-year, randomized, placebo-controlled, double-blind trial (NCT01230424) compared Triamcinolone (40 mg), and saline placebo in 140 patients with symptomatic knee OA. The results

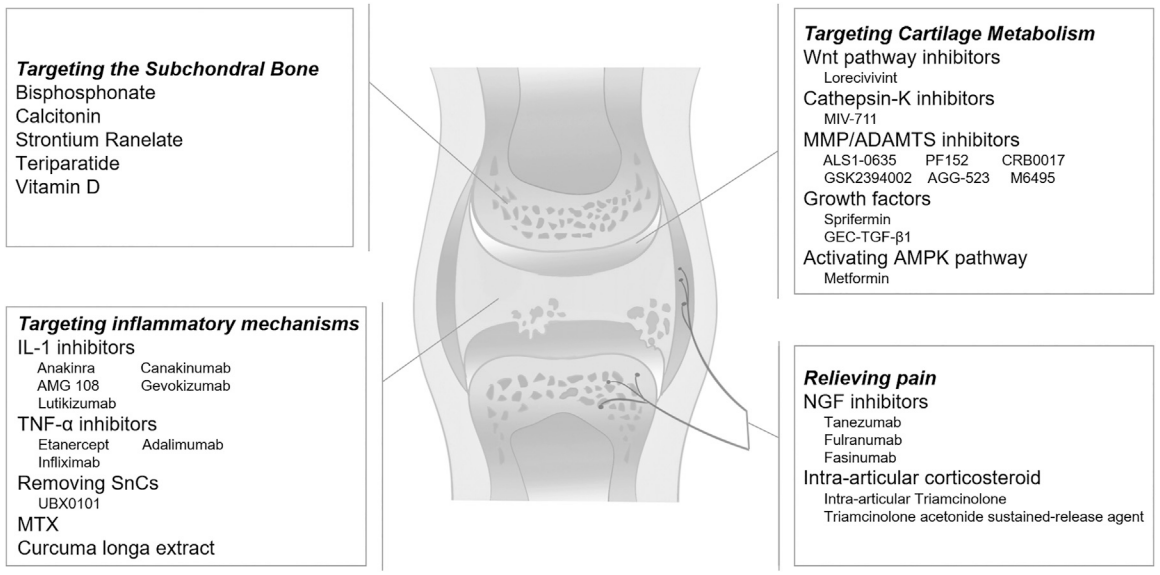


FIGURE 1 | Potential pharmacological therapies for osteoarthritis.

showed that IA Triamcinolone every 3 months for 2 years significantly increased CVL and did not improve knee pain (McAlindon et al., 2017). These findings do not support this long-term treatment for patients with symptomatic knee OA.

EXPERT OPINION

OA is a chronic, painful and disabling arthritis with significant burden on the individual and society. With the population aging and obesity, the incidence of OA is increasing as a leading cause of disability worldwide (Peat and Thomas, 2020). To date, no effective drug is able to inhibit the structural damage or reduce long-term disability, or relieve pain with an acceptable benefit-to-risk profile in OA (Latourte et al., 2020). For these reasons, the OARSI led an effort to submit a White Paper to the FDA in support of the designation of OA as a serious disease in 2016. Actually, OA is a severe disease as RA for their similar disability rates, morbidity, costs, and increased mortality rates (Pincus et al., 2019). In the past few years, it has been realized that a complex interaction of multifactorial mechanisms is involved in the pathophysiology of OA. The heterogeneous condition of OA determines that there is no miracle therapeutic strategy fitting for all patients. Also, this heterogeneity may be the major cause for the failure of clinical trials testing therapeutics intended for structure modification or symptom relief in OA.

Various OA phenotypes and endotypes have been explored to overcome this barrier (Deveza et al., 2019), such as synovial inflammatory phenotype, osteoporotic phenotype, articular cartilage degradation phenotype, metabolic phenotype and so on. However, there are few clinical trials to stratify patients based on these phenotype-guided approaches yet. OA phenotyping would be helpful to therapy selection and expedite the development of investigational tailored drugs directly toward variable courses of OA. Metabolomic studies and innovative machine learning approaches may greatly help to determine the key variables to differentiate specific OA subgroups and progression phenotypes (Carlson et al., 2019; Nelson et al., 2019). Nelson et al. observed that baseline variables as BMLs, osteophytes, medial meniscal extrusion, and urine CTX-II were useful to identify progression OA phenotypes at 48 months, while WOMAC pain, lateral meniscal extrusion, and serum N-terminal pro-peptide of collagen IIA (PIIANP) were associated with non-progression phenotypes (Nelson et al., 2019). Establishing OA phenotypes and then setting up distinctive outcome measures for each phenotype is a way to organize more effective and stratified clinical trials in OA in future (Roman-Blas et al., 2020). For example, the synovitis features detected by MRI or ultrasound (US) have the potential to become the useful outcome measures and could be used in clinical trials of new drugs that target synovitis in OA patients with inflammatory phenotype.

To identify the patient population with disease progression is vital to appropriately power clinical trials. The OA patients in the progressed periods are potentially more responsive to interventions, and these patients might be recruited in DMOAD trials to assess the efficacy of a new drug in the future. Sensitive and valid biomarkers are expected to become useful tools to predict OA progression and understand mechanisms of progression (Roman-Blas et al., 2020). On the other hand, OA may only be retarded at early to mid-stages instead of established or advanced OA. To identify the patient population in the early to mid-stages of the disease is also important. Some studies have proposed using MRI or US for the test of disease-modifying approaches and recruiting patients with early diseases as defined on MRI or US in clinical trials (Eckstein and Le Graverand, 2015; Wang et al., 2021).

There is an unmet need for DMOADs. One approach to develop such drugs is to use imaging-assessed joint structural changes such as loss of cartilage volume/thickness, BMLs and synovitis as primary endpoints. However, these endpoints have not been formally accepted by drug administrations. Recently, several authors from The United States Food and Drug Administration proposed a composite endpoint such as "time to total knee replacement (TKR) or severe pain or severely impaired functioning" which can substantially reduce sample size compared to the use of TKR alone (Kim et al., 2020). The endpoints such as this based on direct measures of patients' functions, feels or survive would be more clinically relevant for development of OA drugs.

A variety of potential therapeutics targeting on inflammation, cellular senescence, cartilage metabolism, subchondral bone remodeling, and peripheral nociceptive pathway are expected to reshape the landscape of OA treatment over the next few years (**Figure 1**). The cartilage destruction is the main characteristic sign of OA. Novel agents targeting articular cartilage molecular mechanisms seem to be most promising. Lorecivivint, MIV-711 and Sprifermin are promising agents as DMOADs to slow disease progression. Long-term RCTs are still needed to confirm the safety and efficacy of these novel OA pharmacotherapy medicines.

AUTHOR CONTRIBUTIONS

XC and SY initiated this review and wrote the manuscript. CD and NY revised our first draft and provided valuable comments. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Science Foundation of Guangzhou First People's Hospital.

REFERENCES

Abou-Raya, A., Abou-Raya, S., and Khadrawe, T. (2018). Retracted: methotrexate in the treatment of symptomatic knee osteoarthritis: randomised placebo-controlled trial. *Ann. Rheum. Dis.* 77 (7), e46. doi:10.1136/annrheumdis-2013-204856

Aitken, D., Laslett, L. L., Pan, F., Haugen, I. K., Otahal, P., Bellamy, N., et al. (2018). A randomised double-blind placebo-controlled crossover trial of HUMira (adalimumab) for erosive hand OsteoaRthritis - the HUMOR trial. *Osteoarthritis Cartilage* 26 (7), 880–887. doi:10.1016/j.joca.2018.02.899

Arden, N. K., Perry, T. A., Bannuru, R. R., Bruyère, O., Cooper, C., Haugen, I. K., et al. (2020). Non-surgical management of knee osteoarthritis: comparison of ESCEO and OARSI 2019 guidelines. *Nat. Rev. Rheumatol.* 17, 59. doi:10.1038/s41584-020-00523-9

Bakker, A. C., van de Loo, F. A. J., van Beuningen, H. M., Sime, P., van Lent, P. L. E. M., van der Kraan, P. M., et al. (2001). Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. *Osteoarthritis Cartilage* 9 (2), 128–136. doi:10.1053/joca.2000.0368

Bally, M., Dendukuri, N., Rich, B., Nadeau, L., Helin-Salmivaara, A., Garbe, E., et al. (2017). Risk of acute myocardial infarction with NSAIDs in real world use: bayesian meta-analysis of individual patient data. *BMJ* 357, j1909. doi:10.1136/bmj.j1909

Berenbaum, F., Blanco, F. J., Guermazi, A., Miki, K., Yamabe, T., Viktrup, L., et al. (2020). Subcutaneous tanezumab for osteoarthritis of the hip or knee: efficacy and safety results from a 24-week randomised phase III study with a 24-week follow-up period. *Ann. Rheum. Dis.* 79 (6), 800–810. doi:10.1136/annrheumdis-2019-216296

Brett, A., Bowes, M. A., Conaghan, P. G., Ladel, C., Guehring, H., Moreau, F., et al. (2020). Automated MRI assessment confirms cartilage thickness modification in patients with knee osteoarthritis: post-hoc analysis from a phase II sprifermin study. *Osteoarthritis Cartilage* 28 (11), 1432–1436. doi:10.1016/j.joca.2020.08.005

Cai, G., Aitken, D., Laslett, L. L., Pelletier, J.-P., Martel-Pelletier, J., Hill, C., et al. (2020). Effect of intravenous zoledronic acid on tibiofemoral cartilage volume Among patients with knee osteoarthritis with bone marrow lesions. *JAMA* 323 (15), 1456–1466. doi:10.1001/jama.2020.2938

Cao, Y., Xu, R. X., and Liu, Z. (2014). A high-throughput quantification method of curcuminoids and curcumin metabolites in human plasma via high-performance liquid chromatography/tandem mass spectrometry. *J. Chromatogr. B* 949-950, 70–78. doi:10.1016/j.jchromb.2013.12.039

Cao, P., Li, Y., Tang, Y., Ding, C., and Hunter, D. J. (2020). Pharmacotherapy for knee osteoarthritis: current and emerging therapies. *Expert Opin. Pharmacother.* 21 (7), 797–809. doi:10.1080/14656566.2020.1732924

Carlson, A. K., Rawle, R. A., Wallace, C. W., Brooks, E. G., Adams, E., Greenwood, M. C., et al. (2019). Characterization of synovial fluid metabolomic phenotypes of cartilage morphological changes associated with osteoarthritis. *Osteoarthritis Cartilage* 27 (8), 1174–1184. doi:10.1016/j.joca.2019.04.007

Cheleschi, S., Cantarini, L., Pascarelli, N. A., Collodel, G., Lucherini, O. M., Galeazzi, M., et al. (2015). Possible chondroprotective effect of canakinumab: an *in vitro* study on human osteoarthritic chondrocytes. *Cytokine* 71 (2), 165–172. doi:10.1016/j.cyto.2014.10.023

Chen, D., Shen, J., Zhao, W., Wang, T., Han, L., Hamilton, J. L., et al. (2017). Osteoarthritis: toward a comprehensive understanding of pathological mechanism. *Bone Res.* 5, 16044. doi:10.1038/boneres.2016.44

Chen, J., Li, J., Li, R., Wang, H., Yang, J., Xu, J., et al. (2017). Efficacy and safety of tanezumab on osteoarthritis knee and hip pains: a meta-analysis of randomized controlled trials. *Pain Med.* 18 (2), pnw262–385. doi:10.1093/pm/pnw262

Cherian, J. J., Parvizi, J., Bramlet, D., Lee, K. H., Romness, D. W., and Mont, M. A. (2015). Preliminary results of a phase II randomized study to determine the efficacy and safety of genetically engineered allogeneic human chondrocytes expressing TGF-β1 in patients with grade 3 chronic degenerative joint disease of the knee. *Osteoarthritis Cartilage* 23 (12), 2109–2118. doi:10.1016/j.joca.2015.06.019

Chevalier, X., and Eymard, F. (2019). Anti-IL-1 for the treatment of OA: dead or alive?. *Nat. Rev. Rheumatol.* 15 (4), 191–192. doi:10.1038/s41584-019-0185-y

Chevalier, X., Goupille, P., Beaulieu, A. D., Burch, F. X., Bensen, W. G., Conrozier, T., et al. (2009). Intraarticular injection of anakinra in osteoarthritis of the knee: a multicenter, randomized, double-blind, placebo-controlled study. *Arthritis Rheum.* 61 (3), 344–352. doi:10.1002/art.24096

Chevalier, X., Ravaud, P., Maheu, E., Baron, G., Rialland, A., Vergnaud, P., et al. (2015). Adalimumab in patients with hand osteoarthritis refractory to analgesics and NSAIDs: a randomised, multicentre, double-blind, placebo-controlled trial. *Ann. Rheum. Dis.* 74 (9), 1697–1705. doi:10.1136/annrheumdis-2014-205348

Childs, B. G., Gluscevic, M., Baker, D. J., Laberge, R.-M., Marquess, D., Dananberg, J., et al. (2017). Senescent cells: an emerging target for diseases of ageing. *Nat. Rev. Drug Discov.* 16 (10), 718–735. doi:10.1038/nrd.2017.116

Chiusaroli, R., Visentini, M., Galimberti, C., Casseler, C., Mennuni, L., Covaceuszach, S., et al. (2013). Targeting of ADAMTS5's ancillary domain with the recombinant mAb CRB0017 ameliorates disease progression in a spontaneous murine model of osteoarthritis. *Osteoarthritis and Cartilage* 21 (11), 1807–1810. doi:10.1016/j.joca.2013.08.015

Cohen, S. B., Proudman, S., Kivitz, A. J., Burch, F. X., Donohue, J. P., Burstein, D., et al. (2011). A randomized, double-blind study of AMG 108 (a fully human monoclonal antibody to IL-1R1) in patients with osteoarthritis of the knee. *Arthritis Res. Ther.* 13 (4), R125. doi:10.1186/ar3430

Conaghan, P. G., Bowes, M. A., Kingsbury, S. R., Brett, A., Guillard, G., Rizoska, B., et al. (2020). Disease-modifying effects of a novel cathepsin K inhibitor in osteoarthritis. *Ann. Intern. Med.* 172 (2), 86–95. doi:10.7326/M19-0675

Conaghan, P. G., Cohen, S. B., Berenbaum, F., Lufkin, J., Johnson, J. R., and Bodick, N. (2018a). Brief report: a phase IIb trial of a novel extended-release microsphere formulation of triamcinolone acetonide for intraarticular injection in knee osteoarthritis. *Arthritis Rheumatol.* 70 (2), 204–211. doi:10.1002/art.40364

Conaghan, P. G., Hunter, D. J., Cohen, S. B., Kraus, V. B., Berenbaum, F., Lieberman, J. R., et al. (2018b). Effects of a single intra-articular injection of a microsphere formulation of triamcinolone acetonide on knee osteoarthritis pain. *J. Bone Jt. Surg.* 100 (8), 666–677. doi:10.2106/JBJS.17.00154

Coryell, P. R., Diekman, B. O., and Loeser, R. F. (2020). Mechanisms and therapeutic implications of cellular senescence in osteoarthritis. *Nat. Rev. Rheumatol.* 17, 47. doi:10.1038/s41584-020-00533-7

Coulombe, J., Faure, H., Robin, B., and Ruat, M. (2004). *In vitro* effects of strontium ranelate on the extracellular calcium-sensing receptor. *Biochem. Biophysical Res. Commun.* 323 (4), 1184–1190. doi:10.1016/j.bbrc.2004.08.209

Crane, J. L., Xian, L., and Cao, X. (2016). Role of TGF-β signaling in coupling bone remodeling. *Methods Mol. Biol.* 1344, 287–300. doi:10.1007/978-1-4939-2966-5_18

da Costa, B. R., Reichenbach, S., Keller, N., Nartey, L., Wandel, S., Jüni, P., et al. (2017). Effectiveness of non-steroidal anti-inflammatory drugs for the treatment of pain in knee and hip osteoarthritis: a network meta-analysis. *Lancet* 390 (10090), e21–e33. doi:10.1016/S0140-6736(17)31744-0

Dakin, P., DiMartino, S. J., Gao, H., Maloney, J., Kivitz, A. J., Schnitzer, T. J., et al. (2019). The efficacy, tolerability, and joint safety of Fasinumab in osteoarthritis pain: a phase IIb/III double-blind, placebo-controlled, randomized clinical trial. *Arthritis Rheumatol.* 71 (11), 1824–1834. doi:10.1002/art.41012

Dejica, V. M., Mort, J. S., Laverty, S., Percival, M. D., Antoniou, J., Zukor, D. J., et al. (2008). Cleavage of type II collagen by cathepsin K in human osteoarthritic cartilage. *Am. J. Pathol.* 173 (1), 161–169. doi:10.2353/ajpath.2008.070494

Denk, F., Bennett, D. L., and McMahon, S. B. (2017). Nerve growth factor and pain mechanisms. *Annu. Rev. Neurosci.* 40, 307–325. doi:10.1146/annurev-neuro-072116-031121

Deshmukh, V., Hu, H., Barroga, C., Bossard, C., Kc, S., Dellamary, L., et al. (2018). A small-molecule inhibitor of the Wnt pathway (SM04690) as a potential disease modifying agent for the treatment of osteoarthritis of the knee. *Osteoarthritis Cartilage* 26 (1), 18–27. doi:10.1016/j.joca.2017.08.015

Deshmukh, V., O'Green, A. L., Bossard, C., Seo, T., Lamangan, L., Ibanez, M., et al. (2019). Modulation of the Wnt pathway through inhibition of CLK2 and DYRK1A by lorecivivint as a novel, potentially disease-modifying approach for knee osteoarthritis treatment. *Osteoarthritis and Cartilage* 27 (9), 1347–1360. doi:10.1016/j.joca.2019.05.006

Deveza, L. A., Nelson, A. E., and Loeser, R. F. (2019). Phenotypes of osteoarthritis: current state and future implications. *Clin. Exp. Rheumatol.* 37 (Suppl. 120), 64–72.

Ding, C., Cicuttini, F., Parameswaran, V., Burgess, J., Quinn, S., and Jones, G. (2009). Serum levels of vitamin D, sunlight exposure, and knee cartilage loss in older adults: the Tasmanian older adult cohort study. *Arthritis Rheum.* 60 (5), 1381–1389. doi:10.1002/art.24486

Duong, L. T., Leung, A. T., and Langdahl, B. (2016). Cathepsin K inhibition: a new mechanism for the treatment of osteoporosis. *Calcif Tissue Int.* 98 (4), 381–397. doi:10.1007/s00223-015-0051-0

Eckstein, F., and Le Graverand, M.-P. H. (2015). Plain radiography or magnetic resonance imaging (MRI): which is better in assessing outcome in clinical trials of disease-modifying osteoarthritis drugs? Summary of a debate held at the World Congress of Osteoarthritis 2014. *Semin. Arthritis Rheum.* 45 (3), 251–256. doi:10.1016/j.semarthrit.2015.06.001

Eckstein, F., Wirth, W., Guermazi, A., Maschek, S., and Aydemir, A. (2015). Brief report: intraarticular sprifermin not only increases cartilage thickness, but also reduces cartilage loss: location-independent post hoc analysis using magnetic resonance imaging. *Arthritis Rheumatol.* 67 (11), 2916–2922. doi:10.1002/art.39265

Eckstein, F., Kraines, J. L., Aydemir, A., Wirth, W., Maschek, S., and Hochberg, M. C. (2020). Intra-articular sprifermin reduces cartilage loss in addition to increasing cartilage gain independent of location in the femorotibial joint: post-hoc analysis of a randomised, placebo-controlled phase II clinical trial. *Ann. Rheum. Dis.* 79 (4), 525–528. doi:10.1136/annrheumdis-2019-216453

Felson, D. T. (2006). Osteoarthritis of the knee. *N. Engl. J. Med.* 354 (8), 841–848. doi:10.1056/NEJMcp051726

Fioravanti, A., Fabbroni, M., Cerase, A., and Galeazzi, M. (2009). Treatment of erosive osteoarthritis of the hands by intra-articular infliximab injections: a pilot study. *Rheumatol. Int.* 29 (8), 961–965. doi:10.1007/s00296-009-0872-0

Fleischmann, R. M., Bliddal, H., Blanco, F. J., Schnitzer, T. J., Peterfy, C., Chen, S., et al. (2019). A phase II trial of lutikizumab, an anti-interleukin-1α/β dual variable domain immunoglobulin, in knee osteoarthritis patients with synovitis. *Arthritis Rheumatol.* 71 (7), 1056–1069. doi:10.1002/art.40840

Fuggle, N., Curtis, E., Shaw, S., Spooner, L., Bruyère, O., Ntani, G., et al. (2019). Safety of opioids in osteoarthritis: outcomes of a systematic review and meta-analysis. *Drugs Aging* 36 (Suppl. 1), 129–143. doi:10.1007/s40266-019-00666-9

Ghouri, A., and Conaghan, P. G. (2019). Treating osteoarthritis pain: recent approaches using pharmacological therapies. *Clin. Exp. Rheumatol.* 37 Suppl 120 (5), 124–129.

Gigout, A., Guehring, H., Froemel, D., Meurer, A., Ladel, C., Reker, D., et al. (2017). Sprifermin (rhFGF18) enables proliferation of chondrocytes producing a hyaline cartilage matrix. *Osteoarthritis Cartilage* 25 (11), 1858–1867. doi:10.1016/j.joca.2017.08.004

Güler-Yüksel, M., Allaart, C. F., Watt, I., Goekoop-Ruiterman, Y. P. M., de Vries-Bouwstra, J. K., van Schaardenburg, D., et al. (2010). Treatment with TNF-α inhibitor infliximab might reduce hand osteoarthritis in patients with rheumatoid arthritis. *Osteoarthritis Cartilage* 18 (10), 1256–1262. doi:10.1016/j.joca.2010.07.011

Ha, C.-W., Noh, M. J., Choi, K. B., and Lee, K. H. (2012). Initial phase I safety of retrovirally transduced human chondrocytes expressing transforming growth factor-beta-1 in degenerative arthritis patients. *Cytotherapy* 14 (2), 247–256. doi:10.3109/14653249.2011.629645

Ha, C. W., Cho, J. J., Elmallah, R. K., Cherian, J. J., Kim, T. W., Lee, M. C., et al. (2015). A multicenter, single-blind, phase IIa clinical trial to evaluate the efficacy and safety of a cell-mediated gene therapy in degenerative knee arthritis patients. *Hum. Gene Ther. Clin. Dev.* 26 (2), 125–130. doi:10.1089/humc.2014.145

Han, W., Fan, S., Bai, X., and Ding, C. (2017). Strontium ranelate, a promising disease modifying osteoarthritis drug. *Expert Opin. Investig. Drugs* 26 (3), 375–380. doi:10.1080/13543784.2017.1283403

Hochberg, M. C., Guermazi, A., Guehring, H., Aydemir, A., Wax, S., Fleuranceau-Morel, P., et al. (2019). Effect of intra-articular sprifermin vs placebo on femorotibial joint cartilage thickness in patients with osteoarthritis. *JAMA* 322 (14), 1360–1370. doi:10.1001/jama.2019.14735

Hochberg, M. C. (2015). Serious joint-related adverse events in randomized controlled trials of anti-nerve growth factor monoclonal antibodies. *Osteoarthritis Cartilage* 23 (Suppl. 1), S18–S21. doi:10.1016/j.joca.2014.10.005

Hochberg, M. C., Tive, L. A., Abramson, S. B., Vignon, E., Verburg, K. M., West, C. R., et al. (2016). When is osteonecrosis not osteonecrosis?: adjudication of reported serious adverse joint events in the tanezumab clinical development program. *Arthritis Rheumatol.* 68 (2), 382–391. doi:10.1002/art.39492

Hunter, D. J., and Bierma-Zeinstra, S. (2019). Osteoarthritis. *Lancet* 393 (10182), 1745–1759. doi:10.1016/S0140-6736(19)30417-9

Jayabalan, P., and Schnitzer, T. J. (2017). Tanezumab in the treatment of chronic musculoskeletal conditions. *Expert Opin. Biol. Ther.* 17 (2), 245–254. doi:10.1080/14712598.2017.1271873

Jeon, O. H., Kim, C., Laberge, R.-M., Demaria, M., Rathod, S., Vasserot, A. P., et al. (2017). Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. *Nat. Med.* 23 (6), 775–781. doi:10.1038/nm.4324

Jeon, O. H., David, N., Campisi, J., and Elisseeff, J. H. (2018). Senescent cells and osteoarthritis: a painful connection. *J. Clin. Invest.* 128 (4), 1229–1237. doi:10.1172/JCI95147

Jin, X., Jones, G., Cicuttini, F., Wluka, A., Zhu, Z., Han, W., et al. (2016). Effect of vitamin D supplementation on tibial cartilage volume and knee pain among patients with symptomatic knee osteoarthritis. *JAMA* 315 (10), 1005–1013. doi:10.1001/jama.2016.1961

Karsdal, M. A., Bay-Jensen, A. C., Lories, R. J., Abramson, S., Spector, T., Pastoureau, P., et al. (2014). The coupling of bone and cartilage turnover in osteoarthritis: opportunities for bone antiresorptives and anabolics as potential treatments?. *Ann. Rheum. Dis.* 73 (2), 336–348. doi:10.1136/annrheumdis-2013-204111

Karsdal, M. A., Byrjalsen, I., Alexandersen, P., Bihlet, A., Andersen, J. R., Riis, B. J., et al. (2015). Treatment of symptomatic knee osteoarthritis with oral salmon calcitonin: results from two phase 3 trials. *Osteoarthritis Cartilage* 23 (4), 532–543. doi:10.1016/j.joca.2014.12.019

Karsdal, M. A., Michaelis, M., Ladel, C., Siebuhr, A. S., Bihlet, A. R., Andersen, J. R., et al. (2016). Disease-modifying treatments for osteoarthritis (DMOADs) of the knee and hip: lessons learned from failures and opportunities for the future. *Osteoarthritis Cartilage* 24 (12), 2013–2021. doi:10.1016/j.joca.2016.07.017

Kim, M. K., Ha, C. W., In, Y., In, S. D., Choi, E. S., Ha, J. K., et al. (2018). A multicenter, double-blind, phase III clinical trial to evaluate the efficacy and safety of a cell and gene therapy in knee osteoarthritis patients. *Hum. Gene Ther. Clin. Dev.* 29 (1), 48–59. doi:10.1089/humc.2017.249

Kim, Y., Levin, G., Nikolov, N. P., Abugov, R., and Rothwell, R. (2020). Concept endpoints informing design considerations for confirmatory clinical trials in osteoarthritis. *Arthritis Care Res*. doi:10.1002/acr.24549

Kingsbury, S. R., Tharmanathan, P., Arden, N. K., Batley, M., Birrell, F., Cocks, K., et al. (2015). Pain reduction with oral methotrexate in knee osteoarthritis, a pragmatic phase iii trial of treatment effectiveness (PROMOTE): study protocol for a randomized controlled trial. *Trials* 16, 77. doi:10.1186/s13063-015-0602-8

Kingsbury, S. R., Tharmanathan, P., Keding, A., Ronaldson, S. J., Grainger, A., Wakefield, R. J., et al. (2018). Hydroxychloroquine effectiveness in reducing symptoms of hand osteoarthritis. *Ann. Intern. Med.* 168 (6), 385–395. doi:10.7326/M17-1430

Kloppenburg, M., Ramonda, R., Bobacz, K., Kwok, W.-Y., Elewaut, D., Huizinga, T. W. J., et al. (2018). Etanercept in patients with inflammatory hand osteoarthritis (EHOA): a multicentre, randomised, double-blind, placebo-controlled trial. *Ann. Rheum. Dis.* 77 (12), 1757–1764. doi:10.1136/annrheumdis-2018-213202

Kloppenburg, M., Peterfy, C., Haugen, I. K., Kroon, F., Chen, S., Wang, L., et al. (2019). Phase IIa, placebo-controlled, randomised study of lutikizumab, an anti-interleukin-1α and anti-interleukin-1β dual variable domain immunoglobulin, in patients with erosive hand osteoarthritis. *Ann. Rheum. Dis.* 78 (3), 413–420. doi:10.1136/annrheumdis-2018-213336

Kraus, V. B., Conaghan, P. G., Aazami, H. A., Mehra, P., Kivitz, A. J., Lufkin, J., et al. (2018). Synovial and systemic pharmacokinetics (PK) of triamcinolone acetonide (TA) following intra-articular (IA) injection of an extended-release microsphere-based formulation (FX006) or standard crystalline suspension in patients with knee osteoarthritis (OA). *Osteoarthritis and Cartilage* 26 (1), 34–42. doi:10.1016/j.joca.2017.10.003

Kroon, F., Bay-Jensen, A., Wittoek, R., Verbruggen, G., Smolen, J., Kloppenburg, M., et al. (2020). Etanercept therapy leads to reductions in matrix metalloproteinase-3 in patients with erosive hand osteoarthritis. *Scand. J. Rheumatol.* 49 (2), 167–168. doi:10.1080/03009742.2019.1657493

Kuttapitiya, A., Assi, L., Laing, K., Hing, C., Mitchell, P., Whitley, G., et al. (2017). Microarray analysis of bone marrow lesions in osteoarthritis demonstrates upregulation of genes implicated in osteochondral turnover, neurogenesis and inflammation. *Ann. Rheum. Dis.* 76 (10), 1764–1773. doi:10.1136/annrheumdis-2017-211396

Lacy, S. E., Wu, C., Ambrosi, D. J., Hsieh, C.-M., Bose, S., Miller, R., et al. (2015). Generation and characterization of ABT-981, a dual variable domain immunoglobulin (DVD-IgTM) molecule that specifically and potently neutralizes both IL-1α and IL-1β. *MAbs* 7 (3), 605–619. doi:10.1080/19420862.2015.1026501

Lane, N. E., and Corr, M. (2017). Anti-NGF treatments for pain - two steps forward, one step back?. *Nat. Rev. Rheumatol.* 13 (2), 76–78. doi:10.1038/nrrheum.2016.224

Larkin, J., Lohr, T. A., Elefante, L., Shearin, J., Matico, R., Su, J.-L., et al. (2015). Translational development of an ADAMTS-5 antibody for osteoarthritis disease modification. *Osteoarthritis and Cartilage* 23 (8), 1254–1266. doi:10.1016/j.joca.2015.02.778

Latourte, A., Kloppenburg, M., and Richette, P. (2020). Emerging pharmaceutical therapies for osteoarthritis. *Nat. Rev. Rheumatol.* 16 (12), 673–688. doi:10.1038/s41584-020-00518-6

Lee, W., Ruijgrok, L., Boxma-de Klerk, B., Kok, M. R., Kloppenburg, M., Gerards, A., et al. (2018). Efficacy of hydroxychloroquine in hand osteoarthritis: a randomized, double-blind, placebo-controlled trial. *Arthritis Care Res.* 70 (9), 1320–1325. doi:10.1002/acr.23471

LeGrand, A., Fermor, B., Fink, C., Pisetsky, D. S., Weinberg, J. B., Vail, T. P., et al. (2001). Interleukin-1, tumor necrosis factor ?, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci**.** *Arthritis Rheum.* 44 (9), 2078–2083. doi:10.1002/1529-0131(200109)44:9<2078::aid-art358>3.0.co;2-j

Leopoldino, A. O., Machado, G. C., Ferreira, P. H., Pinheiro, M. B., Day, R., McLachlan, A. J., et al. (2019). Paracetamol versus placebo for knee and hip osteoarthritis. *Cochrane Database Syst. Rev.* 2, D13273. doi:10.1002/14651858.CD013273

Li, H., Ding, X., Terkeltaub, R., Lin, H., Zhang, Y., Zhou, B., et al. (2020). Exploration of metformin as novel therapy for osteoarthritis: preventing cartilage degeneration and reducing pain behavior. *Arthritis Res. Ther.* 22 (1). doi:10.1186/s13075-020-2129-y

Li, J., Zhang, B., Liu, W.-X., Lu, K., Pan, H., Wang, T., et al. (2020). Metformin limits osteoarthritis development and progression through activation of AMPK signalling. *Ann. Rheum. Dis.* 79 (5), 635–645. doi:10.1136/annrheumdis-2019-216713

Lindström, E., Rizoska, B., Henderson, I., Terelius, Y., Jerling, M., Edenius, C., et al. (2018a). Nonclinical and clinical pharmacological characterization of the potent and selective cathepsin K inhibitor MIV-711. *J. Transl Med.* 16 (1), 125. doi:10.1186/s12967-018-1497-4

Lindström, E., Rizoska, B., Tunblad, K., Edenius, C., Bendele, A. M., Maul, D., et al. (2018b). The selective cathepsin K inhibitor MIV-711 attenuates joint pathology in experimental animal models of osteoarthritis. *J. Transl Med.* 16 (1), 56. doi:10.1186/s12967-018-1425-7

Liu, W., Feng, M., Jayasuriya, C. T., Peng, H., Zhang, L., Guan, Y., et al. (2020). Human osteoarthritis cartilage-derived stromal cells activate joint degeneration through TGF-beta lateral signaling. *FASEB j.* 34 (12), 16552–16566. doi:10.1096/fj.202001448R

Loeser, R. F., Goldring, S. R., Scanzello, C. R., and Goldring, M. B. (2012). Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum.* 64 (6), 1697–1707. doi:10.1002/art.34453

Lohmander, L. S., Hellot, S., Dreher, D., Krantz, E. F. W., Kruger, D. S., Guermazi, A., et al. (2014). Intraarticular sprifermin (recombinant human fibroblast growth factor 18) in knee osteoarthritis: a randomized, double-blind, placebo-controlled trial. *Arthritis Rheumatol.* 66 (7), 1820–1831. doi:10.1002/art.38614

Lories, R. J., Corr, M., and Lane, N. E. (2013). To Wnt or not to Wnt: the bone and joint health dilemma. *Nat. Rev. Rheumatol.* 9 (6), 328–339. doi:10.1038/nrrheum.2013.25

Lu, C.-H., Chung, C.-H., Lee, C.-H., Hsieh, C.-H., Hung, Y.-J., Lin, F.-H., et al. (2018). Combination COX-2 inhibitor and metformin attenuate rate of joint replacement in osteoarthritis with diabetes: a nationwide, retrospective, matched-cohort study in Taiwan. *PLoS One* 13 (1), e0191242. doi:10.1371/journal.pone.0191242

Ma, C. H., Lv, Q., Yu, Y. X., Zhang, Y., Kong, D., Niu, K. R., et al. (2015). Protective effects of tumor necrosis factor-α blockade by adalimumab on articular cartilage and subchondral bone in a rat model of osteoarthritis. *Braz. J. Med. Biol. Res.* 48 (10), 863–870. doi:10.1590/1414-431X20154407

Macica, C., Liang, G., Nasiri, A., and Broadus, A. E. (2011). Genetic evidence of the regulatory role of parathyroid hormone-related protein in articular chondrocyte maintenance in an experimental mouse model. *Arthritis Rheum.* 63 (11), 3333–3343. doi:10.1002/art.30515

Malfait, A.-M., and Schnitzer, T. J. (2013). Towards a mechanism-based approach to pain management in osteoarthritis. *Nat. Rev. Rheumatol.* 9 (11), 654–664. doi:10.1038/nrrheum.2013.138

Malfait, A. M., and Tortorella, M. D. (2019). The "elusive DMOAD": aggrecanase inhibition from laboratory to clinic. *Clin. Exp. Rheumatol.* 37 Suppl 120 (5), 130–134.

McAlindon, T. E., and Bannuru, R. R. (2010). OARSI recommendations for the management of hip and knee osteoarthritis: the semantics of differences and changes. *Osteoarthritis Cartilage* 18 (4), 473–475. doi:10.1016/j.joca.2010.02.011

McAlindon, T., LaValley, M., Schneider, E., Nuite, M., Lee, J. Y., Price, L. L., et al. (2013). Effect of vitamin D supplementation on progression of knee pain and cartilage volume loss in patients with symptomatic osteoarthritis. *JAMA* 309 (2), 155–162. doi:10.1001/jama.2012.164487

McAlindon, T. E., LaValley, M. P., Harvey, W. F., Price, L. L., Driban, J. B., Zhang, M., et al. (2017). Effect of intra-articular triamcinolone vs saline on knee cartilage volume and pain in patients with knee osteoarthritis. *JAMA* 317 (19), 1967–1975. doi:10.1001/jama.2017.5283

Miller, R. E., Miller, R. J., and Malfait, A.-M. (2014). Osteoarthritis joint pain: the cytokine connection. *Cytokine* 70 (2), 185–193. doi:10.1016/j.cyto.2014.06.019

Miller, R. E., Tran, P. B., Ishihara, S., Larkin, J., and Malfait, A. M. (2016). Therapeutic effects of an anti-ADAMTS-5 antibody on joint damage and mechanical allodynia in a murine model of osteoarthritis. *Osteoarthritis Cartilage* 24 (2), 299–306. doi:10.1016/j.joca.2015.09.005

Miller, R. E., Block, J. A., and Malfait, A.-M. (2017). Nerve growth factor blockade for the management of osteoarthritis pain: what can we learn from clinical trials and preclinical models? *Curr. Opin. Rheumatol.* 29 (1), 110–118. doi:10.1097/BOR.0000000000000354

Miller, R. E., Block, J. A., and Malfait, A.-M. (2018). What is new in pain modification in osteoarthritis?. *Rheumatology (Oxford)* 57 (Suppl. l_4), iv99–iv107. doi:10.1093/rheumatology/kex522

Millerand, M., Berenbaum, F., and Jacques, C. (2019). Danger signals and inflammaging in osteoarthritis. *Clin. Exp. Rheumatol.* 37 Suppl 120 (Suppl. 1205), 48–56.

Moore, E. E., Bendele, A. M., Thompson, D. L., Littau, A., Waggie, K. S., Reardon, B., et al. (2005). Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. *Osteoarthritis Cartilage* 13 (7), 623–631. doi:10.1016/j.joca.2005.03.003

Nelson, A. E., Fang, F., Arbeeva, L., Cleveland, R. J., Schwartz, T. A., Callahan, L. F., et al. (2019). A machine learning approach to knee osteoarthritis phenotyping: data from the FNIH Biomarkers Consortium. *Osteoarthritis Cartilage* 27 (7), 994–1001. doi:10.1016/j.joca.2018.12.027

Nwosu, L. N., Gowler, P. R. W., Burston, J. J., Rizoska, B., Tunblad, K., Lindström, E., et al. (2018). Analgesic effects of the cathepsin K inhibitor L-006235 in the monosodium iodoacetate model of osteoarthritis pain. *Pain Rep.* 3 (6), e685. doi:10.1097/PR9.0000000000000685

Ohtori, S., Orita, S., Yamauchi, K., Eguchi, Y., Ochiai, N., Kishida, S., et al. (2015). Efficacy of direct injection of Etanercept into knee joints for pain in moderate and severe knee osteoarthritis. *Yonsei Med. J.* 56 (5), 1379–1383. doi:10.3349/ymj.2015.56.5.1379

Onakpoya, I. J., Spencer, E. A., Perera, R., and Heneghan, C. J. (2017). Effectiveness of curcuminoids in the treatment of knee osteoarthritis: a systematic review and meta-analysis of randomized clinical trials. *Int. J. Rheum. Dis.* 20 (4), 420–433. doi:10.1111/1756-185X.13069

Onuora, S. (2014). Sprifermin shows cartilage-protective effects in knee OA. *Nat. Rev. Rheumatol.* 10 (6), 322. doi:10.1038/nrrheum.2014.68

Oo, W. M., and Hunter, D. J. (2019). Disease modification in osteoarthritis: are we there yet?. *Clin. Exp. Rheumatol.* 37 Suppl 120 (5), 135–140.

Oo, W. M., Yu, S. P.-C., Daniel, M. S., and Hunter, D. J. (2018). Disease-modifying drugs in osteoarthritis: current understanding and future therapeutics. *Expert Opin. Emerging Drugs* 23 (4), 331–347. doi:10.1080/14728214.2018.1547706

Orita, S., Koshi, T., Mitsuka, T., Miyagi, M., Inoue, G., Arai, G., et al. (2011). Associations between proinflammatory cytokines in the synovial fluid and radiographic grading and pain-related scores in 47 consecutive patients with osteoarthritis of the knee. *BMC Musculoskelet. Disord.* 12, 144. doi:10.1186/1471-2474-12-144

Peat, G., and Thomas, M. J. (2020). Osteoarthritis year in review 2020: epidemiology & therapy. *Osteoarthritis Cartilage* 29, 180–189. doi:10.1016/j.joca.2020.10.007

Pelletier, J. P., Roubille, C., Raynauld, J. P., Abram, F., Dorais, M., Delorme, P., et al. (2015). Disease-modifying effect of strontium ranelate in a subset of patients from the Phase III knee osteoarthritis study SEKOIA using quantitative MRI: reduction in bone marrow lesions protects against cartilage loss. *Ann. Rheum. Dis.* 74 (2), 422–429. doi:10.1136/annrheumdis-2013-203989

Piecha, D., Weik, J., Kheil, H., Becher, G., Timmermann, A., Jaworski, A., et al. (2010). Novel selective MMP-13 inhibitors reduce collagen degradation in bovine articular and human osteoarthritis cartilage explants. *Inflamm. Res.* 59 (5), 379–389. doi:10.1007/s00011-009-0112-9

Pincus, T., Castrejon, I., Yazici, Y., Gibson, K. A., Bergman, M. J., and Block, J. A. (2019). Osteoarthritis is as severe as rheumatoid arthritis: evidence over 40 years according to the same measure in each disease. *Clin. Exp. Rheumatol.* 37 Suppl 120 (5), 7–17.

Reginster, J.-Y., Badurski, J., Bellamy, N., Bensen, W., Chapurlat, R., Chevalier, X., et al. (2013). Efficacy and safety of strontium ranelate in the treatment of knee osteoarthritis: results of a double-blind, randomised placebo-controlled trial. *Ann. Rheum. Dis.* 72 (2), 179–186. doi:10.1136/annrheumdis-2012-202231

Reginster, J.-Y., Reiter-Niesert, S., Bruyère, O., Berenbaum, F., Brandi, M.-L., Branco, J., et al. (2015). Recommendations for an update of the 2010 European regulatory guideline on clinical investigation of medicinal products used in the treatment of osteoarthritis and reflections about related clinically relevant outcomes: expert consensus statement. *Osteoarthritis Cartilage* 23 (12), 2086–2093. doi:10.1016/j.joca.2015.07.001

Reker, D., Kjelgaard-Petersen, C. F., Siebuhr, A. S., Michaelis, M., Gigout, A., Karsdal, M. A., et al. (2017). Sprifermin (rhFGF18) modulates extracellular matrix turnover in cartilage explants *ex vivo*. *J. Transl Med.* 15 (1), 250. doi:10.1186/s12967-017-1356-8

Roemer, F. W., Aydemir, A., Lohmander, S., Crema, M. D., Marra, M. D., Muurahainen, N., et al. (2016). Structural effects of sprifermin in knee osteoarthritis: a post-hoc analysis on cartilage and non-cartilaginous tissue alterations in a randomized controlled trial. *BMC Musculoskelet. Disord.* 17, 267. doi:10.1186/s12891-016-1128-2

Roman-Blas, J. A., Mendoza-Torres, L. A., Largo, R., and Herrero-Beaumont, G. (2020). Setting up distinctive outcome measures for each osteoarthritis phenotype. *Ther. Adv. Musculoskelet.* 12, 1759720X20937966. doi:10.1177/1759720X20937966

Rünger, T. M., Adami, S., Benhamou, C. L., Czerwiński, E., Farrerons, J., Kendler, D. L., et al. (2012). Morphea-like skin reactions in patients treated with the cathepsin K inhibitor balicatib. *J. Am. Acad. Dermatol.* 66 (3), e89–e96. doi:10.1016/j.jaad.2010.11.033

Salminen-Mankonen, H., Morko, J., and Vuorio, E. (2007). Role of cathepsin K in normal joints and in the development of arthritis. *Curr. Drug Targets* 8 (2), 315–323. doi:10.2174/138945007779940188

Sampson, E. R., Hilton, M. J., Tian, Y., Chen, D., Schwarz, E. M., Mooney, R. A., et al. (2011). Teriparatide as a chondroregenerative therapy for injury-induced osteoarthritis. *Sci. Transl. Med.* 3 (101), 101ra93. doi:10.1126/scitranslmed.3002214

Scanzello, C. R., and Loeser, R. F. (2015). Editorial: inflammatory activity in symptomatic knee osteoarthritis: not all inflammation is local. *Arthritis Rheumatol.* 67 (11), 2797–2800. doi:10.1002/art.39304

Schett, G., Dayer, J.-M., and Manger, B. (2016). Interleukin-1 function and role in rheumatic disease. *Nat. Rev. Rheumatol.* 12 (1), 14–24. doi:10.1038/nrrheum.2016.166

Schnitzer, T. J., Easton, R., Pang, S., Levinson, D. J., Pixton, G., Viktrup, L., et al. (2019). Effect of tanezumab on joint pain, physical function, and patient global assessment of osteoarthritis among patients with osteoarthritis of the hip or knee. *JAMA* 322 (1), 37–48. doi:10.1001/jama.2019.8044

Schnute, M. E., O'Brien, P. M., Nahra, J., Morris, M., Howard Roark, W., Hanau, C. E., et al. (2010). Discovery of (pyridin-4-yl)-2H-tetrazole as a novel scaffold to identify highly selective matrix metalloproteinase-13 inhibitors for the treatment of osteoarthritis. *Bioorg. Med. Chem. Lett.* 20 (2), 576–580. doi:10.1016/j.bmcl.2009.11.081

Sennett, M. L., Meloni, G. R., Farran, A. J. E., Guehring, H., Mauck, R. L., and Dodge, G. R. (2018). Sprifermin treatment enhances cartilage integration in an *in vitro* repair model. *J. Orthop. Res.* 36 (10), 2648–2656. doi:10.1002/jor.24048

Shen, L., and Ji, H. F. (2012). The pharmacology of curcumin: is it the degradation products?. *Trends Mol. Med.* 18 (3), 138–144. doi:10.1016/j.molmed.2012.01.004

Siebuhr, A. S., Werkmann, D., Bay-Jensen, A.-C., Thudium, C. S., Karsdal, M. A., Serruys, B., et al. (2020). The anti-ADAMTS-5 Nanobody M6495 protects cartilage degradation ex vivo. *Int. J. Mol. Sci.* 21 (17), 5992. doi:10.3390/ijms21175992

Sohn, D., Sokolove, J., Sharpe, O., Erhart, J. C., Chandra, P. E., Lahey, L. J., et al. (2012). Plasma proteins present in osteoarthritic synovial fluid can stimulate cytokine production via Toll-like receptor 4. *Arthritis Res. Ther.* 14 (1), R7. doi:10.1186/ar3555

Sota, J., Vitale, A., Vitale, A., Insalaco, A., Sfriso, P., Lopalco, G., et al. (2018). Safety profile of the interleukin-1 inhibitors anakinra and canakinumab in real-life clinical practice: a nationwide multicenter retrospective observational study. *Clin. Rheumatol.* 37 (8), 2233–2240. doi:10.1007/s10067-018-4119-x

Tat, S. K., Pelletier, J.-P., Mineau, F., Caron, J., and Martel-Pelletier, J. (2011). Strontium ranelate inhibits key factors affecting bone remodeling in human osteoarthritic subchondral bone osteoblasts. *Bone* 49 (3), 559–567. doi:10.1016/j.bone.2011.06.005

Thysen, S., Luyten, F. P., and Lories, R. J. (2015). Loss of Frzb and Sfrp1 differentially affects joint homeostasis in instability-induced osteoarthritis. *Osteoarthritis Cartilage* 23 (2), 275–279. doi:10.1016/j.joca.2014.10.010

Tu, L., Zheng, S., Cicuttini, F., Jin, X., Han, W., Zhu, Z., et al. (2020). Effects of vitamin D supplementation on disabling foot pain in patients with symptomatic knee osteoarthritis. *Arthritis Care Res.* doi:10.1002/acr.24371

Usami, Y., Gunawardena, A. T., Iwamoto, M., and Enomoto-Iwamoto, M. (2016). Wnt signaling in cartilage development and diseases: lessons from animal studies. *Lab. Invest.* 96 (2), 186–196. doi:10.1038/labinvest.2015.142

Vaysbrot, E. E., Osani, M. C., Musetti, M.-C., McAlindon, T. E., and Bannuru, R. R. (2018). Are bisphosphonates efficacious in knee osteoarthritis? A meta-analysis of randomized controlled trials. *Osteoarthritis Cartilage* 26 (2), 154–164. doi:10.1016/j.joca.2017.11.013

Verbruggen, G., Wittoek, R., Cruyssen, B. V., and Elewaut, D. (2012). Tumour necrosis factor blockade for the treatment of erosive osteoarthritis of the interphalangeal finger joints: a double blind, randomised trial on structure modification. *Ann. Rheum. Dis.* 71 (6), 891–898. doi:10.1136/ard.2011.149849

Vincent, T. L. (2020). Peripheral pain mechanisms in osteoarthritis. *Pain* 161 (Suppl. 1), S138–S146. doi:10.1097/j.pain.0000000000001923

Wang, C. C., Wang, C. T., Tsai, K. L., Chou, C. L., Chao, J. K., Huang, H. Y., et al. (2021). Effect of ultrasound-detected synovitis on therapeutic efficacy of hyaluronic acid injection for symptomatic knee osteoarthritis. *Rheumatology (Oxford)*. doi:10.1093/rheumatology/keab020

Wang, J., Ma, J., Gu, J.-H., Wang, F.-Y., Shang, X. S., Tao, H. R., et al. (2017). Regulation of type II collagen, matrix metalloproteinase-13 and cell proliferation by interleukin-1β is mediated by curcumin via inhibition of NF-κB signaling in rat chondrocytes. *Mol. Med. Rep.* 16 (2), 1837–1845. doi:10.3892/mmr.2017.6771

Wang, S. X., Abramson, S. B., Attur, M., Karsdal, M. A., Preston, R. A., Lozada, C. J., et al. (2017). Safety, tolerability, and pharmacodynamics of an anti-interleukin-1α/β dual variable domain immunoglobulin in patients with osteoarthritis of the knee: a randomized phase 1 study. *Osteoarthritis Cartilage* 25 (12), 1952–1961. doi:10.1016/j.joca.2017.09.007

Wang, X., Cicuttini, F., Jin, X., Wluka, A. E., Han, W., Zhu, Z., et al. (2017). Knee effusion-synovitis volume measurement and effects of vitamin D supplementation in patients with knee osteoarthritis. *Osteoarthritis Cartilage* 25 (8), 1304–1312. doi:10.1016/j.joca.2017.02.804

Wang, Y., Hussain, S. M., Wluka, A. E., Lim, Y. Z., Abram, F., Pelletier, J.-P., et al. (2019). Association between metformin use and disease progression in obese people with knee osteoarthritis: data from the Osteoarthritis Initiative-a prospective cohort study. *Arthritis Res. Ther.* 21 (1). doi:10.1186/s13075-019-1915-x

Wang, Z., Jones, G., Winzenberg, T., Cai, G., Laslett, L. L., Aitken, D., et al. (2020). Effectiveness of curcuma longa extract for the treatment of symptoms and effusion-synovitis of knee osteoarthritis. *Ann. Intern. Med.* 173 (11), 861–869. doi:10.7326/M20-0990

Yang, X., Chen, L., Xu, X., Li, C., Huang, C., and Deng, C. X. (2001). TGF-β/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. *J. Cell Biol.* 153 (1), 35–46. doi:10.1083/jcb.153.1.35

Yazici, Y., McAlindon, T. E., Fleischmann, R., Gibofsky, A., Lane, N. E., Kivitz, A. J., et al. (2017). A novel Wnt pathway inhibitor, SM04690, for the treatment of moderate to severe osteoarthritis of the knee: results of a 24-week, randomized, controlled, phase 1 study. *Osteoarthritis Cartilage* 25 (10), 1598–1606. doi:10.1016/j.joca.2017.07.006

Yu, D. G., Ding, H. F., Mao, Y. Q., Liu, M., Yu, B., Zhao, X., et al. (2013). Strontium ranelate reduces cartilage degeneration and subchondral bone remodeling in rat osteoarthritis model. *Acta Pharmacol. Sin* 34 (3), 393–402. doi:10.1038/aps.2012.167

Zhang, R. K., Li, G. W., Zeng, C., Lin, C. X., Huang, L. S., Huang, G. X., et al. (2018). Mechanical stress contributes to osteoarthritis development through the activation of transforming growth factor beta 1 (TGF-β1). *Bone Jt. Res.* 7 (11), 587–594. doi:10.1302/2046-3758.711.BJR-2018-0057.R1

Zhang, W., Nuki, G., Moskowitz, R. W., Abramson, S., Altman, R. D., Arden, N. K., et al. (2010). OARSI recommendations for the management of hip and knee osteoarthritis. *Osteoarthritis Cartilage* 18 (4), 476–499. doi:10.1016/j.joca.2010.01.013

Zheng, S., Jin, X., Cicuttini, F., Wang, X., Zhu, Z., Wluka, A., et al. (2017). Maintaining vitamin D sufficiency is associated with improved structural and symptomatic outcomes in knee osteoarthritis. *Am. J. Med.* 130 (10), 1211–1218. doi:10.1016/j.amjmed.2017.04.038

Zheng, S., Tu, L., Cicuttini, F., Han, W., Zhu, Z., Antony, B., et al. (2019). Effect of vitamin D supplementation on depressive symptoms in patients with knee osteoarthritis. *J. Am. Med. Directors Assoc.* 20 (12), 1634–1640. doi:10.1016/j.jamda.2018.09.006

Zhu, Z., Yu, Q., Leng, X., Han, W., Li, Z., Huang, C., et al. (2020). Can low-dose methotrexate reduce effusion-synovitis and symptoms in patients with mid- to late-stage knee osteoarthritis? Study protocol for a randomised, double-blind, and placebo-controlled trial. *Trials* 21 (1), 795. doi:10.1186/s13063-020-04687-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 11 May 2021
doi: 10.3389/fphar.2021.635034

Reduction of Matrix Metallopeptidase 13 and Promotion of Chondrogenesis by Zeel T in Primary Human Osteoarthritic Chondrocytes

Christelle Sanchez[1], Kathrin Hemmer[2], Natascha Krömmelbein[2], Bernd Seilheimer[2], Jean-Emile Dubuc[1,3], Christophe Antoine[4] and Yves Henrotin[1,4,5]*

[1]MusculoSKeletal Innovative Research Lab, University of Liège, Center for Interdisciplinary Research on Medicines, Liège, Belgium, [2]Heel GmbH, Baden-Baden, Germany, [3]Division of Orthopedics and Musculoskeletal Trauma, Cliniques Universitaires de St Luc, Brussels, Belgium, [4]Artialis SA, Liège, Belgium, [5]Physical Therapy and Rehabilitation Department, Princess Paola Hospital, Vivalia, Marche-en-Famenne, Belgium

Objectives: Zeel T (Ze14) is a multicomponent medicinal product. Initial preclinical data suggested a preventive effect on cartilage degradation. Clinical observational studies demonstrated that Ze14 reduced symptoms of osteoarthritis (OA), including stiffness and pain. This study aimed to explore these effects further to better understand the mode of action of Ze14 on human OA chondrocytes *in vitro*.

Methods: Primary chondrocytes were obtained from the knees of 19 OA patients and cultured either as monolayers or in alginate beads. The cultures were treated with 20% or 10% (v/v) Ze14 or placebo. For RNA-seq, reads were generated with Illumina NextSeq5000 sequencer and aligned to the human reference genome (UCSC hg19). Differential expression analysis between Ze14 and placebo was performed in R using the DESeq2 package. Protein quantification by ELISA was performed on selected genes from the culture medium and/or the cellular fractions of primary human OA chondrocyte cultures.

Results: In monolayer cultures, Ze14 20% (v/v) significantly modified the expression of 13 genes in OA chondrocytes by at least 10% with an adjusted p-value < 0.05: EGR1, FOS, NR4A1, DUSP1, ZFP36, ZFP36L1, NFKBIZ, and CCN1 were upregulated and ATF7IP, TXNIP, DEPP1, CLEC3A, and MMP13 were downregulated after 24 h Ze14 treatment. Ze14 significantly increased (mean 2.3-fold after 24 h, $p = 0.0444$ and 72 h, $p = 0.0239$) the CCN1 protein production in human OA chondrocytes. After 72 h, Ze14 significantly increased type II collagen pro-peptide production by mean 27% ($p = 0.0147$). For both time points CCN1 production by OA chondrocytes was correlated with aggrecan

OPEN ACCESS

Edited by:
Per-Johan Jakobsson, Karolinska Institutet (KI), Sweden

Reviewed by:
Carole L Wilson, University of Wisconsin-Madison, United States
Cinzia Parolini, University of Milan, Italy

****Correspondence:***
Christelle Sanchez
christelle.sanchez@uliege.be

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *29 November 2020*
Accepted: *26 February 2021*
Published: *11 May 2021*

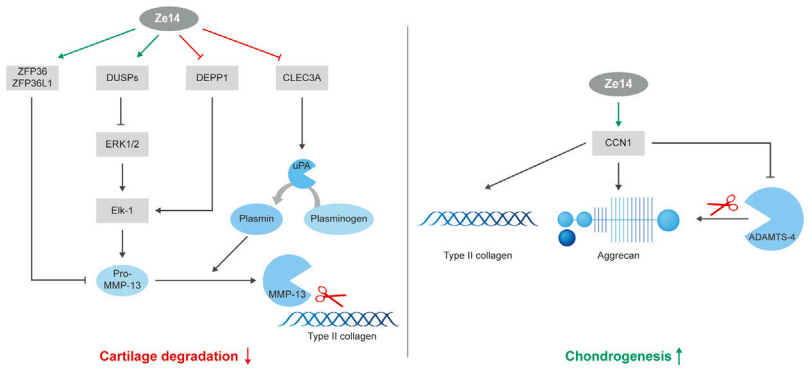
Citation:
Sanchez C, Hemmer K, Krömmelbein N, Seilheimer B, Dubuc J-E, Antoine C and Henrotin Y (2021) Reduction of Matrix Metallopeptidase 13 and Promotion of Chondrogenesis by Zeel T in Primary Human Osteoarthritic Chondrocytes. Front. Pharmacol. 12:635034. doi: 10.3389/fphar.2021.635034

Abbreviations: ADAMTS-4, a disintegrin and metalloproteinase with thrombospondin motifs 4; AP, alkaline phosphatase; CM, cells with associated matrix; CYR61, cysteine-rich angiogenic inducer 61; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; EGR, early growth response; FBS, fetal bovine serum; FDR, false discovery rate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL, interleukin; LDH, lactate dehydrogenase; MMP, matrix metallopeptidase; NGS, next-generation sequencing; OA, osteoarthritis; padj, p-value adjusted; RA, rheumatoid arthritis; RT-PCR, reverse transcription-polymerase chain reaction; RIN, RNA integrity number; RNA, ribonucleic acid; ROS, reactive oxygen species; SD, standard deviation; SE, standard error; TKR, total knee replacement; TNF, tumor necrosis factor.

(r = 0.66, p = 0.0004) and type II collagen pro-peptide (r = 0.64, p = 0.0008) production. In alginate beads cultures, pro-MMP-13 was decreased by Ze14 from day 7–14 (from –16 to –25%, p < 0.05) and from day 17–21 (–22%, p = 0.0331) in comparison to controls.

Conclusion: Ze14 significantly modified the expression of DUSP1, DEPP1, ZFP36/ZFP36L1, and CLEC3A, which may reduce MMP13 expression and activation. Protein analysis confirmed that Ze14 significantly reduced the production of pro-MMP-13. As MMP-13 is involved in type II collagen degradation, Ze14 may limit cartilage degradation. Ze14 also promoted extracellular matrix formation arguably through CCN1 production, a growth factor well correlated with type II collagen and aggrecan production.

Keywords: osteoarthritis, cartilage, chondrogenesis, zeel T, MMP13, multitarget



GRAPHICAL ABSTRACT |

INTRODUCTION

Osteoarthritis is the most common form of arthritis, affecting millions of people worldwide. It is a serious disorder primarily affecting weight bearing joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses, including proinflammatory pathways of innate immunity. The disease manifests first as a molecular dysregulation (abnormal joint tissue metabolism) progressing to anatomical derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation, and loss of normal joint function) (Kraus et al., 2015). It primarily affects the elderly population. Due to an increasing number of OA patients, finding a disease-modifying OA drug, defined as "a drug that inhibits structural disease progression and also improves symptoms and/or function" (Barr and Conaghan, 2013), remains an urgent unmet need. Currently, despite the guidelines, first-line treatment of OA in daily medical practice remains with analgesics and anti-inflammatory drugs, although it is recognized that the long-term use of these drugs is linked to severe adverse effects (Bannuru et al., 2019). The recommendations published by medical and scientific societies are indeed unanimous: their use must be limited. Therefore, it is essential to find alternative treatments to manage the symptoms of patients with OA as well as to inhibit structural disease progression (Geenen et al., 2018; Rausch Osthoff et al., 2018; Bannuru et al., 2019). Due to the complexity of the disease, multitarget treatments seem to be more suitable to target the multiple pathological pathways associated with OA. Zeel T (Ze14) is a multicomponent medicinal product composed of plant and organ extracts. Clinical observational studies demonstrated that Ze14 reduced symptoms of OA, including stiffness and pain, and was generally well-tolerated with a good safety profile (Gottwald and Weiser, 2000; Lesiak et al., 2001). Initial preclinical data suggested a preventive effect of Ze14 on cartilage degradation (Stancikova, 1999); however, Ze14's mode of action is still poorly understood. Due to its promising multitarget nature and the lack of effective treatment options for OA patients, investigating the mode of action of Ze14 in OA pathophysiology may provide important insights explaining at least some effects observed in the clinical studies with OA patients.

Recently, transcriptome analysis was used to investigate the effects of natural or chemically produced molecules on chondrocyte transcriptome *in vitro* (Gouze et al., 2006; James et al., 2007; Comblain et al., 2016; Aury-Landas et al., 2017).

Technologies like Next Generation Sequencing RNA-seq generate an unbiased view of the transcriptome offering a wider dynamic range, high sensitivity, and accurate results on all genes expressed by cells (Wang et al., 2014; Liu et al., 2015; Li et al., 2016).

This exploratory study aimed to investigate the mode of action of Ze14 in OA by comparing the transcriptomic profile of human OA chondrocytes treated with Ze14 or saline as control. The transcriptomic profile of OA chondrocytes was determined from primary cell cultures treated with Ze14 at two concentrations, with or without the addition of IL-1β to activate inflammatory pathways. Human primary cells retain the morphological and functional characteristics of their tissue of origin. Thus, we chose this primary OA cell model to reflect the pathological processes of knee OA, also considering patient to patient variations. Affected genes of the most relevant disease pathways were then confirmed by protein analysis in additional chondrocyte cultures.

MATERIAL AND METHODS

Ethical Statement

Articular cartilage samples from 19 patients with knee OA were obtained at the time of total knee joint replacement (TKR) surgery. All participants have signed the informed patient consent, and the protocol was approved by the ethical committee of the Catholic University of Louvain (B403201214793 amendment n°2). All procedures followed the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, revised in 2000. The Supplementary File S1 gives an overview of the patients' characteristics.

Study Medication

Zeel T (Ze14) injection solution was manufactured and bottled in 2.0 mL glass ampoules by Heel GmbH, Germany, according to GMP standards. The study medication was packaged, shipped, and labeled by Heel GmbH, Germany. The full composition of Ze14 injection solution is provided in the Supplementary File 2. Each 1.1 mL ampoule of the saline control contained 0.9% sodium chloride for injection.

Study Design

The cartilage samples from the patients were divided into two groups, one for sequencing and transcriptome generation, and another for protein quantification by ELISA. The harvested tissue was enzymatically digested and seeded in monolayer cultures or suspended in alginate beads depending on the analysis. Supplementary File 3 gives a detailed overview of the study design and explains the distribution of tissue samples for the primary cultures, number of treatment conditions including controls, time points of sample collection for analyses, and performed analyses. In short, for transcriptome analysis, chondrocytes were treated with Ze14 or saline control with or without the presence of IL-1β to induce the inflammatory state. Two concentrations of Ze14, 10% (v/v) and 20% (v/v) were used. For protein analysis, chondrocytes were seeded as monolayers or suspended in alginate beads to investigate chondrocyte hypertrophy. Both cultures were treated with either 20% (v/v) Ze14 or saline control in medium.

Sample Collection

Full-depth articular cartilage was excised and immersed in Dulbecco's Modified Eagle Medium (DMEM) (with phenol red and 4.5 g/L glucose) supplemented with N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) (10 mM), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) (all from Lonza, Belgium). After three washes, chondrocytes were released from cartilage by sequential enzymatic digestions with 0.5 mg/mL hyaluronidase type IV S (Sigma-Aldrich, Belgium) for 30 min at 37°C, 1 mg/mL pronase E (Merck, Belgium) for 1 h at 37°C and 0.5 mg/mL clostridial collagenase IA (Sigma-Aldrich, Belgium) for 16–20 h at 37°C. The enzymatically isolated cells were then filtered through a nylon mesh (70 μm), washed three times and counted.

Chondrocyte Monolayer Culture

Chondrocytes were dispersed in a suspension of 0.1×10^6 cells/mL in DMEM supplemented with 10% fetal bovine serum (Biowest, France), 10 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine (Lonza, Belgium), 20 μg/mL proline and 50 μg/mL ascorbic acid (Sigma-Aldrich, Belgium) and seeded in 6-well plates at the density of 0.2×10^6 cells/well. Chondrocytes were cultured in monolayer for 5–7 days until 95% confluence. Only primary cultures were used to ensure the stability of the chondrocyte phenotype. Chondrocytes were then cultured 24 h in DMEM supplemented with 1% fetal bovine serum, 10 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine, 20 μg/mL proline and 50 μg/mL ascorbic acid. Afterward, the culture medium was replaced by fresh culture medium containing either 20% (v/v) saline (control), 20% (v/v) Ze14 or 10% (v/v) Ze14 (lower concentration was achieved as follows: 8 parts medium: 1 part saline: 1 part Ze14; corresponding to half of the Ze14 concentration, used only for the transcriptomic study), with or without the addition of human IL-1β 10^{-11} M (Roche, Belgium) (Comblain et al., 2016). Cells were incubated for 24 h (transcriptome analysis only) or 72 h (transcriptome and protein analysis). Each culture condition was carried out in triplicates.

For transcriptome analysis, cells were scrapped after 24 h of incubation, three wells were pooled, and ribonucleic acid (RNA) extraction was performed using an RNeasy mini kit according to the instructions of the manufacturer (Qiagen, Netherlands). Cell lysates were stored frozen at −80°C until RNA extraction.

For LDH release assay, conditioned culture media was collected after 24 h of incubation and assayed immediately. Cells were scraped and homogenized in 500 μL of Tris-HCl buffer by ultrasonic dissociation for 20 s at 4°C, to measure total LDH content. Remaining conditioned culture media was stored at −20°C until further analysis.

For protein analyses, conditioned culture media was collected after 24 h and 72 h of incubation and was then stored at −20°C until further analysis. Three wells were used per condition. Cells

were trypsinized and homogenized in 500 µL of Tris-HCl buffer by ultrasonic dissociation for 20 s at 4°C to measure total DNA content after 24 h and 72 h of incubation.

Chondrocyte Alginate Beads Culture

Chondrocytes were suspended in alginate beads (as described (Sanchez et al., 2002)) and cultured for 28 days in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 mM HEPES, penicillin (100 U/mL) and streptomycin (100 µg/mL), 200 µg/mL glutamine, 50 µg/mL ascorbic acid, and 2 mM proline to induce hypertrophy (Pesesse et al., 2013). Alginate beads containing chondrocytes were placed in 24-well plates, nine beads per well. Six wells were used per time point and treatment: three wells per condition were used for the analyses of the alkaline phosphatase activity and the protein pro-MMP-13 production, and three other wells for RNA extraction and type X collagen gene expression.

Ze14 or saline control was added at 20% (v/v) in the culture medium with every culture medium replacement (from day 0 to day 24). The culture medium was changed twice a week.

At each time point (Day 3-7-14-17-21-24-28), cell culture was stopped, and supernatant was collected and stored at −20°C for further analysis. Additionally, supernatant was collected on day 10. The beads of each well were rinsed in NaCl 0.9% and then dissolved in 1 mL of 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. With this method, 2 fractions were collected: the supernatant containing macromolecules that originated from further-removed matrix (FRM) and pellet-containing cells with their associated matrix (CM). These two fractions CM and FRM were kept separately at −20°C until analysis. The supernatant from the FRM was not used for the analyses. CM of 3 wells was homogenized in 500 µL of Tris-HCl buffer by ultrasonic dissociation for 20 s at 4°C to measure total DNA content and alkaline phosphatase activity. The CM of the remaining 3 wells were homogenized in Lysis buffer for ribonucleic acid (RNA) extraction using RNeasy mini kit according to the instructions of the manufacturer (Qiagen, Netherlands). Cell lysates were stored frozen at −80°C until RNA extraction.

LDH Viability Test

Cell viability was estimated by quantifying the release of LDH into the culture supernatant as previously described (Mathy-Hartert et al., 2009). In brief, a sample of the supernatant or dilutions of standard solution (LDH from rabbit muscle, from Roche, Belgium) was mixed with Tris buffer (10 mM Tris-HCl (pH 8.5), 0.1% bovine serum albumin) containing 800 mM lactate. Then, colorimetric reagent, 1.6 mg/mL iodonitrotetrazolium chloride (Sigma-Aldrich, Belgium), 4 mg/mL nicotinamide adenine dinucleotide (Roche Diagnostics, Belgium), and 0.4 mg/mL phenazine methosulfate (Sigma-Aldrich, Belgium), was added, and the solution was discoloured red after 10 min of incubation at room temperature. The percentage of cell death was obtained by comparing the LDH release into the supernatant to the total LDH concentration (cell and supernatant).

DNA Quantification

DNA content of all cell cultures was measured according to a fluorometric method (Labarca and Paigen, 1980). In brief, 200 µL of 2 µg/mL Hoechst dye solution (Sigma Aldrich, Belgium)−in a buffer containing 50 mM PO_4 and 2 M NaCl− was added to 50 µL of sample. After 30 min incubation time in the dark, 356 nm excitation/458 nm emission was read in a spectrophotometer. Placental DNA (Sigma Aldrich, Belgium) was used for the standard curve.

RNA Extraction

Total RNA was extracted using an RNeasy mini kit (Qiagen, Belgium) according to the instructions of the manufacturer. The yield of the extracted RNA was determined spectrophotometrically by measuring the optical density at 260 nm. For transcriptome analysis, the purity and quality of extracted RNA was further evaluated using an RNA Nano 6000 Bioanalyzer Agilent (Santa Clara, United States) according to the manufacturer's instructions. High-quality RNA with RNA quality indicator scores (RIN) of >8 were used.

RNA-Seq and Differential Gene Expression Analysis

One µg of RNA from each culture condition was used for this analysis. Libraries were prepared with the Illumina Truseq stranded mRNA sample prep kit according to the manufacturer's instructions. Based on poly(A) selection of mRNAs, the coding strand information was kept. Sixty (60) libraries were generated. Poly(A) plus RNA was enriched using oligo (dT) beads followed by fragmentation and reverse transcription. Afterward, the 5′ and 3′ ends of cDNA fragments were prepared to ensure efficient ligation of "Y" adapters containing a unique barcode and primer binding sites. Finally, ligated cDNAs were PCR-amplified to be ready for cluster generation and sequencing.

Sequencing was performed on Illumina NextSeq5000, Single-Read 75 bp read length, high output mode 2017–2018 (Maximum Reads per Run: 400 million clusters), 60 libraries per run, and using 3 runs per library, generating around 20 M reads per sample (Chaitankar et al., 2016). Denaturated NGS library fragments were flowed across a flow cell and hybridized on a lawn of complementary Illumina adapter oligos. Complementary fragments were extended, amplified via bridge amplification PCR and denaturated, resulting in clusters of identical single-stranded library fragments. Fragments were primed and sequenced utilizing reversible terminator nucleotides. Base pairs were identified after laser excitation and fluorescence detection.

Raw data was demultiplexed into individual libraries. After filtering out reads mapping to rRNA, tRNA, mitochondrial RNA, and other contaminants, (e.g. adapters, etc.) using bowtie2, reads were aligned onto the human reference genome (UCSC hg19 annotation) and quantified with Star to give the Counts file. Quality control of sequencing reads was assessed with FASTQC and quality control after mapping with Picard tools. Compilation of tool metrics was performed with MultiQC.

Differential expression analysis was made in R (version 3.4.3 (2017–11–30), https://www.R-project.org/) using the DESeq2 package (1.18.1) and R code design = ~ Patient + Treatment. Analysis was performed with treatment as contrast:

- Saline control vs Ze14 20% (v/v)
- Saline control vs Ze14 10% (v/v)
- Saline control vs Ze14 20% (v/v), both with IL-1β
- Saline control vs Ze14 10% (v/v), both with IL-1β

Alternatively, the interaction "Patient:Treatment" was added to the R code design to evaluate if the treatment effect varied from patient to patient. False Discovery Rate of 0.05 was used to assess the statistical significance.

Alkaline Phosphatase Activity

The enzymatic activity of alkaline phosphatase normalized to the DNA content of the respective well was analyzed in the alginate beads cultures according to the previously described method (Sanchez et al., 2005). In brief, 50 µL of cell extract were incubated with 100 µL of p-nitrophenylphosphate (liquid p-NPP, ready to use, Sigma Aldrich, Belgium). In the presence of ALP, p-NPP is transformed to p-nitrophenol and inorganic phosphate. p-nitrophenol absorbance is measured at 405 nm, after 10 min of incubation at 37°C. A standard preparation of p-nitrophenol was used for calibration. Results were expressed in nmoles of p-nitrophenol released per min and per µg of DNA.

Quantitative Real-Time RT-PCR Gene Expression

Reverse transcription was executed by using sensiscript kit according to the instructions of the manufacturer (Qiagen, Belgium), and cDNA was kept at −20°C. Polymerase chain reaction was performed by using the Rotor Gene (Qiagen, Belgium)—SYBR premix Ex Taq (Takara, Belgium). The PCR template source was either first-strand cDNA (10 ng) or purified DNA standard. The PCR program comprised an initial denaturation step at 95°C for 10 s followed by 40 cycles of denaturation at 95°C for 5 s and then an annealing/extension step at 60°C for 25 s. Followed by an ending melting step from 65°C to 96°C with a 1°C increase each second. The following primer sequences were used to amplify the desired cDNA: Hypoxanthine-guanine phosphoribosyltransferase forward 5′-TGTAATGAC CAGTCAACAGGG-3′ and reverse 5′-TGCCTGACCAAGGAA AGC-3′ and COL10A1 forward 5′-GGGAGTGCCATCATCG-3′ and reverse 5′-AGGGTGGGGTAGAGTT-3′. HPRT was used as an internal standard and the ratio of genes to HPRT was calculated. After HPRT normalization for COL10A1, relative expression was calculated.

ELISA for CCN1, Pro-MMP-13, Aggrecan and Type II Collagen Pro-Peptide

Protein amount of aggrecan was assessed from the supernatant and the cell pellet while the protein amount of type II collagen pro-peptide, CCN1, and pro-MMP-13 was only measured from the supernatant, by specific enzyme amplified sensitivity immunoassays (Aggrecan: Diasource, Belgium PG EASIA KAP1461, batch 1902–2260; CCN1: CYR61 R&Dsystems UK DuoSet DY4055, batch P161032; collagenase 3 precursor -pro-MMP-13-: R&Dsystems UK DuoSet DY913, batch P196342; type II collagen pro-peptide: R&Dsystems UK DuoSet DY7589–05, batch P151876). Aggrecan, type II collagen pro-peptide, and CCN1 were assayed from chondrocytes in monolayer cultures, and pro-MMP-13 was analyzed from chondrocytes of alginate beads hypertrophic cultures. Protein content was normalized to the DNA content.

Statistical Analysis

For the transcriptome analysis, DESeq2 Bioconductor package was used for normalization, principal component analysis, and differential gene expression. DESeq2 differential gene analysis was based on the hypothesis that most genes were not differentially expressed (Anders and Huber, 2010; Love et al., 2014). The method was based on the negative binomial distribution model. Within the DESeq2 package, and with the *estimateSizeFactorsForMatrix* function, scaling factors were calculated for each run. After dividing gene counts by each scaling factor, DESeq2 values were calculated as the total of rescaled gene counts of all runs.

The amplitude of changes is represented either in the log2 Fold Change format (classical representation from DESeq2, where "0" means "no change," "1" means "2-fold induction" and "−1" means "2-fold decrease") or in Fold Change (where "1" means "no change," "2" means "2-fold induction" and "0.5" means "2-fold decrease").

Along with the standard *p*-value, an adjusted *p*-value (padj) was calculated. The adjustment methods included the Bonferroni correction ("bonferroni") in which the *p*-values were multiplied by the number of comparisons. Less conservative corrections were also included by Holm ("holm") (Holm, 1979), Hochberg ("hochberg") (Hochberg, 1988), Hommel ("hommel") (Hommel, 1988), Benjamini and Hochberg ("BH" or its alias "fdr") (Benjamini and Hochberg, 1995), and Benjamini and Yekutieli ("BY") (Benjamini and Yekutieli, 2001), respectively. The "BH" (aka "fdr") and "BY" method of Benjamini, Hochberg, and Yekutieli control the false discovery rate, the expected proportion of false discoveries among the rejected hypotheses.

For the protein analyses, results were statistically analyzed using GraphPad Prism 6.0. Before calculating the statistical difference, a Kolmogorov-Smirnov normality test and a ROUT outlier test were performed. For monolayer experiments, either a ratio paired *t*-test, or a Wilcoxon matched-pairs signed-rank test was used. For alginate beads experiments, paired one-way ANOVA was used to compare Ze14 to saline control at each time point. Furthermore, for cumulative pro-MMP-13 production, two-way ANOVA was used to evaluate the kinetic effect of the treatment. For assessing the correlation between two factors, Pearson's test was used for normal data distribution, and Spearman's test was used non-normal distribution.

RESULTS

Cell Viability and RNA-Seq Database Analysis

Cell viability was higher than 98% and was not affected by IL-1β and/or Ze14 treatment, either at 10% or 20% (v/v)

TABLE 1 | Differentially expressed genes in chondrocytes treated with Ze14 20% (v/v) compared to saline control using the DESeq2 R package designed for RNA-seq differential gene expression paired-analysis. baseMean is the mean of normalized counts of all samples, normalizing for sequencing depth. padj: False Discovery Rate (FDR) adjusted *p*-value (for details see material and method, statistics paragraph).

	GeneName	baseMean	log2 Fold Change	Fold Change	*p*-value	padj
ATF7IP	Activating transcription factor 7 interacting protein	1249	–0.1585	0.896	2.01E-05	0.0153
DEPP1	DEPP1, autophagy regulator	1649	–0.1792	0.883	2.71E-05	0.0189
CLEC3A	C-type lectin domain family 3 member A	1132	–0.1902	0.877	7.14E-06	0.0108
CCN1	Cellular communication network factor 1	26,310	0.1845	1.136	4.81E-06	0.0087
DUSP1	Dual specificity phosphatase 1	738	0.2363	1.178	8.38E-05	0.0448
EGR1	Early growth response 1	1468	0.9481	1.929	1.93E-05	0.0153
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	188	0.9058	1.874	4.44E-05	0.0269
MMP13	Matrix metallopeptidase 13	566	–0.2924	0.817	2.73E-07	0.0008
NFKBIZ	NFKB inhibitor zeta	685	0.2116	1.158	1.61E-05	0.0146
NR4A1	Nuclear receptor subfamily 4 group a member 1	201	0.5149	1.429	8.76E-06	0.0111
TXNIP	Thioredoxin interacting protein	2047	–0.1696	0.889	2.29E-07	0.0008
ZFP36	ZFP36 ring finger protein	599	0.2327	1.175	4.24E-05	0.0269
ZFP36L1	ZFP36 ring finger protein like 1	4000	0.1950	1.145	1.67E-08	0.0002

(Supplementary File 4). RNA-seq experiments were performed with a sequencing depth of 16.75 ± 0.52 million copies of genes per sample. IL-1β 10^{-11} M drastically modified the gene expression pattern of human OA chondrocytes. Principal component analysis (PCA) with regularized-logarithm transformation showed that the large variance between the samples was related to the IL-1β stimulation, explaining 86.9% of the total variance (PC1) (Supplementary File 5). The database was then split into basal and IL-1β-treated samples to continue the analysis.

Ze14 Modulated the Expression of 13 Genes Under Basal Conditions

Under basal conditions, chondrocytes expressed genes representing a well-differentiated and mature chondrogenic cell type as confirmed by the top-count genes, with a high level of type II collagen (COL2A1–third-most counted, 6-times more than COL1A1) and aggrecan (ACAN–eighth-most counted) gene expression (Supplementary File 6).

According to the DESeq2 analysis and in basal condition, Ze14 20% (v/v) significantly modified the expression of 13 genes by at least ± 10% of Fold Change with a padj <0.05 (**Table 1**; **Figure 1A**, full results in Supplementary File 6). Ze14 10% (v/v) failed to significantly modify gene expression.

The treatment dose-response of each patient sample is illustrated in the Supplementary File 7A (upregulated DEGs) and 7B (downregulated DEGs).

IL-1β Induced the Expression of Proinflammatory Genes in Human OA Chondrocytes

IL-1β treatment induced a strong upregulation of proinflammatory cytokines including chemokines. Top 12 cytokines produced by OA chondrocytes were in descending order of expression: CXCL8 (~20.000-fold increase), IL-6, CXCL1, CCL20, CXCL6, CCL2, CXCL3, CXCL2, CXCL5, CCL5, IL-11, and CCL8. CXCL12 and CXCL14 were downregulated by IL-1β. Complete results of differentially expressed genes (DEGs) with IL-1β treatment are in Supplementary File 8. In presence of IL-1β 10^{-11} M, Ze14 20% (v/v) modified the expression of lipopolysaccharide binding protein (LBP, +17%, padj = 0.0126) and E-selectin (SELE, −11%, padj = 0.0252) (**Figure 1B**). Furthermore, Ze14 also increased the expression of CCN1 (+12%, p = 0.0026) and EGR1 (+46%, p = 0.0014) and reduced the expression of two genes encoding key enzymes of the kynurenine pathway of tryptophan degradation, kynureninase (KYNU, −16%, $p = 2.84*10^{-7}$) and indoleamine 2,3-dioxygenase 1 (IDO1, −21% $p = 5.96*10^{-5}$) that had been upregulated by IL-1β (5.78 and 8.81 log2 Fold Change, respectively). Complete results are in the Supplementary Files 7C and 9.

Ze14 Promoted Extracellular Matrix Formation Through CCN1 Production

Cellular communication network factor 1 (CCN1), also known as CYR61, is a growth factor-inducible immediate-early gene, induced notably by TGF-β, shown to be important in chondrogenesis (Chijiiwa et al., 2015). The stimulating effect of Ze14 20% (v/v) on CCN1 production was confirmed by immunoassay. CCN1 was assayed in the culture supernatant of six monolayer chondrocyte cultures from six independent OA patients. Ze14 significantly increased (2.3-fold ± 1.2 after 24 h, p = 0.0444 and 2.3-fold ± 1.0 after 72 h, p = 0.0239) the CCN1 protein production by human OA chondrocytes (**Figure 2A**). Because CCN1 inhibits the activity of ADAMTS-4, an important enzyme involved in aggrecan degradation, and has been shown to increase aggrecan and type II collagen synthesis in chondrocytes (Wong et al., 1997; Chijiiwa et al., 2015), we also quantified the protein production of both molecules in these cell cultures. After 72 h, Ze14 20% (v/v) slightly but not significantly increased aggrecan production (14 ± 19%, p = 0.1117, **Figure 2B**) and significantly increased type II collagen propeptide production by 27 ± 20% (p = 0.0147, **Figure 2C**). For both time points, CCN1 production by OA chondrocytes was positively

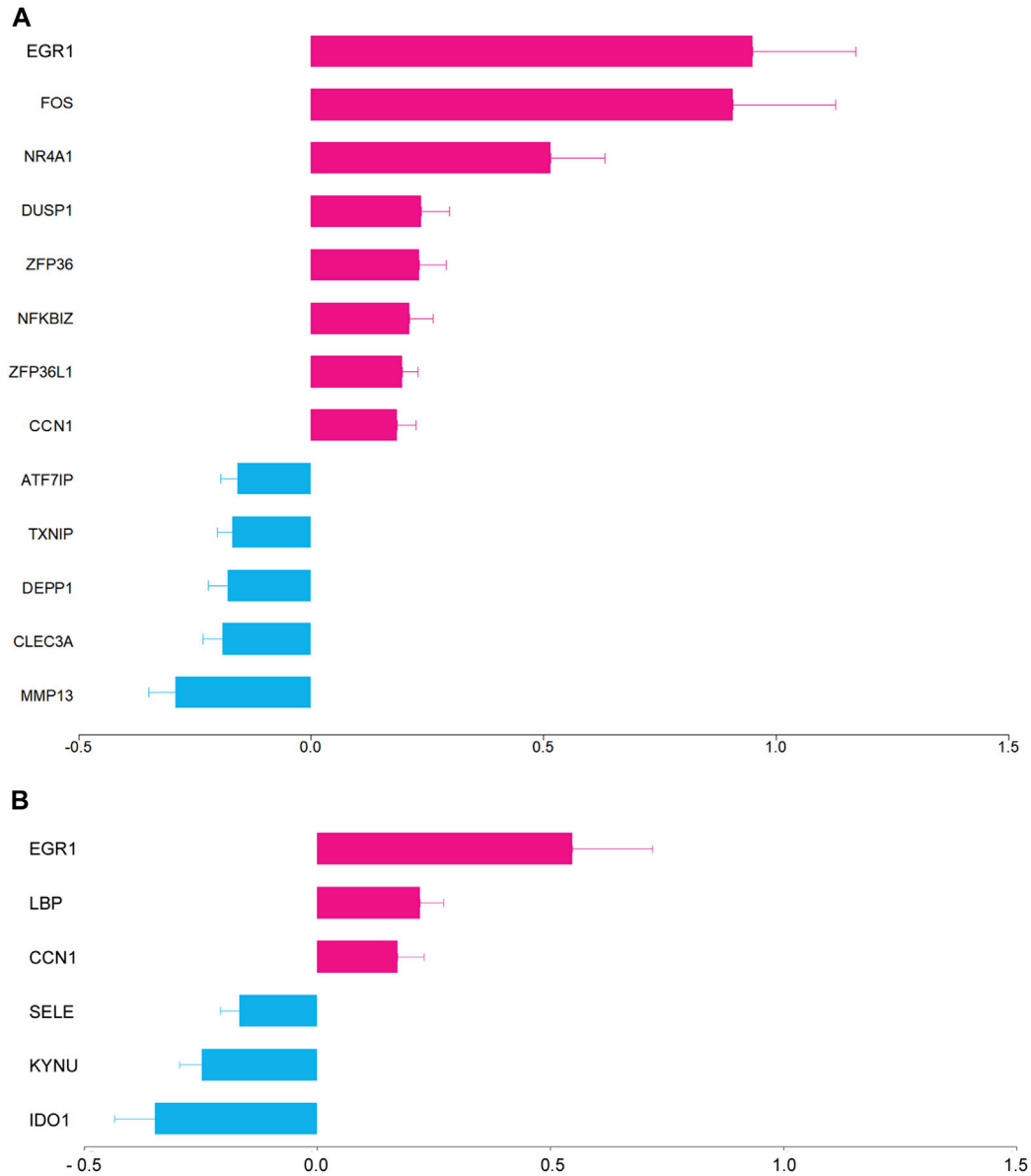


FIGURE 1 | Differentially expressed genes by Ze14 20% (v/v) with Fold Change >1.1 or <0.9, expressed as log2 Fold Change (mean ± SE, $n = 10$). **(A)**: without IL-1β treatment, **(B)**: with IL-1β 10^{-11} M treatment.

and significantly correlated with aggrecan (r = 0.66, $p = 0.0004$, **Figure 2D**) and type II collagen pro-peptide (r = 0.64, $p = 0.0008$, **Figure 2E**) production.

Ze14 Decreased the Production of Pro-MMP-13 in a Hypertrophy-Independent Pathway

RNA-seq revealed that Ze14 acts on matrix metallopeptidase 13 as well as on several independent genes involved in MMP13 regulation and activation (**Figure 3A**). MMP-13 is a major enzyme involved in OA pathology: it is both a marker for hypertrophy in chondrocytes and is known to function as an extracellular matrix-degrading enzyme in OA joints (Li et al., 2017). To investigate MMP-13 production during hypertrophic chondrocyte differentiation, we cultured primary OA chondrocytes for 28 days in the alginate beads model in the presence of 10% FBS. We have previously shown that articular chondrocytes become hypertrophic between 21 and 28 days of culture in these conditions (Pesesse et al., 2013; Pesesse et al., 2014). Fresh Ze14 20% (v/v) was added to the culture media twice a week during these 28 days.

In the alginate beads model, pro-MMP-13 production highly increased between day 3 and day 14 and then decreased independent of the treatment (**Figure 4A**). Interestingly, pro-MMP-13 was significantly decreased in Ze14-treated cultures from day 7–14 (from −16 to −25%, $p < 0.05$) and from day 17–21 (−22%,

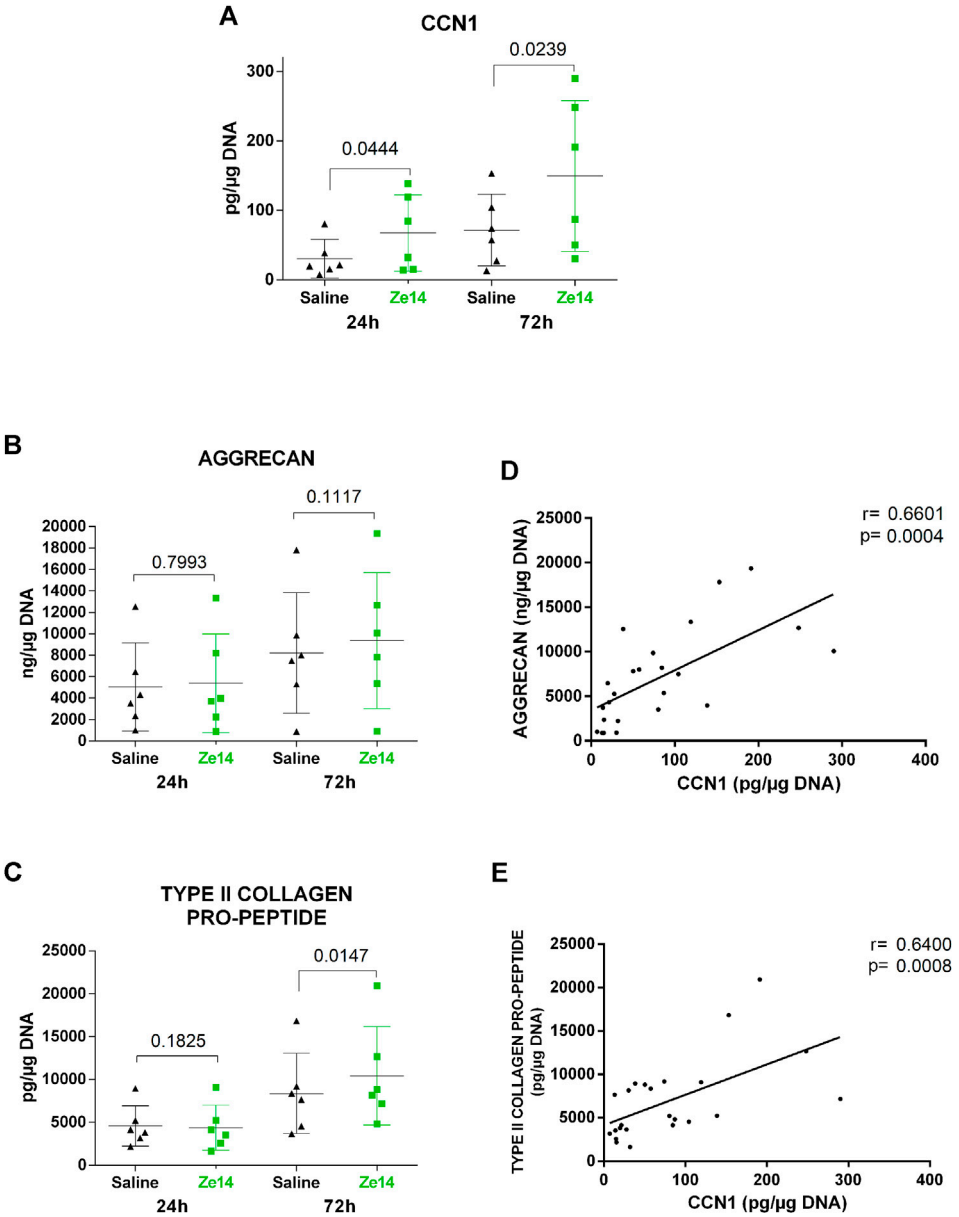


FIGURE 2 | Effect of Ze14 on CCN1, aggrecan and type II collagen pro-peptide production by OA chondrocytes cultured as monolayer (n = 6). Saline-treated cells served as controls. Results are shown as mean ± SD of the cultures (n = 6). Each dot represents individual culture means. Data were statistically analyzed using paired t-test, correlations were analyzed using Pearson's for aggrecan and Spearman's for type II collagen pro-peptide.

p = 0.0331) in comparison to saline-treated control cultures. The cumulative pro-MMP-13 production over 28 days was significantly lower in Ze14-treated chondrocyte cultures than in saline-treated control cultures ($p < 0.0001$, **Figure 4B**).

To analyze the Ze14 effect on the terminal hypertrophy differentiation, we investigated AP activity and type X collagen gene expression. These markers of hypertrophy increased with culture time, confirming that an induction of hypertrophy took place in these experimental conditions (data not shown). Ze14 did not modify AP activity or COL10A1 expression (Supplementary File 10).

DISCUSSION

In this study, we aimed to identify molecular pathways in human OA chondrocytes treated with Ze14. We used the latest NGS technology to generate the transcriptome, capturing dynamic changes in chondrocytes induced by the treatment, compared to saline control. We extended our transcript-level analysis to the protein level by directly measuring proteins with ELISA. Here we demonstrate that Ze14 promotes extracellular matrix formation arguably through CCN1 production and reduced pro-MMP-13, which may limit cartilage degradation. These findings may

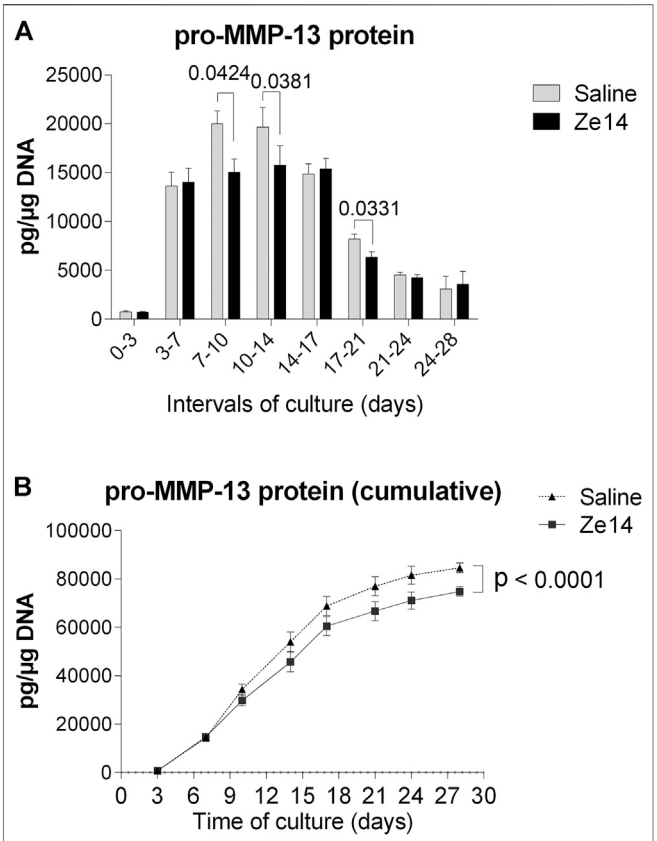


FIGURE 3 | Effects of Ze14 20% (v/v) on pro-MMP-13 production by chondrocytes in alginate beads culture. Saline-treated cells served as controls. Results are shown as mean ± SD of the cultures (n = 4). Data were statistically analyzed using paired one-way ANOVA **(A)** or two-way ANOVA **(B)**.

explain some effects observed in the clinical studies with OA patients.

Most of Ze14 DEGs were involved in MMP-13, also known as collagenase 3, regulation pathways (**Figure 4A**), including expression of MMP13 itself. It is known that MMP13 is significantly overexpressed in the joints and articular cartilage in patients with OA, and is a major proteinase involved in the degradation of type II collagen, a key constituent of the extracellular matrix (Li et al., 2017). Indeed, Ze14 modified DUSP1, DEPP1, ZFP36, ZFP36L1, and CLEC3A, which are signaling factors regulating MMP13 expression and activation. Furthermore, long-term (28 days) treatment with Ze14 significantly reduced the production of pro-MMP-13, the inactive precursor of MMP-13. The data analysis suggests that Ze14 potentially limits type II collagen degradation by reducing MMP-13 production and could have a beneficial effect on cartilage degradation.

Other notable results of this study are the stimulating effects of Ze14 on type II collagen and CCN1 production in human OA chondrocytes showing that Ze14 has proanabolic properties on cartilage.

During chondrogenic differentiation, chondrocytes express a mature chondrogenic phenotype (a set of characteristics specific to mature chondrocytes present in healthy cartilage). In adult cartilage, it involves the synthesis of matrix components, mainly type II collagen and aggrecan, two molecules characteristic of cartilage extracellular matrix, without changing the cell number. CCN1 is a growth factor-inducible immediate-early gene, directly involved in chondrogenesis: in micromass culture, purified recombinant CCN1 protein promoted chondrogenesis demonstrated by the expression of type II collagen, increased [35 S] sulfate incorporation, and larger alcian-blue staining of cartilage nodules used to assess aggrecan. Aggrecan is the major proteoglycan of the cartilage matrix. (Wong et al., 1997; Chijiiwa et al., 2015). Furthermore, CCN1 inhibits the activity of aggrecanase-1, also known as ADAMTS-4, an important

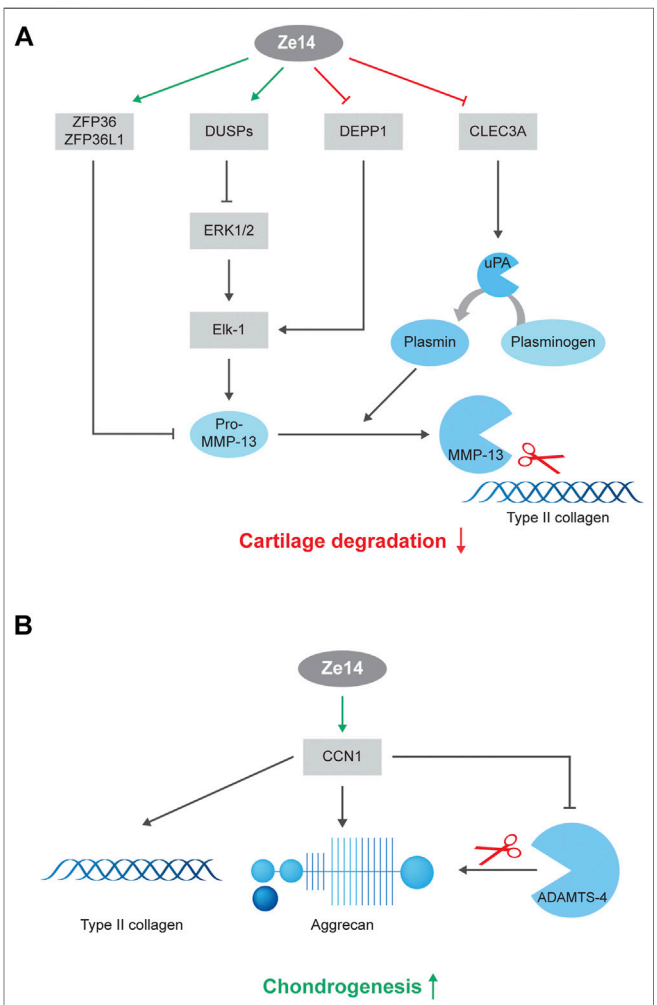


FIGURE 4 | (A): Mechanisms of action of Ze14 on MMP-13 production by chondrocytes: Ze14 significantly modified the expression of DUSP1, DEPP1, ZFP36, ZFP36L1 and CLEC3A, which are pathway mediators involved in MMP13 expression and activation, and reduced the production of pro-MMP-13. This may limit cartilage degradation. Upregulation and downregulation by Ze14 are indicated in green and red color, respectively. **(B)**: Mechanisms of action of Ze14 on chondrogenesis: Ze14 has a proanabolic effect on chondrocytes arguably through CCN1 production. Ze14 increased type II collagen and CCN1 production. Green color indicate upregulation by Ze14.

enzyme involved in aggrecan degradation. Arguably, by increasing CCN1, Ze14 could prevent aggrecan degradation and increase the synthesis of type II collagen and aggrecan (proanabolic effect). Interestingly, we found that type II collagen and aggrecan production were correlated with CCN1 production, suggesting that CCN1 could be a signaling factor involved in Ze14's anabolic effect on chondrocytes. The correlation between aggrecan and CCN1 has previously also been observed using alcian-blue staining (Wong et al., 1997).

These results demonstrate that Ze14 increases cartilage matrix formation in OA chondrocytes, arguably via CCN1, promoting chondrogenesis (**Figure 4B**). That is an important finding since chondrocytes lose their "healthy" chondrogenic properties and change into hypertrophic, catabolic, or fibroblastic chondrocytes as OA develops and progresses. However, in our *in vitro* hypertrophy model, Ze14 failed to stop or delay the terminal hypertrophic differentiation of chondrocytes. One reason could be that activation of hypertrophic differentiation pathways in chondrocytes obtained from our patients might have already been too advanced. Using healthy chondrocytes from young donors could reveal different outcomes. Furthermore, additional studies are needed to investigate the effect of Ze14 on fibroblastic chondrocytes.

Interestingly, Ze14 increased the expression of two key signaling factors involved in chondrogenesis: Early Growth Response (EGR) 1 and FOS. SOX9 is upregulated through the induction of EGR1, EGR3 and FOS mRNA (Spaapen et al., 2013). SOX9 is the transcription factor involved in chondrogenesis known to increase the expression of type II collagen and aggrecan in chondrocytes (Oh et al., 2010). Arguably, the upregulation of EGR1 and FOS by Ze14 has a beneficial effect on chondrogenesis and potentially leads to an increased anabolism.

Finally, we have observed that Ze14 may modulate the harmful effect of IL-1β on chondrocyte metabolism. IL-1β is considered as one of the major proinflammatory mediators in OA. Besides, IL-1β is a potent stimulatory and deleterious cytokine, and IL-1β stimulation of chondrocytes is the most widely used *in vitro* model in OA. In the presence of IL-1β 10^{-11} M, Ze14 20% (v/v) increased CCN1, LBP, and EGR1, and decreased expression of E-selectin and of two enzymes of the kynurenine pathway of tryptophan degradation, kynureninase and indoleamine 2,3-dioxygenase 1.

E-selectin is an adhesion molecule mediating the initial rolling of leukocytes along the surface of the vascular endothelium before firm adhesion and migration of the leukocytes occurs. It has been reported that both P- and E-selectin are expressed on the vascular endothelium of the synovium in rheumatoid arthritis (RA), and high levels of soluble E-selectin are detectable in the synovial fluid in RA. E-selectin plays an important role early in the development of adjuvant-induced arthritis in the rat (Issekutz et al., 2001). In our study, E-selectin was highly upregulated with IL-1β (12.6 log2 Fold Change) and was reduced by Ze14 treatment. Therefore, we assume that the initial rolling and the subsequent recruitment of leukocytes is inhibited in the presence of Ze14, leading to a reduction of inflammation. To confirm the potential effect on inflammation, further investigations would be necessary.

Another important effect of Ze14 during IL-1β-treatment was observed on two enzymes of the kynurenine pathway of tryptophan degradation, kynureninase and indoleamine 2,3-dioxygenase 1. These enzymes were highly upregulated with IL-1β stimulation (5.78 and 8.81 log2 Fold Change, respectively), and were decreased after Ze14 treatment (−16% and −21%). Elevated tryptophan metabolism and kynurenine levels have also been shown in primary synovial cell cultures in response to elevated interferon-γ, suggesting altered or increased tryptophan metabolism in response to inflammatory cytokines associated with arthritis (Malone et al., 1994). In this instance, further understanding of the impact of disease progression on tryptophan/kynurenine metabolism could benefit from further analysis of this pathway in OA.

In conclusion, in primary OA chondrocyte cultures, Ze14 promoted extracellular matrix formation probably through CCN1 production, a growth factor well-correlated with type II collagen and aggrecan production. Ze14 also significantly modified the expression of DUSP1, DEPP1, ZFP36, ZFP36L1, and CLEC3A, which are pathway mediators involved in MMP13 expression and activation. Long-term treatment with Ze14 significantly decreased pro-MMP-13 production, which is the inactive precursor of the metallopeptidase 13 involved in type II collagen degradation. For confirmation whether the effect of Ze14 on pro-MMP-13 has indeed an impact on type II collagen and cartilage matrix degradation, further investigations would be necessary, such as the analysis of protein activation (pro-MMP-13 to active MMP-13), the determination of MMP-13 enzymatic activity, and the analysis of type II collagen degradation biomarkers *in vivo*.

Our analyses show that Ze14 is a multitarget medication reducing several characteristics of OA: it decreased pro-MMP-13, potentially inhibiting catabolism, and demonstrated anabolic properties probably driven by CCN1, leading to a global net increase of chondrogenesis, characterized by a stimulation of type II collagen synthesis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Gene Expression Omnibus (GEO) repository, under accession number GSE162510 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE162510).

AUTHOR CONTRIBUTIONS

CS, KH, NK, BS, CA, and YH conceived and planned the experiments. CS carried out the experiments. JD contributed to sample preparation. CS analyzed the results and performed the statistical analysis. CS, KH, BS, and YH wrote the manuscript in consultation with NK, JD, and CA. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by Heel GmbH.

ACKNOWLEDGMENTS

We would like to thank the staff of the GIGA ULiège Genomic Next Generation Sequencing platform for performing the RNA sequencing and Benoit Charloteaux for his help in RNA-seq data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.635034/full#supplementary-material

REFERENCES

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol.* 11, R106. doi:10.1186/gb-2010-11-10-r106

Aury-Landas, J., Bazille, C., Allas, L., Bouhout, S., Chesneau, C., Leclercq, S., et al. (2017). Anti-inflammatory and chondroprotective effects of the S-adenosylhomocysteine hydrolase inhibitor 3-Deazaneplanocin A, in human articular chondrocytes. *Sci. Rep.* 7, 6483. doi:10.1038/s41598-017-06913-6

Bannuru, R. R., Osani, M. C., Vaysbrot, E. E., Arden, N. K., Bennell, K., Bierma-Zeinstra, S. M. A., et al. (2019). OARSI guidelines for the non-surgical management of knee, hip, and polyarticular osteoarthritis. *Osteoarthritis and Cartilage* 27, 1578–1589. doi:10.1016/j.joca.2019.06.011

Barr, A. J., and Conaghan, P. (2013). Disease-modifying osteoarthritis drugs (DMOADs): what are they and what can we expect from them? *Medicographia* 35, 189–196. doi:10.1214/AOS/1013699998

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B (Methodological)* 57, 289–300. doi:10.1111/j.2517-6161.1995.tb02031.x

Benjamini, Y., and Yekutieli, D. (2001). The control of the false discovery rate in multiple testing under dependency. *Ann. Statist.* 29, 1165–1188.

Chaitankar, V., Karakülah, G., Ratnapriya, R., Giuste, F. O., Brooks, M. J., and Swaroop, A. (2016). Next generation sequencing technology and genomewide data analysis: perspectives for retinal research. *Prog. Retin. Eye Res.* 55, 1–31. doi:10.1016/j.preteyeres.2016.06.001

Chijiiwa, M., Mochizuki, S., Kimura, T., Abe, H., Tanaka, Y., Fujii, Y., et al. (2015). CCN1 (Cyr61) is overexpressed in human osteoarthritic cartilage and inhibits ADAMTS-4 (aggrecanase 1) activity. *Arthritis Rheumatol.* 67, 1557–1567. doi:10.1002/art.39078

Comblain, F., Dubuc, J. E., Lambert, C., Sanchez, C., Lesponne, I., Serisier, S., et al. (2016). Identification of targets of a new nutritional mixture for osteoarthritis management composed by curcuminoids extract, hydrolyzed collagen and green tea extract. *PLoS One* 11, e0156902. doi:10.1371/journal.pone.0156902

Geenen, R., Overman, C. L., Christensen, R., Åsenlöf, P., Capela, S., Huisinga, K. L., et al. (2018). EULAR recommendations for the health professional's approach to pain management in inflammatory arthritis and osteoarthritis. *Ann. Rheum. Dis.* 77, 797–807. doi:10.1136/annrheumdis-2017-212662

Gottwald, R., and Weiser, M. (2000). Treatment of osteoarthritis of the knee with Zeel T. *Med. Biol.* 13, 109–113.

Gouze, J.-N., Gouze, E., Popp, M. P., Bush, M. L., Dacanay, E. A., Kay, J. D., et al. (2006). Exogenous glucosamine globally protects chondrocytes from the arthritogenic effects of IL-1beta. *Arthritis Res. Ther.* 8, R173. doi:10.1186/ar2082

Hochberg, Y. (1988). A sharper Bonferroni procedure for multiple tests of significance. *Biometrika* 75, 800–802. doi:10.1093/biomet/75.4.800

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* 6, 65–70. doi:10.2307/4615733

Hommel, G. (1988). A stagewise rejective multiple test procedure based on a modified Bonferroni test. *Biometrika* 75, 383–386. doi:10.1093/biomet/75.2.383

Issekutz, A. C., Mu, J. Y., Liu, G., Melrose, J., and Berg, E. L. (2001). E-selectin, but not P-selectin, is required for development of adjuvant-induced arthritis in the rat. *Arthritis Rheum.* 44, 1428–1437. doi:10.1002/1529-0131(200106)44:6<1428::aid-art238>3.0.co;2-u

James, C. G., Ulici, V., Tuckermann, J., Underhill, T. M., and Beier, F. (2007). Expression profiling of Dexamethasone-treated primary chondrocytes identifies targets of glucocorticoid signalling in endochondral bone development. *BMC Genomics* 8, 205. doi:10.1186/1471-2164-8-205

Kraus, V. B., Blanco, F. J., Englund, M., Karsdal, M. A., and Lohmander, L. S. (2015). Call for standardized definitions of osteoarthritis and risk stratification for clinical trials and clinical use. *Osteoarthritis and Cartilage* 23, 1233–1241. doi:10.1016/j.joca.2015.03.036

Labarca, C., and Paigen, K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102, 344–352. doi:10.1016/0003-2697(80)90165-7

Lesiak, A., Gottwald, R., and Weiser, M. (2001). The effectiveness of Zeel T treatment in intraarticular and intramuscular injections in osteoarthritis [in Polish]. *Medyzyna Biol.* 2, 30–36.

Li, H., Wang, D., Yuan, Y., and Min, J. (2017). New insights on the MMP-13 regulatory network in the pathogenesis of early osteoarthritis. *Arthritis Res. Ther.* 19, 248. doi:10.1186/s13075-017-1454-2

Li, J., Hou, R., Niu, X., Liu, R., Wang, Q., Wang, C., et al. (2016). Comparison of microarray and RNA-Seq analysis of mRNA expression in dermal mesenchymal stem cells. *Biotechnol. Lett.* 38, 33–41. doi:10.1007/s10529-015-1963-5

Liu, Y., Morley, M., Brandimarto, J., Hannenhalli, S., Hu, Y., Ashley, E. A., et al. (2015). RNA-Seq identifies novel myocardial gene expression signatures of heart failure. *Genomics* 105, 83–89. doi:10.1016/j.ygeno.2014.12.002

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550. doi:10.1186/s13059-014-0550-8

Malone, D. G., Dolan, P. W., Brown, R. R., Kalayoglu, M. V., Arend, R. A., Byrne, G. I., et al. (1994). Interferon gamma induced production of indoleamine 2,3 dioxygenase in cultured human synovial cells. *J. Rheumatol.* 21, 1011–1019.

Mathy-Hartert, M., Jacquemond-Collet, I., Priem, F., Sanchez, C., Lambert, C., and Henrotin, Y. (2009). Curcumin inhibits pro-inflammatory mediators and metalloproteinase-3 production by chondrocytes. *Inflamm. Res.* 58, 899–908. doi:10.1007/s00011-009-0063-1

Oh, C. D., Maity, S. N., Lu, J. F., Zhang, J., Liang, S., Coustry, F., et al. (2010). Identification of SOX9 interaction sites in the genome of chondrocytes. *PLoS One* 5, e10113. doi:10.1371/journal.pone.0010113

Pesesse, L., Sanchez, C., Delcour, J.-P., Bellahcène, A., Baudouin, C., Msika, P., et al. (2013). Consequences of chondrocyte hypertrophy on osteoarthritic cartilage: potential effect on angiogenesis. *Osteoarthritis and Cartilage* 21, 1913–1923. doi:10.1016/j.joca.2013.08.018

Pesesse, L., Sanchez, C., Walsh, D. A., Delcour, J.-P., Baudouin, C., Msika, P., et al. (2014). Bone sialoprotein as a potential key factor implicated in the pathophysiology of osteoarthritis. *Osteoarthritis and Cartilage* 22, 547–556. doi:10.1016/j.joca.2014.01.010

Rausch Osthoff, A.-K., Niedermann, K., Braun, J., Adams, J., Brodin, N., Dagfinrud, H., et al. (2018). 2018 EULAR recommendations for physical activity in people with inflammatory arthritis and osteoarthritis. *Ann. Rheum. Dis.* 77, 1251–1260. doi:10.1136/annrheumdis-2018-213585

Sanchez, C., Mateus, M. M., Defresne, M. P., Crielaard, J. M., Reginster, J. Y., and Henrotin, Y. E. (2002). Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1beta and nonsteroidal antiinflammatory drugs. *J. Rheumatol.* 29, 772–782.

Sanchez, C., Deberg, M. A., Piccardi, N., Msika, P., Reginster, J.-Y. L., and Henrotin, Y. E. (2005). Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1β and oncostatin M pre-treated non-sclerotic osteoblasts. *Osteoarthritis and Cartilage* 13, 979–987. doi:10.1016/j.joca.2005.03.008

Spaapen, F., van den Akker, G. G., Caron, M. M., Prickaerts, P., Rofel, C., Dahlmans, V. E., et al. (2013). The immediate early gene product EGR1 and polycomb group proteins interact in epigenetic programming during chondrogenesis. *PLoS One* 8, e58083. doi:10.1371/journal.pone.0058083

Stancikova, M. (1999). Inhibition of leukocyte elastase activity *in vitro* with Zeel T, Zeel comp., and their various potentiated constituents. *Biol. Medizin* 28, 83–84.

Wang, C., Gong, B., Bushel, P. R., Thierry-Mieg, J., Thierry-Mieg, D., Xu, J., et al. (2014). The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. *Nat. Biotechnol.* 32, 926–932. doi:10.1038/nbt.3001

Wong, M., Kireeva, M. L., Kolesnikova, T. V., and Lau, L. F. (1997). Cyr61, product of a growth factor-inducible immediate-early gene, regulates chondrogenesis in mouse limb bud mesenchymal cells. *Developmental Biol.* 192, 492–508. doi:10.1006/dbio.1997.8766

Conflict of Interest: KH and BS are employed by Heel GmbH. NK was employed by Heel GmbH at the time of study conception and implementation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 26 May 2021
doi: 10.3389/fphar.2021.675470

Total Flavonoids of Rhizoma Drynariae Promotes Differentiation of Osteoblasts and Growth of Bone Graft in Induced Membrane Partly by Activating Wnt/β-Catenin Signaling Pathway

Shuyuan Li[1,2], *Hongliang Zhou*[1,2], *Cheng Hu*[1,2], *Jiabao Yang*[1,2], *Jinfei Ye*[1,2], *Yuexi Zhou*[1,2], *Zige Li*[1,2], *Leilei Chen*[3]* *and Qishi Zhou*[4]*

[1]*Guangzhou University of Chinese Medicine, Guangzhou, China,* [2]*Lingnan Medical Research Center of Guangzhou University of Chinese Medicine, Guangzhou, China,* [3]*Third Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, China,* [4]*First Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, China*

OPEN ACCESS

Edited by:
Raquel Largo,
Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain

Reviewed by:
Maria Teresa Esposito,
University of Roehampton London, United Kingdom
Nasiara Karim,
University of Malakand, Pakistan

***Correspondence:**
Leilei Chen
yutian_1010@sina.com
Qishi Zhou
zhouqishi@139.com

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *03 March 2021*
Accepted: *06 May 2021*
Published: *26 May 2021*

Citation:
Li S, Zhou H, Hu C, Yang J, Ye J, Zhou Y, Li Z, Chen L and Zhou Q (2021) Total Flavonoids of Rhizoma Drynariae Promotes Differentiation of Osteoblasts and Growth of Bone Graft in Induced Membrane Partly by Activating Wnt/β-Catenin Signaling Pathway. Front. Pharmacol. 12:675470. doi: 10.3389/fphar.2021.675470

Total flavonoids of Rhizoma drynariae (TFRD), a Chinese medicine, is widely used in the treatment of fracture, bone defect, osteoporosis and other orthopedic diseases, and has achieved good effects. Purpose of this trial was to explore efficacy of TFRD on bone graft's mineralization and osteoblasts' differentiation in Masquelet induced membrane technique in rats. Forty male Sprague-Dawley rats were randomly divided into high dose group (H-TFRD), middle dose group (M-TFRD), low dose group (L-TFRD) and control group (control). The critical size bone defect model of rats was established with 10 rats in each group. Polymethyl methacrylate (PMMA) spacer was implanted into the defect of right femur in rats. After the formation of the induced membrane, autogenous bone was implanted into the induced membrane. After 12 weeks of bone graft, bone tissues in the area of bone graft were examined by X-ray, Micro-CT, hematoxylin-eosin (HE) and Masson trichrome staining to evaluate the growth of the bone graft. The β-catenin, c-myc, COL1A1, BMP-2 and OPN in bone graft were quantitatively analyzed by Western blot and Immunohistostaining. Osteoblasts were cultured in the medium containing TFRD. Cell Counting Kit-8 (CCK-8) method, Alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining, Western blot, RT-PCR and other methods were used to detect the effects of TFRD on the proliferation of osteoblasts and the regulation of Wnt/β-catenin signaling pathway. *In vivo* experiments showed that the growth and mineralization of bone graft in TFRD group was better. Moreover, the expression of Wnt/β-catenin and osteogenesis-related proteins in bone tissue of TFRD group was more than that in other groups. *In vitro* experiments indicated that osteoblasts proliferated faster, activity of ALP was higher,

Abbreviations: TFRD, Total flavonoids of Rhizome drynariae; TCF, T-cell factor; LEF, lymphoid enhancer factor; HE, Hematoxylin-eosin; Dkk1, Recombinant Human Dickkopf-Related Protein 1; BMSC, bone marrow derived mesenchymal stem cell; DMEM, dulbecco's modified eagle medium; PBS, Phosphate-buffered saline; PMMA, Polymethyl methacrylate; BMD, bone mineral density; ALP, alkaline phosphatase; ARS: Alizarin red S; COL1A1, collagen type I alpha 1; BMP-2, bone morphogenetic protein 2; OPN, osteopontin.

number of mineralized nodules and proteins related to osteogenesis were more in TFRD group. But blocking Wnt/β-catenin signaling pathway could limit these effects. Therefore, TFRD could promote mineralization of bone graft and differentiation of osteoblasts in a dose-dependent manner during growing period of the bone graft of induced membrane technique, which is partly related to the activation of Wnt/β-catenin signaling pathway.

Keywords: total flavonoids of rhizoma drynariae, induced membrane, Wnt/β-catenin, bone defect, osteogenic efficacy

INTRODUCTION

Critical-sized defects (CSDs) refers to a bone defect that cannot be healed naturally or treated with a standard cancellous bone graft (Lasanianos et al., 2010). Generally, length of bone loss is more than 2 to 2.5 times the diameter of the affected bone (Wiese and Pape, 2010). It is usually caused by trauma, osteomyelitis and bone tumor resection. Most of the traditional treatments are Papineau bone grafting (Masquelet and Bégué, 2010), vascularized free fibula graft (Osterman and Bora, 1984) and Ilizarov technique (Aronson et al., 1989). Induced membrane technique, also known as masquelet technique, is a new technique for the reconstruction of large bone defects (Masquelet et al., 2000), which mainly includes two steps. In the first step, the bone segments involved by inflammation are thoroughly removed and a fibrous membrane is induced around the bone defect by implanting a Polymethyl methacrylate (PMMA) spacer. In the second step, the induced membrane was opened after 6–8 weeks, the spacer was removed, and autogenous bone was implanted into the induced membrane. Because of its advantages of simple operation, easy fixation and a wide range of indications, it has been continuously concerned by scholars all over the world. Although the successful rate of induced membrane technique is high, it also has the problem of long healing time. Studies have shown that the postoperative bone healing time of induced membrane technique is from 3 to 94 months (Pelissier et al., 2004).

Bone formation is a series of complex physiological and pathological processes, including intramembranous ossification and endochondral ossification. Previous studies have shown that the bone formation function of osteoblasts and the bone resorption function of osteoclasts play a key role in the process of bone formation and remodeling (Raggatt and Partridge, 2010; Chen et al., 2018a). Osteoblasts originate from bone mesenchymal stem cells (BMSCs) and differentiate from BMSCs under the action of osteoblast differentiation factor. Osteoblasts are not only the main effector cells of mechanical stress in bone tissue, but also the main functional cells of bone formation responsible for the synthesis, secretion and mineralization of bone matrix. Its differentiation and proliferation mainly determines the bone mass. Therefore, the regulation of osteoblasts has become an vital target to promote bone formation.

Wnt/β-catenin signaling pathway is an important regulatory mechanism involved in osteoblasts' differentiation (Krishnan, 2006; Luyten et al., 2009; Milat and Ng, 2009), which is essential for bone development, bone mass maintenance and bone remodeling. When Wnt protein binds to specific frizzled transmembrane receptors and the Low-density lipoprotein receptor related protein (LRP, Lrp5/6) co-receptor on cell surface, β-catenin is released in the cytoplasm and no protein degradation occurs. The accumulated β-catenin is transferred to the nucleus, where it binds to T-cell factor 4 (TCF-4) or lymphoid enhancer factor 1 (LEF-1) to activate downstream target genes such as c-myc, cyclinD, Runx2 transcription (Canalis, 2013). CyclinD 1, β-catenin and c-myc are the main functional molecules in Wnt/β-catenin. Dickkopfs (Dkks) bind and sequester the Lrp5/6 and Krm1/2 membrane complex to inhibit Wnt activity (Monroe et al., 2012). Dkk1 is a secretory Wnt inhibitor with good specificity and is active in many tissues (Pinzone et al., 2009). Data from different animal models have confirmed that Dkk1 can inhibit Wnt signaling pathway and thus inhibit bone formation. Anti-Dkk-1 neutralizing antibodies against the epitopes necessary for LRP-5 and LRP-6 binding to Dkk-1 increased bone mass in normal mice (Glantschnig et al., 2020). The experimental model of multiple myeloma showed that anti-Dkk-1 antibody treatment reversed the inhibitory effect of Dkk-1 on osteoblasts' differentiation and bone formation, thus reducing bone loss (Fulciniti et al., 2009).

Total flavonoids of Rhizoma Drynariae (TFRD) is an effective ingredient extracted from the dried root of traditional Chinese medicine Rhizoma Drynariae. Nowadays, TFRD has been developed into a postmarketing Chinese medicine called Qianggu capsule (drug approval number: Z20030007, Qi-Huang Pharmaceutical Co. Ltd., Beijing, China) (Sun et al., 2016). TFRD has been wildely used in many Asian countries including China, Korea, and Japan for the treatment of diverse orthopedic diseases such as fracture, osteoporosis, bone defects, arthritis, etc., and has pharmacological activities to promote osteogenesis, anti-inflammation and anti-oxidative damage (Kuo et al., 2014; Song et al., 2016; Yang et al., 2017; Chen et al., 2018b; Jiang et al., 2018). Animal experiments showed that TFRD could increase the number of bone trabeculae and bone mineral density (BMD), improve the morphology of bone tissue, promote new bone formation and increase biomechanical strength in bone defect or osteoporotic rats, and no systemic side effects such as infection were found (Wong et al., 2007; Guo et al., 2019). In addition, *in vitro* experiments showed that TFRD could accelerate the differentiation and mineralization of osteoblasts (Chen et al., 2011) and inhibit the bone resorption of osteoclasts (Jeong et al., 2003). However, its specific mechanism is not clear in terms of microstructure and cellular and molecular biology.

Idea of this trial was to explore effects of TFRD on mineralization of bone graft and osteoblasts' differentiation in Masquelet induced membrane from the point of view of Wnt/β-catenin signaling pathway. Moreover, it is also hoped to provide experimental basis for TFRD on promoting bone formation and mineralization of induced membrane technique, shortening the treatment cycle of bone healing and improving the quality of osteogenesis.

MATERIALS AND METHODS

Main Materials and Reagents

Total flavonoids of Rhizoma drynariae (TFRD) were purchased from Beijing Qihuang Pharmaceutical Manufacturing Co., Ltd. (National Medicine Permit No. Z20030007, number of production: 04080081, the content of TFRD ≥80%); 4% paraformaldehyde fix solution (Guangzhou Dianzhong Trading Co., Ltd., China, batch number: IS013); Penicillin Sodium for injection (Shandong Lukang Co., Ltd., China, 1.6 million units per bottle, national pharmaceutical standard H37020080); Polymethyl methacrylate (PMMA, Heraeus Company of Germany, batch number: 90914791); Recombinant Human Dickkopf-Related Protein 1/Dkk1(Absin Bioscience Inc., Shanghai, China abs0435); Phosphate-buffered saline (PBS), dulbecco's modified eagle medium (DMEM)/high glucose, trypsin, fetal bovine serum (FBS), Penicillin-Streptomycin, Cell Counting Kit-8 (CCK-8), lipopoly saccharide (LPS), dimethyl lsulfoxide (DMSO) and concanavalin A (ConA) were acquired from Beijing Suo Laibao Technology Co., Ltd. (Beijing, China); Alizarin Red S Staining Quantification Assay, Alkaline phosphatase staining solution, and Alkaline phosphatase Assay Kit were acquired from Guangzhou Haoma Biotechnology Co., Ltd. (Gunagzhou, China).

Experimental Animals and Groups

Forty healthy male Sprague-Dawley (SD) rats of 10–12 weeks old, weighing 250–310 g (280.3 ± 21.4 g), were selected and provided by Guangdong Medical Experimental Animal Center. License No.: SCXK (Yue) 2018-0002, Experimental Animal Certificate No.44007200064529. All the selected experimental animals were raised in the SPF animal room of Guangzhou University of traditional Chinese Medicine (the laboratory temperature was 22–24°C, the humidity was 60–70%, and the light and dark cycle was 12 h/12 h), feeding feed was provided by the Experimental Animal Center of Guangzhou University of Chinese Medicine. The experiment was carried out one week after feeding. According to the method of random number table, the experimental animals were randomly divided into four groups: high dose group (H-TFRD), middle dose group (M-TFRD), low dose group (L-TFRD) and control group, with 10 rats in each group. All protocols were approved by Institutional Animal Care and Ethics Committee of Guangzhou University of Chinese Medicine.

The Establishment of Animal Models

Surgical procedure was performed as described in earlier work by Gouron et al., with slight modifications (Gouron et al., 2017). Before the experiment, the rats fasted for 12 h, and 40,000 U of penicillin was injected intramuscularly to prevent infection. Anesthesia was given intraperitoneally with 3% pentobarbital (1.5 ml/kg). After the anesthesia took effect, the right hindlimb was shaved to prepare the skin. The rats were taken from the left recumbent position to expose the right hindlimb, sterilized and covered with aseptic towels. The first stage operation: the skin and fascia were cut longitudinally from the lateral greater trochanter to the lateral condyle of the femur, and the subcutaneous muscles were separated to expose the lateral side of the femur. Place a custom six-hole plate on the anterolateral side of the femur. After drilling, two cortical self-tapping screws were used to fix the plate at the distal and proximal ends, and a wire saw was used to cut the bone at the center of the femoral shaft, the length of which was 6 mm. The bone defect area was filled with a PMMA spacer of 6 mm length. After repeated washing with normal saline, the incision was sutured layer by layer (**Figure 1A**). The second stage operation: six weeks after the first stage operation, two caudal vertebrae were taken from the middle or distal segment of the rat tailbone and cut into particles for bone grafting. The skin, subcutaneous tissue and induced membrane were cut longitudinally along the incision of the first stage operation. After removing the PMMA spacer, the prepared bone particles were filled into the bone defect area (**Figure 1B**). Finally, the induced membrane, fascia and skin were sutured.

Within 3 days after stage Ⅰ and stage Ⅱ, 40,000 u of penicillin was injected intramuscularly every day to prevent infection.

Intervention Measures

TFRD was added to distilled water to make a certain concentration of solution. The equivalent dose of TFRD was calculated according to the body surface area. The rats in the high, middle and low dose groups were given TFRD of 0.44, 0.22, and 0.11 g/kg/day respectively, and the rats in the control group were given the same volume of normal saline. In the course of the experiment, the weight was weighed every 2 weeks, and the dose was adjusted in time according to the change of body weight. From the second day after stage II surgery, the rats were administered orally with TFRD until the bone samples were collected after 12 weeks.

X-Ray, Micro-CT Analyses in Area of Bone Graft

Three rats were taken from each group to analyse by X-ray of femur after 12 weeks of bone graft. The growth and mineralization of bone graft, the bone resorption, loosening or prolapse of steel plates and screws were observed. After X-ray analysis, the rats were killed under excessive anesthesia, the right femur was removed and put into the Micro-CT sample tube for Micro-CT examination. After the completion of the scan, the scanning results were analyzed by CT-An software, and the area of bone graft was manually selected to establish a three-dimensional region of interest and analysis. Micro-CT parameters include Tissue Volume (TV), Bone Volume (BV), Bone Volume Fraction (BV/TV), Bone Surface Fraction (BS/BV), Structural Model Index (SMI),

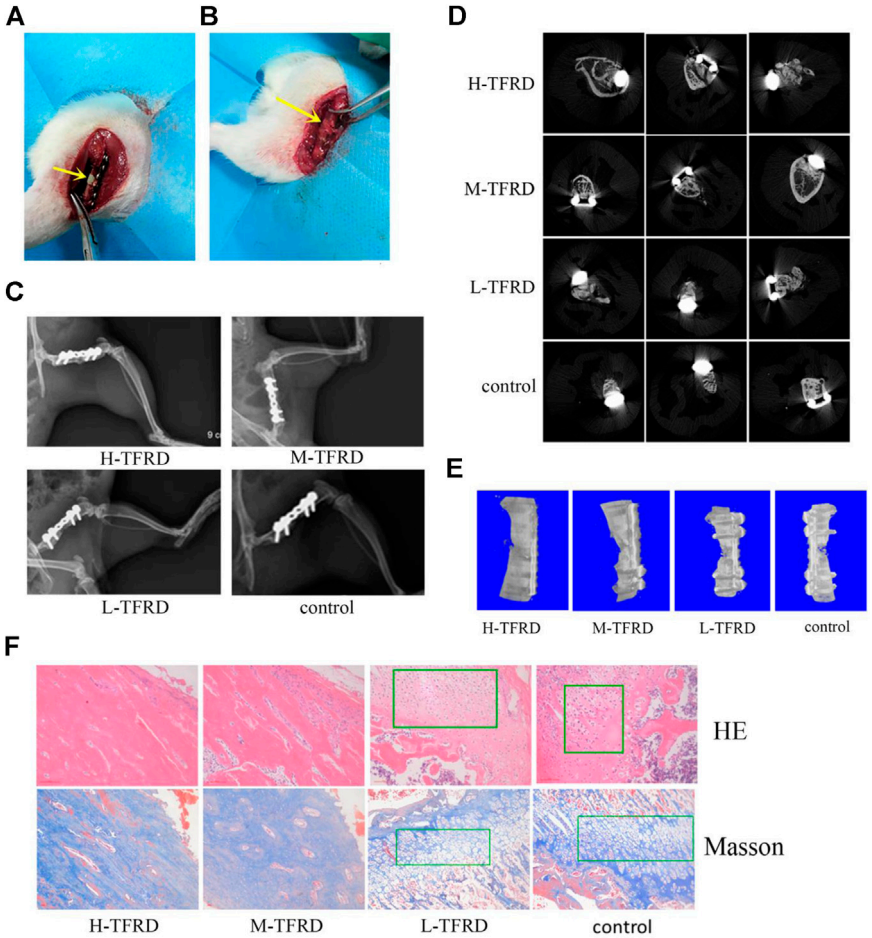


FIGURE 1 | TFRD accelerates the growth and mineralization of bone graft. **(A)** The yellow arrow in the picture refers to the 6 mm bone defect constructed in the right femur of rats during the first stage operation. PMMA spacer was implanted in this area to induce formation of biofilm. **(B)** The yellow arrow refers to the area of bone graft in the right femur of rats at the second stage operation. **(C)** X-ray was performed on the right femur of rats. Among them, the amount of callus and cortical bone shaping in the H-TFRD and M-TFRD groups were more obvious than those in the L-TFRD and control groups. **(D)** was the result of Micro-CT cross-sectional scanning of the bone graft in the right femur of rats. **(E)** was the results of three-dimensional reconstruction of the right femur of rats. **(F)** shows the histological and structural characteristics of bone graft in the right femur of rats (magnification, × 200). The green boxes show the cartilage area, and other parts in pictueres show the osteogenic area.

Trabecular Number (Tb.N), Trabecular Thickness (Tb.Th), Trabecular Separation/Spacing (Tb.Sp), Connectivity Density (Conn.D.). After the detection, the three-dimensional reconstruction of the bone graft area of the femur was carried out.

Histological Analysis

After X-ray and Micro-CT analysis, the bone tissues in the induced membrane was cut and treated with decalcification, dehydration and paraffin embedding, then the tissue slicer was used for continuous slicing with a thickness of 5 μm. After baking at 68°C in a constant temperature baking machine, the bone tissue sections were stained with Masson trichrome and hematoxylin-eosin (HE) staining solution. After sealing, the osteogenic process was observed and evaluated under biological microscope (Olympus, BX53, Japan).

Immunohistostaining

The paraffin sections of bone tissues were deparaffinized, rehydrated, and then incubated in the citrate antigen retrieval solution (Beijing Solarbio Science and Technology Co.,Ltd., C1031, China) for 5 min. After quenching endogenous peroxidase activity with 3% H_2O_2 for 8 min, the slides were incubated with anti-BMP-2 antibody (ab214821, Abcam, United States, 1:100), anti-COL1A1 antibody (ab270993, Abcam, United States, 1:100) and anti-OPN antibody (ab228748, Abcam, United States, 1:100) at 4°C overnight. On the next day, the slides were incubated at 37°C for 20 min with goat anti-rabbit IgG (A32731, Invitrogen, United States, 1:500). After 3,3′-diaminobenzidine (DAB) (Gene Tech Company Ltd., GK5007, China) staining, the slides were counterstained with hematoxylin for 3 min at room temperature, dehydrated in a series of 70–100% alcohol baths and cleared in a xylene bath. The

slides were mounted with neutral balsam and observed using a biological microscope (Olympus, BX53, Japan).

Extraction and Identification of Osteoblasts

Five suckling rats of SD rats were killed, soaked in 75% alcohol for 2 min, the calvaria was taken under strict sterile conditions, the connective tissue attached to the bone surface was removed, PBS was washed repeatedly until the bone tissue was whitened, the bone tissue was cut to the size of 1 mm × 1 mm with scissors, the phosphate buffer (PBS) was rinsed to the bone tissue whitening, and the bone tissue fragments were placed in a centrifuge tube and digested with 0.25% trypsin. After 30 min, trypsin was discarded, 0.1% type II collagenase of 8 ml was added, digested for 1 h, the Supernatant fluid was collected and transferred to another centrifugal tube, and centrifuged by 1000 r/min for 10 min 0.1% type II collagenase was added to the centrifuge tube containing bone tissue for 1 h, and the Supernatant fluid was collected and centrifuged to collect cells; added to the prepared cell culture medium. The cells were inoculated in the 25 cm^2 culture bottle at the concentration of 2 × 10^4 /ml and cultured in incubator (37°C, 5% CO_2). The adhesion and growth of cells were observed every day. After the cells were pasted to the bottom, they were digested and passaged with trypsin. The third generation osteoblasts were used in the experiment. After the cells adhered to the wall, the culture flasks containing primary osteoblasts and passage osteoblasts were observed and photographed under inverted fluorescence microscope. Osteoblasts were identified by morphological observation and ALP staining.

Preparation and Grouping of Culture Medium

According to the composition of the medium, they were divided into the following four groups: control group, Dkk1 group, TFRD group and TFRD + Dkk1 group. Osteoblasts were cultured in DMEM (high glucose) containing 10% volume of FBS and 1% volume of Penicillin-Streptomycin. Besides, The culture medium of TFRD group contained different doses of TFRD (0, 12.5, 25, 50, 100, and 200 ug/ml, respectively). The concentration of TFRD in the TFRD + Dkk1 group was 100 ug/ml. In the Dkk1 group and TFRD + Dkk1 group, the Recombinant Human Dickkopf-related protein 1 (Dkk1) was added to each medium at a concentration of 0.4 μg/ml. The third generation of osteoblasts were cultured in various pre-prepared media. In the process of osteoblasts culture, the medium was changed every 2 days, and the growing status of the cells was observed.

Analysis of the Proliferation Rate of Osteoblasts

Proliferation of cells in each group was detected by Cell Counting Kit-8 (CCK-8) method. Osteoblast suspension (100 μl/well) was inoculated in 96-well culture plate and cultured in different media. CCK eight solution was added to each well and incubated in the incubator for 4 h. The absorbance (O.D value) at 450 nm was determined by enzyme labeling instrument.

Analysis of Alkaline Phosphatase Activity

After 3, 6, 9, and 12 days of cell culture, the activity of ALP of osteoblasts was measured by Para-nitrophenyl phosphate (pNPP). The culture medium was removed, and 0.5% Triton X-100 cell lysate (50 μl) was added at 4°C for 1 h. The 20 μl lysate was taken on a 96-well plate and operated according to the operation table of the Alkaline phosphatase Assay Kit. The OD value was determined by enzyme labeling instrument at 405 nm wavelength. According to the OD value of the sample, the activity value of ALP (U/L) was read on the ALP standard curve.

Alkaline Phosphatase and Alizarin Red S Staining

After 6 and 21 days of culture, the medium was removed. Osteoblasts were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Using PBS to wash away 4% paraformaldehyde and add ALP staining solution or 1% alizarin red S solution for 20 min. Finally, the amount of ALP or mineralized nodules was observed under the microscope.

Immunofluorescent Analysis

After the predetermined time of cells culture (3, 6, 9 days), culture medium was quickly absorbed, and dish was washed with cold PBS for 3 times. The cells were fixed with 4% paraformaldehyde for 30 min and washed with PBS for 3 times. Adding 0.25% Triton X100 to plate at 37°C for 10 min and washing plate for 3 times. After drying, adding sealed serum at 37°C for 30 min. Then, cells were briefly washed with PBS and incubated with anti-β-catenin antibody (ab32572, abcam, United States, 1:250) at 4°C overnight. Then, the primary antibody was sucked out and washed by PBS for 3 times. Under the condition of avoiding light, the secondary antibody (goat anti-rabbit IgG, A32731, Invitrogen, United States, 1:500) with FITC labeling was added. After 1 h, the nuclei of cells were stained with DAPI for 10 min. The expression and localization of β-catenin were observed under fluorescence microscope.

Protein Extraction and Western Blot Analysis

The total proteins of bone tissues and osteoblasts was extracted, and the protein content was measured by BCA assay kit. The proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membrane. After the end of the film transfer, each blot were blocked with 5% skim milk for 1 h. Then the primary antibody was added and incubated at 4°C overnight. After the incubation membrane was washed, the secondary antibody was added. ECL kit was used for photoluminescence development, and GAPDH (ab8245, abcam, United States, 1:1,000) was used as the reference protein. The grayscale values of each band were analyzed by ImageJ software. The information of primary antibodies was listed as follows: β-catenin (ab32572, abcam, United States, 1:5,000), TCF (ab185736, abcam, United States, 1:1,000), LEF-1 (ab137872, abcam, United States, 1:1,000), cyclinD (#2978, Cell Signaling

TABLE 1 | Prime sequences for RT-PCR.

gene	Primer sequence
β-catenin	F: 5′-AGGGCAATCCTGAGGAAGAAGA-3′
	R: 5′-TGCGTGAAGGACTGGGAAAA-3′
TCF	F: 5′-CGAGGAGGTCACATCAGTGG-3′
	R: 5′-AGGGACAGCACCTCATCTGTA-3′
LEF-1	F: 5′-ACACGGACAGCGACCTAATG-3′
	R: 5′-CTGCGCTCTCCTTTAGCGTA-3′
cyclinD	F: 5′-CCCACGATTTCATCGAACA-3′
	R: 5′-GGGTGGGTTGGAAATGAAC-3′
c-myc	F: 5′-ACTGCGGTCTCCTAAAGGTCG-3′
	R: 5′-GACCTGGGGAAGCAGCAAC-3′
Runx2	F: 5′- CGGAATGCCTCTGCTGTTAT-3′
	R: 5′- TTCCCGAGGTCCATCTACTG-3′
ACTB	F: 5′-TCAGCAAGCAGGAGTACGATG-3′
	R: 5′-GTGTAAAACGCAGCTCAGTAACA-3′

Technology, United States, 1:1,500), c-myc (ab32072, abcam, Unites States, 1:1,000), Runx2 (ab236639, abcam, United States, 1:1,000), COL1A1 (ab270993, abcam, United States, 1:1,000), BMP-2 (ab214821, Abcam, United States, 1:1,000), OPN (ab228748, abcam, United States, 1:1,000).

RNA Isolation and Real-time PCR

The total RNA in bone tissues and osteoblasts was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, United States) and reverse transcribed into cDNA by PrimeScript RT reagent Kit (Japan, RR037A). Then, the RT-PCR analysis was carried out through using the Prime Script^{TM}RT reagent Kit SYBR (Takara, DRR047A). ACTB was used as the reference gene. If the amplification efficiency of target gene and reference gene is close to 100%, $2^{-\triangle\triangle Ct}$ method was used for RT-PCR analysis. If not, PFAFFL method is more appropriate (Bustin et al., 2009). In this study, the amplification efficiency of all genes was close to 100%, so we used $2^{-\triangle\triangle Ct}$ method to analyze the relative expression level. The primer sequence of β-catenin, TCF, LEF-1, cyclinD, c-myc, Runx2 and ACTB was list in **Table 1**.

Statistical Analysis

All the data were analyzed by Stata 12.0 software, and the metrological data such as β-catenin, TCF and LEF protein content were expressed as means ± standard deviation (SD). After satisfying the normal distribution, the mean among the four groups were compared by one-way ANOVA, $p < 0.05$ was considered statistically significant.

RESULTS

TFRD Accelerates the Growing Rate of Bone Graft

After 12 weeks of bone graft, new bone could be seen in the femoral bone defect area of different doses of TFRD. In the H-TFRD and M-TFRD groups, the continuous callus filled with defects, the volume of callus was larger, and the cortical bone was basically molded. In the L-TFRD and control groups, the volume of callus in the area of bone defect was small, only partially passed through the area of bone defect, and the cortical bone had not been completely molded. The above results showed that TFRD could promote the growth and mineralization of bone graft in the induced membrane, especially in the H-TFRD and M-TFRD groups (**Figure 1C**).

TFRD Enhances Mineralization of Bone Graft

The parameters of Micro-CT showed that the Bone Volume Fraction (BV/TV), Trabecular Number (Tb.N), Trabecular Thickness (Tb.Th), Connectivity Density (Conn.D.) in the H-TFRD and M-TFRD groups were significantly higher than those in the L-TFRD and control groups ($p < 0.05$). The Bone Surface Fraction (BS/BV), Structural Model Index (SMI), Trabecular Separation/Spacing (Tb.Sp) were smaller than those in L-TFRD and control groups, and the difference was statistically significant ($p < 0.05$). The above result suggested that TFRD could promote the formation of bone trabeculae of the right femur of rats 12 weeks after bone grafting (**Table 2**). Images of cross-sectional scan and three-dimensional reconstruction of micro-CT indicated that H-TFRD and M-TFRD groups had more bone mass and better effects of mineralization, and bone defects in L-TFRD and control groups had not been completely healed (**Figures 1D,E**).

Histological and Structural Characteristics of Bone Graft

According to the results of HE and Masson trichromatic staining of bone tissues 12 weeks after bone graft in the femur of rats, the cartilage and osteogenic area could be clearly seen in four groups, which showed a typical process of endochondral ossification. Among them, the osteogenic area in H-TFRD and M-TFRD groups were larger than that in L-TFRD and control groups, and the cortical bone was more fully molded. The L-TFRD and control groups were still dominated by cartilage at this stage. The results suggested that the TFRD could promote the process of intrachondral bone formation in the induced membrane, and the formation of bone at H-TFRD and M-TFRD groups was faster and the effect was better (**Figure 1F**).

TFRD Promotes Expression of Wnt/β-Catenin and Osteogenesis-Related Proteins in Bone Graft

Wnt/β-catenin signaling pathway plays an crucial role in promoting osteoblasts' differentiation and bone shape (Krishnan, 2006; Luyten et al., 2009; Milat and Ng, 2009). We found that TFRD could significantly promotes the expression of β-catenin and c-myc in bone tissues in a dose-dependent manner (**Figure 2**). Furthermore, there are many proteins related to bone formation in the process of osteoblast differentiation, such as bone morphogenetic protein 2 (BMP-2), collagen type I alpha 1 (COL1A1) and osteopontin (OPN). According to results of Western blot of bone tissues, the average protein level of

TABLE 2 | Comparison of the parameters of bone structure in the area of bone graft in each group. Each value was presented as the mean ± SD. $^{*}p < 0.05$ Vs. the control group; $^{\#}p < 0.05$ Vs. the L-TFRD.

group	n	BV/TV/%	BS/BV/%	SMI	Tb.N/mm^{-1}	Tb.Th/mm	Tb.Sp/mm	Conn.D./1/mm^{3}
H-TFRD	3	27.85 ± 1.33$^{*\#}$	16.33 ± 1.47$^{*\#}$	1.53 ± 0.52$^{*\#}$	2.63 ± 0.06$^{*\#}$	0.25 ± 0.05$^{*\#}$	0.25 ± 0.04$^{*\#}$	7.18 ± 0.48$^{*\#}$
M-TFRD	3	27.40 ± 0.95$^{*\#}$	15.80 ± 2.19$^{*\#}$	1.47 ± 0.59$^{*\#}$	2.64 ± 0.42$^{*\#}$	0.28 ± 0.03$^{*\#}$	0.27 ± 0.03$^{*\#}$	7.29 ± 0.73$^{*\#}$
L-TFRD	3	16.00 ± 2.48	21.65 ± 1.71	2.71 ± 0.39	1.59 ± 0.30	0.13 ± 0.02	0.46 ± 0.05	3.33 ± 0.85
Control	3	14.43 ± 1.07	22.50 ± 2.10	2.80 ± 0.39	1.72 ± 0.16	0.12 ± 0.01	0.42 ± 0.03	3.73 ± 0.56

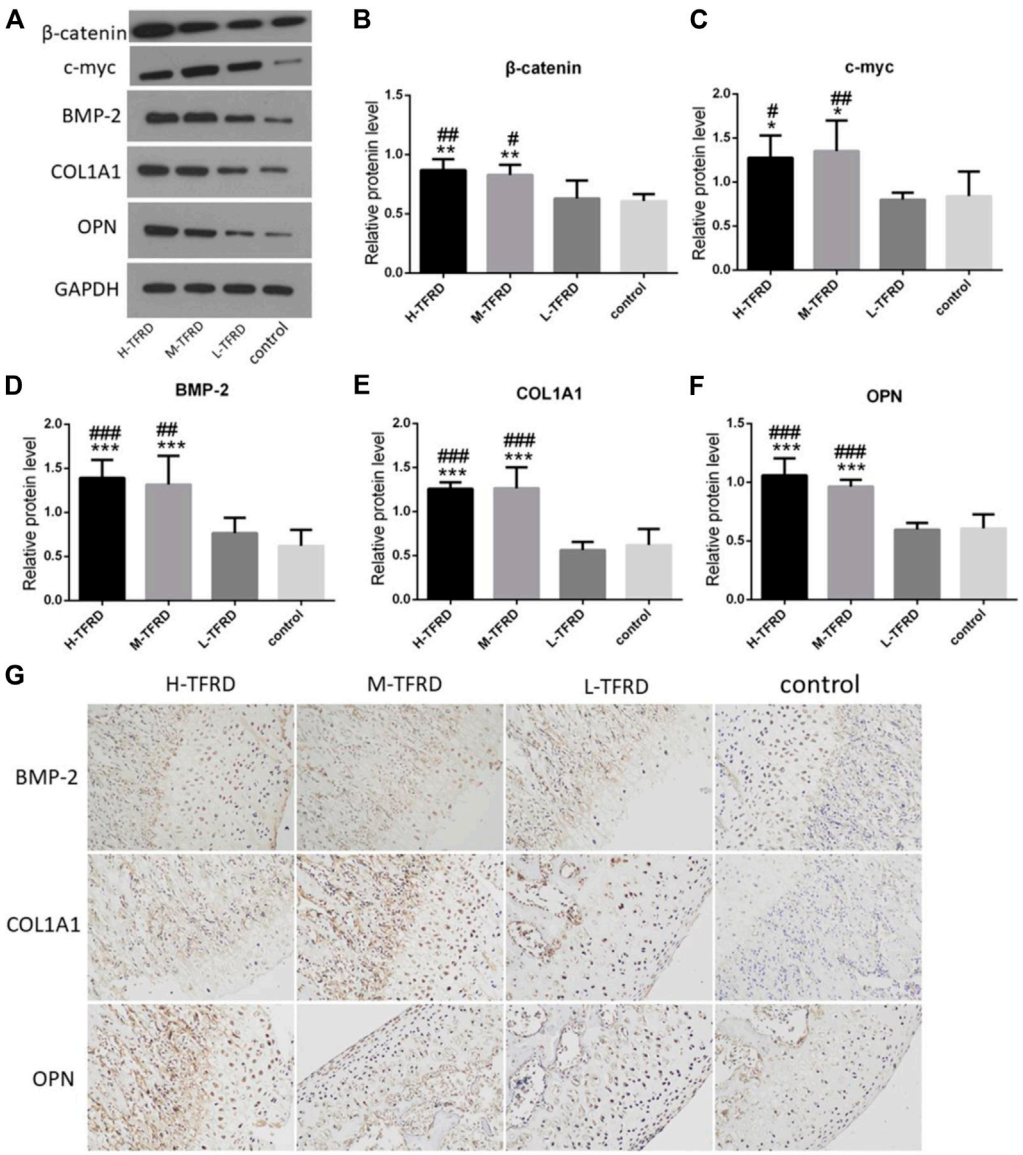


FIGURE 2 | TFRD promotes expression of Wnt/β-catenin and osteogenesis-related proteins in the bone graft. **(A)** is the Western Blot band of β-catenin, c-myc, BMP-2, COL1A1 and OPN respectively. **(B–F)** is the gray value of β-catenin, c-myc, BMP-2, COL1A1 and OPN. Each value was presented as the mean ± SD. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. the control group; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. the L-TFRD group. **(G)** Immunohistostaining was performed to evaluate the protein level of BMP-2, COL1A1 and OPN in bone graft area (magnification, × 200). The intensity and area of three osteogenesis-related proteins in the H-TFRD and M-TFRD groups were higher than those in the L-TFRD and control groups.

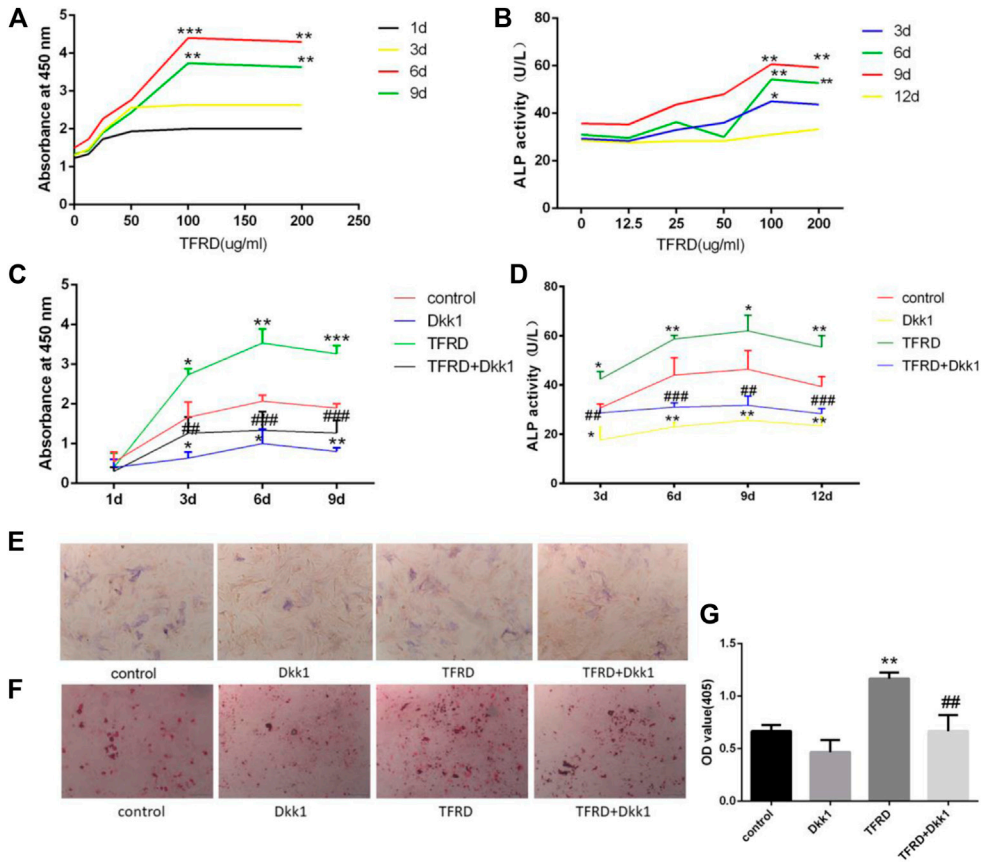


FIGURE 3 | TFRD promotes proliferation and mineralization of osteoblast by Wnt/β-catenin signaling pathway. **(A)** is the effect of different concentrations of TFRD on the proliferation of osteoblasts in different periods. ***$p < 0.001$, **$p < 0.01$vs. 0, 12.5, 25, 50 ug/ml. **(B)** is the effect of different concentrations of TFRD on the activity of ALP of osteoblasts in different periods. **$p < 0.01$, *$p < 0.05$ vs. 0, 12.5, 25, and 50 ug/ml. (c) is trend and comparison proliferation of osteoblasts in different groups. **(D)** is activity of ALP of osteoblasts in different groups. **(E,F)** The ALP and ARS staining of osteoblasts was performed (magnification, ×100). **(G)** Quantitative determination of ALP activity and the production of mineralized nodules. **(C–G)** N = 3/group. Each value was presented as the mean ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. the control group; $^{###}p < 0.001$, $^{##}p < 0.01$ vs. the TFRD group.

BMP-2, COL1A1 and OPN in the H-TFRD and M-TFRD groups was higher than that in the L-TFRD group and control groups ($p < 0.001$ or $p < 0.01$) (**Figure 2**). The result of immunohistochemical staining also showed that the positive expression level of BMP-2, COL1A1 and OPN in the H-TFRD and M-TFRD groups were also significantly higher than those in the L-TFRD group and control groups (**Figure 2G**),which was consistent with the result of Western Blot. Therefore, we speculated that TFRD may promote osteoblasts' differentiation and expression of osteogenesis-related proteins partly by activating Wnt/β-catenin signaling pathway, then promote bone healing in induced membrane.

TFRD Increases the Number of Osteoblasts and the Activity of ALP in a Dose-dependent Manner

In order to determine the effect of the concentration of TFRD on the proliferation of osteoblasts, we used different concentrations of TFRD to culture osteoblasts *in vitro*. The *in vitro* results confirmed that the proliferation rate and activity of ALP from osteoblasts were significantly enhanced in a dose-dependent manner after TFRD treatment, and the proliferative rate and activity of ALP were the highest when the dose of TFRD was 100 μg/ml (**Figures 3A,B**). Thus, the optimal concentration of TFRD was 100 μg/ml. The follow-up experiments were carried out with the culture medium containing TFRD of 100 μg/ml.

TFRD Promotes the Proliferation of Osteoblasts by Wnt/β-catenin Signaling Pathway

On the whole, the proliferation of osteoblasts in different groups had a certain rule: during 6 days, osteoblasts' proliferation increased gradually. The proliferation of osteoblasts entered the plateau on the 6th to 9th day, then decreased. On the 3rd day, 6th day and 9th day, there were significant differences in the increment rate of osteoblasts. The sequence of osteoblasts' increment rate of each group was as follows: TFRD > control > TFRD + Dkk1 > Dkk1 (**Figure 3C**). Because Dkk1 is a specific

inhibitor of Wnt/β-catenin signalinging pathway, and the increment rate of osteoblasts in TFRD + Dkk1 group was lower than that in TFRD group, the effect that TFRD promoted the proliferation of osteoblasts should be related to the activation of Wnt/β-catenin signaling pathway.

TFRD Increases the Activity and Amount of ALP of Osteoblasts by Wnt/β-catenin Signaling Pathway

The activity of ALP of osteoblasts in each group also suggested a certain rule: the ALP activity of osteoblasts showed an upward trend during 9 days of cells culture, especially within 3–6 days. From 9 to 12 days, the activity of ALP tended to a downward trend. In our detected time, the activity value of osteoblasts was in the following order: TFRD > control > TFRD + Dkk1 > Dkk1, and the difference was statistically significant (**Figure 3D**). In order to further analyze the amount of ALP expression in each group, we performed ALP staining after 6 days of osteoblasts culture. The positive region of ALP showed grayish-brown flake deposition in the cytoplasm. The positive region of ALP was the most in the TFRD group, followed by the control group and the TFRD + Dkk1 group, the Dkk1 group was the least (**Figure 3E**). TFRD could increase the activity and amount of ALP in osteoblasts, but Dkk1 decrease the expression of ALP in osteoblasts.

TFRD Promotes the Maturation of Osteoblasts

The formation of mineralized nodules is one of the important signs in the process of osteoblasts' maturation. According to the results of ARS staining of osteoblasts, deep red mineralized nodules were observed in all groups of osteoblasts after 21 days of cell culture, and there were significant differences in the number of mineralized nodules of different groups. Among them, the number of mineralized nodules in the TFRD group was the most, followed by the control and TFRD + Dkk1 group, and the number of mineralized nodules in the Dkk1 group was the least (**Figures 3F,G**). The results showed that TFRD could promote the maturation of osteoblasts by activating Wnt/β-catenin signaling pathway.

TFRD Upregulates Wnt/β-catenin Signaling Pathway on Osteoblasts

According to the results of Western blot, there were significant differences in the expression of β-catenin, TCF, LEF, cyclin D, c-myc and Runx2 of different groups after 6 days of cells culture. The average expression of signaling pathway-related proteins in the TFRD group was higher than that in the control and TFRD + Dkk1 group, indicating that TFRD could promote the activation of Wnt/β-catenin signaling pathway in osteoblasts, but the effect of up-regulation can be contained by Dkk1. At the same time, the control group was higher than the Dkk1 group (**Figure 4**), demonstrating that Dkk1 had a definite blocking effect on Wnt/β-catenin signaling pathway. In addition, RT-PCR results showed that there were significant differences in the relative mRNA expressions of β-catenin, TCF, LEF, cyclinD, c-myc and Runx2 among different groups. The relative expression of mRNA related to Wnt/β-catenin signaling pathway on osteoblasts was generally consistent with the results of Western blot (**Figure 5**).

TFRD Upregulates Wnt/β-Catenin Signaling Pathway during Different Periods

In order to dynamically observe the regulatory effect of TFRD on Wnt/β-catenin signaling pathway, the expression of β-catenin protein in different periods was detected by immunofluorescence. The β-catenin was observed in all groups after 3, 6, and 9 days of osteoblasts culture, and was positive in cell membrane, cytoplasm and nucleus, showing high-intensity green fluorescence. The expression region of β-catenin gradually increased with the extension of time. Generally speaking, the fluorescence intensity of β-catenin was more obvious on the 9th day, and was weaker on the 3rd day and 6th day. From the comparison among the groups, the most positive areas of fluorescence signal were found in the TFRD group, followed by the control group. The expression of β-catenin in TFRD + Dkk1 group and Dkk1 group was the least at different stages (**Figures 6A–C**). These results suggested that TFRD could continuously upregulate Wnt/β-catenin signaling pathway.

TFRD Promotes Expression of Osteogenesis-Related Proteins

There are many proteins related to bone formation in the process of osteoblast differentiation, such as collagen type I alpha 1 (COL1A1), bone morphogenetic protein 2 (BMP-2) and osteopontin (OPN). We found that TFRD significantly increased the expression of the three osteogenic marker proteins vs. the control group. With the intervention of dkk1, the expression of three osteogenic proteins significantly decreased (**Figure 6D**). It proved that TFRD may promote the secretion of osteogenesis-related proteins partly by activating Wnt/β-catenin signaling pathway on osteoblasts, then plays the role of osteogenesis.

DISCUSSION

Although Masquelet technique has achieved a high successful rate in clinical practices, the composition and characteristics of induced membrane and mechanism of its bone healing are not clear. According to the diamond concept of bone healing (Giannoudis et al., 2007; Giannoudis et al., 2008), including osteoblasts, bone conduction scaffolds, blood vessels, osteogenic factors, and the stable mechanical environment, osteoblasts are essential for the growth and mineralization of bone. It is well known that bone formation of osteoblasts and bone resorption of osteoclasts play a key role in the process of bone formation and remodeling. In the process of bone

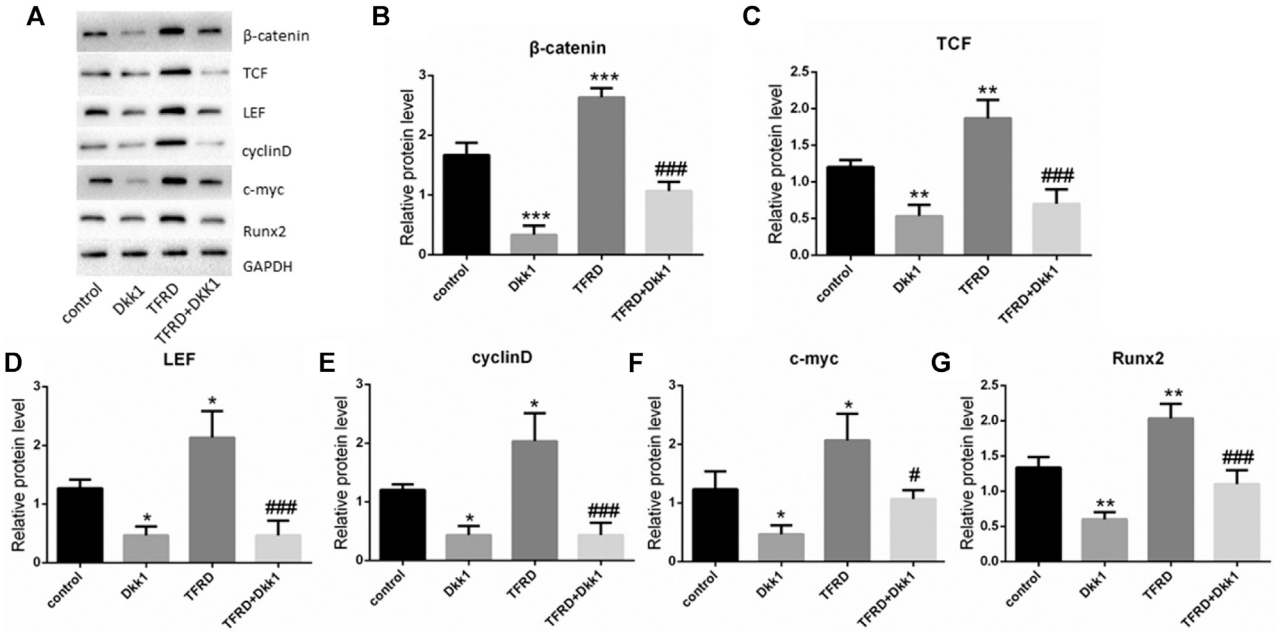


FIGURE 4 | Effect of TFRD on proteins related to Wnt/β-catenin signaling pathway in osteoblasts. **(A)** The relative protein expression levels of β-catenin, TCF, LEF, cyclinD, c-myc and Runx2 were detected by Western blot. **(B–G)** Semi quantitative analysis of protein expression. N = 3/group. Each value was presented as the mean ± SD. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. the control group; $^{###}p < 0.001$, $^{#}p < 0.05$ vs. the TFRD group.

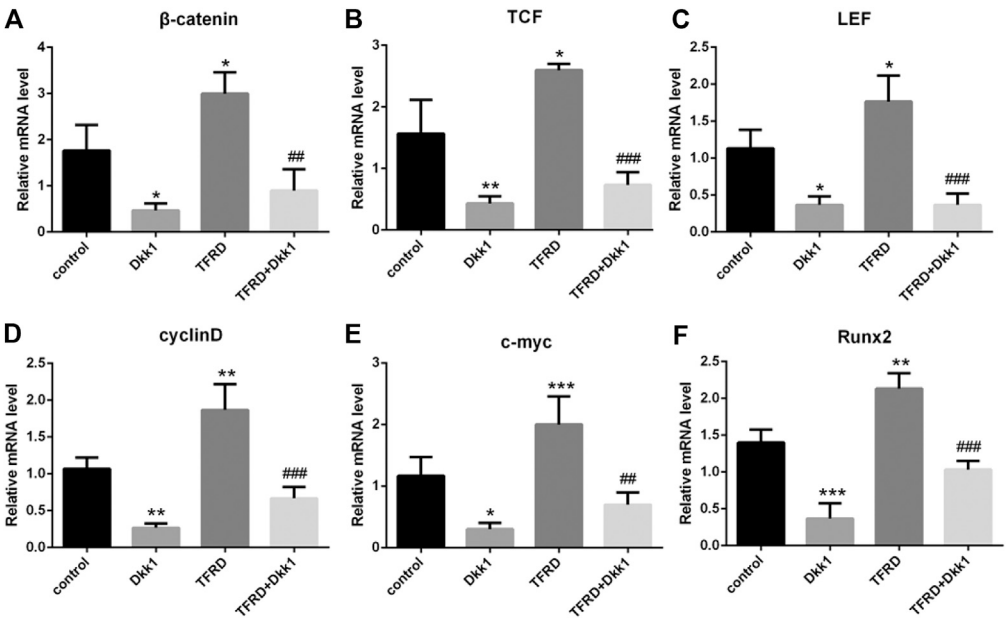


FIGURE 5 | Effect of TFRD on mRNA related to Wnt/β-catenin signaling pathway in osteoblasts. **(A–F)** The relative mRNA levels of β-catenin, TCF, LEF, cyclinD, c-myc and Runx2 were detected by RT-PCR. N = 3/group. Each value was presented as the mean ± SD. $^{***}p < 0.001$, $^{**}p < 0.01$,$^{*}p < 0.05$ vs. the control group; $^{###}p < 0.001$, $^{##}p < 0.01$ vs. the TFRD group.

formation, osteoblasts go through four stages: osteoblast proliferation, extracellular matrix maturation, extracellular matrix mineralization and osteoblast apoptosis. Therefore, promoting the proliferation, differentiation and mineralization of osteoblasts and increasing the secretion of osteogenesis-related proteins have become one of the studying idea to accelerate the speed of bone healing in induced membrane.

TFRD promoting bone formation has become a unique method for the treatment of fracture and osteoporosis (Qian, 2015), but the specific mechanism is not clear. An *in vivo* trail

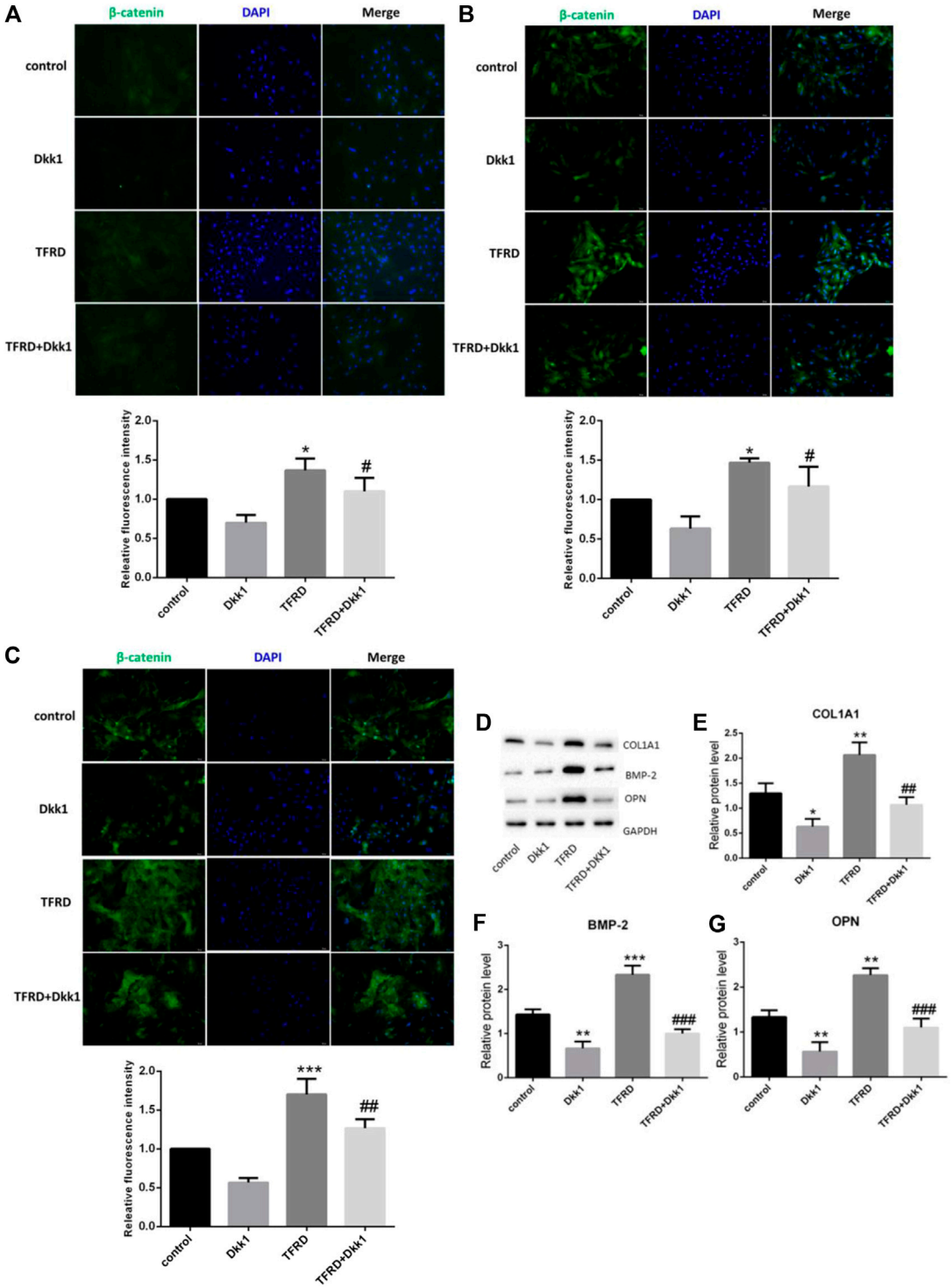


FIGURE 6 | Effect of TFRD on the expression of β-catenin and osteogenic-associated proteins in osteoblasts. **(A–C)** Detection of β-catenin fluorescence in different periods (3, 6, 9 days). The β-catenin was positive in cell membrane, cytoplasm and nucleus, showing high-intensity green fluorescence. Blue fluorescence indicates the nuclei counterstained with DAPI. Values are presented as the mean ± SD of three independent experiments. $^{***}p < 0.001$, $^{*}p < 0.05$ vs. the control group; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. the TFRD group (magnification, × 200). **(D)** The relative protein levels of COL1A1, BMP-2 and OPN were detected by Western blot. **(E–G)** Semi quantitative analysis of protein expression. N = 3/group. Each value was presented as the mean ± SD. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. the control group; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ vs. the TFRD group.

indicated that TFRD could increase BMD, mechanical strength, Bone Volume (BV), Bone Volume Fraction (BV/TV), Trabecular Number (Tb.N), Trabecular Thickness (Tb.Th) and decreased Trabecular Separation (Tb.Sp) in osteoporotic rats (Song et al., 2017). Yao *et al.* found that after taking TFRD, chickens with Tibial dyschondroplasia (TD) recovered their walking ability earlier, repair and arrangement of chondrocytes were more regular, the vascular invasion of cartilage area was earlier, and the expression level of BMP-2 and Runx2 were higher (Yao et al., 2018). Both BMP-2 and Runx2 are essential regulatory genes for bone formation and differentiation (Chenard et al., 2012; Nishimura et al., 2012). Most of these trails focus on the effect of TFRD on improving fracture and osteoporosis, but there are few reports on promoting the growth of bone graft after secondary operation of masquelet technique. Our results pointed that remodeling ability of the bone graft in TFRD group was better than that in control group in terms of histology and imaging results.

Studies have shown that bone formation is related to the activation of osteogenesis-related signaling pathways in BMSCs and osteoblasts, including Wnt/β-catenin signaling pathway, MAPK signaling pathway, Smad signaling pathway and so on (Heo et al., 2018). Among these signaling pathways, the most important pathway is Wnt/β-catenin signaling pathway (Huang et al., 2014). With the activation of Wnt/β-catenin signaling pathway on osteoblasts, osteoblasts enter the mitotic phase. At this stage, the differentiation and proliferation of osteoblasts is accelerated, the synthesis of ALP is increased, and calcification is initiated, thus promoting bone formation. The content of ALP in cells represents the degree and state of cell differentiation and is an early specific marker of extracellular matrix maturation (Nguyen et al., 2003). The local use of β-catenin enhancer can promote the proliferation and differentiation of osteoblasts, and then promotes new bone formation (Liu et al., 2010). In a experiment of transgenic mice, it was found that the expression level of β-catenin was directly related to bone formation, and the loss of β-catenin expression directly led to the decrease of osteoblast differentiation and the disturbance of bone formation (Huang et al., 2014). In the Wnt/β-catenin signaling pathway, Dkk1 is one of the vital antagonists, which can specifically inhibit the classical Wnt signaling pathway (Kawano and Kypta, 2003). *In vitro* experiments have confirmed that TFRD can induce the proliferation and differentiation of BMSCs and osteoblasts and inhibit the early apoptosis of osteoblasts (Zhang et al., 2009; Guo et al., 2012). In order to further confirm the specific mechanism of TFRD on osteoblasts, we cultured osteoblasts *in vitro*. The results suggested that TFRD could promote the proliferation and ALP activity of osteoblasts in a dose-dependent manner. After the intervention of the best dose of TFRD (100 ug/ml), the proliferation rate, the activity of ALP and the number of mineralized nodules of osteoblasts in the TFRD group were significantly higher than those in the control group, indicating that TFRD could significantly promote the proliferation and mineralization of osteoblasts. However, this promoting effect of TFRD can be specifically blocked by Dkk1, implicating that the reason that TFRD promotes osteoblasts' proliferation and mineralization should be related to the up-regulation of Wnt/β-catenin signaling pathway. In addition, the osteoblasts' increment rate and the expression of pathway protein in the TFRD + Dkk1 group were still higher than those in the Dkk1 group, suggesting that TFRD could reverse the inhibitory effect of Dkk1 on Wnt/β-catenin signaling pathway to some extent.

Moreover, we also explored expression of osteogenic-related proteins, including COL1A1, BMP-2 and OPN, induced by TFRD. COL1A1 is responsible for the synthesis of type 1 collagen, thus ensuring that bones and cartilage are resistant to tension, shear and compression (Palomo et al., 2017). Abnormal collagen production can lead to bone-related diseases, such as Paget disease and osteoporosis (Li et al., 2019). BMP-2 is highly involved in inducing mesenchymal cells to differentiate into osteoblasts and promoting osteoblasts to produce bone matrix (Susperregui et al., 2008). OPN can stimulate osteoblasts' adhesion, proliferation and calcification, and mediate the changes of bone metabolism caused by mechanical stress (Chatakun et al., 2014). Our experimental results *in vitro* and *in vivo* presented that the expression of these proteins in TFRD group was increased significantly with the increase of TFRD dose. After the intervention of Dkk1, the expression of three proteins decreased, which further confirmed that TFRD promoted the expression of osteogenic-related proteins on osteoblasts by activating Wnt/β-catenin signaling pathway.

Wnt/β-catenin signaling has become an essential pathway for regulating bone formation and bone resorption. However, it is well-known that the aberrant activation of Wnt/β-catenin pathway is the basis of progression of various types of malignant tumors, including colorectal cancer, liver cancer, gastric cancer, lung cancer, breast cancer and so on (Zhao et al., 2018; Zhao et al., 2020; Jiang et al., 2021; Liu et al., 2021; Zheng et al., 2021). TFRD could up-regulate the Wnt/β-catenin signaling pathway, which may potentially aggravate the development of cancers in patients or animals in theory. The failure to verify the safety of TFRD in animals with cancer is one of the limitations of this study. However, we believe that the side effects of medicine, including carcinogenicity, gastrointestinal toxicity, hepatorenal toxicity and so on, are related to population, dose, mode of administration, time and other factors. In the course of this study, two weeks after Masquelet surgery, there were no significant changes in spirit, diet, activity, body weight and skin of these rats. At the end of the experiment, the chest, abdomen, limbs and other parts of the rats did not touch abnormal masses and tumors. In the range of dose and time of our study, TFRD was relatively safe for non-cancer rats. According to the current reports, after TFRD acted on the rat models of bone defect, fracture, osteoporosis and osteonecrosis of femoral head, no carcinogenic and other adverse reactions were found (Song et al., 2016; Song et al., 2017; Yao et al., 2018; Guo et al., 2019; Shen et al., 2020; Lv et al., 2021). The doses of administration in these articles can be summarized as 75–440 mg/kg/d (oral administration) and 20 mg/kg/day (intraperitoneal injection), respectively. Moreover, the mechanism of tumors' progression is very complex, which is not only one

mechanism of aberrant activation of Wnt/β-catenin signaling pathway. TFRD may also up-regulate other protective pathways that inhibit cancers, thus inhibiting the development of cancers. Therefore, whether TFRD has side effects such as aggravating the progression of cancers remains to be determined.

CONCLUSION

TFRD could promote the growth and mineralization of bone graft in the induced membrane, which is related to the fact that TFRD should promote osteoblasts' differentiation, mineralization and expression of osteogenesis-related proteins partly by activating Wnt/β-catenin signaling pathway. However, whether TFRD also upregulates other signaling pathways, whether there is a synergistic effect between these signaling pathways, and side effects of TFRD is the direction of our follow-up studies.

DATA AVAILABILITY STATEMENT

All relevant data regarding the study is included in this article and any supplementary data is available from the corresponding author upon request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Ethics Committee of Guangzhou University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

Designed the experiments: SL, QZ. Performed the experiments: SL, HZ, CH, JYa, JYe, YZ, ZL. Analyzed the data and wrote the manuscript: SL, LC, QZ.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 81674001).

ACKNOWLEDGMENTS

The authors are grateful to all participants. We thank Lingnan Medical Research Center of Guangzhou University of Chinese Medicine for providing associated facilities.

REFERENCES

Aronson, J., Johnson, E., and Harp, J. H. (1989). Local Bone Transportation for Treatment of Intercalary Defects by the Ilizarov Technique. *Clin. Orthopaedics Relat. Res.*, 243, 71–79. doi:10.1097/00003086-198906000-00011

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* 55 (4), 611–622. doi:10.1373/clinchem.2008.112797

Canalis, E. (2013). Wnt Signalling in Osteoporosis: Mechanisms and Novel Therapeutic Approaches. *Nat. Rev. Endocrinol.* 9 (10), 575–583. doi:10.1038/nrendo.2013.154

Chatakun, P., Núñez-Toldrà, R., Díaz López, E. J., Gil-Recio, C., Martínez-Sarrà, E., Hernández-Alfaro, F., et al. (2014). The Effect of Five Proteins on Stem Cells Used for Osteoblast Differentiation and Proliferation: a Current Review of the Literature. *Cell. Mol. Life Sci.* 71 (1), 113–142. doi:10.1007/s00018-013-1326-0

Chen, X., Wang, Z., Duan, N., Zhu, G., Schwarz, E. M., and Xie, C. (2018a). Osteoblast--osteoclast Interactions. *Connect. Tissue Res.* 59 (2), 99–107. doi:10.1080/03008207.2017.1290085

Chen, L.-l., Lei, L.-h., Ding, P.-h., Tang, Q., and Wu, Y.-m. (2011). Osteogenic Effect of Drynariae Rhizoma Extracts and Naringin on MC3T3-E1 Cells and an Induced Rat Alveolar Bone Resorption Model. *Arch. Oral Biol.* 56 (12), 1655–1662. doi:10.1016/j.archoralbio.2011.06.008

Chen, L., Tao, Z. S, Chen, H., Zhou, K., and Zhou, D. S. (2018b). Combined Treatment with Alendronate and Drynaria Rhizome Extracts : Effect on Fracture Healing in Osteoporotic Rats. *Z. Gerontol. Geriatr.* 51 (8), 875–881. doi:10.1007/s00391-017-1326-z

Chenard, K. E., Teven, C. M., He, T. C., and Reid, R. R. (2012). Bone Morphogenetic Proteins in Craniofacial Surgery: Current Techniques, Clinical Experiences, and the Future of Personalized Stem Cell Therapy. *J. Biomed. Biotechnol.* 2012, 601549–601610. doi:10.1155/2012/601549

Fulciniti, M., Tassone, P., Hideshima, T., Vallet, S., Nanjappa, P., Ettenberg, S. A., et al. (2009). Anti-DKK1 mAb (BHQ880) as a Potential Therapeutic Agent for Multiple Myeloma. *Blood* 114 (2), 371–379. doi:10.1182/blood-2008-11-191577

Giannoudis, P. V., Einhorn, T. A., and Marsh, D. (2007). Fracture Healing: the diamond Concept. *Injury* 38, S3–S6. doi:10.1016/s0020-1383(08)70003-2

Giannoudis, P. V., Einhorn, T. A., Schmidmaier, G., and Marsh, D. (2008). The diamond Concept - Open Questions. *Injury* 39, S5–S8. doi:10.1016/s0020-1383(08)70010-x

Glantschnig, H., Hampton, R. A., Lu, P., Zhao, J. Z., Vitelli, S., Huang, L., et al. (2020). Generation and Selection of Novel Fully Human Monoclonal Antibodies that Neutralize Dickkopf-1 (DKK1) Inhibitory Function *In Vitro* and Increase Bone Mass *In Vivo*. *J. Biol. Chem.* 285 (51), 40135–40147. doi:10.1074/jbc.M110.166892

Gouron, R., Petit, L., Boudot, C., Six, I., Brazier, M., Kamel, S., et al. (2017). Osteoclasts and Their Precursors Are Present in the Induced-Membrane during Bone Reconstruction Using the Masquelet Technique. *J. Tissue Eng. Regen. Med.* 11 (2), 1–8. doi:10.1002/term.1921

Guo, W., Shi, K., Xiang, G., Lu, D., Dou, H., Xie, C., et al. (2019). Effects of Rhizoma Drynariae Cataplasm on Fracture Healing in a Rat Model of Osteoporosis. *Med. Sci. Monit.* 25, 3133–3139. doi:10.12659/msm.914568

Guo, Y., Li, P. Y., and Deng, H. (2012). Effects of Total Flavonoids of Rhizoma Drynariae on Wnt/β-Catenin Signal Pathway during Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells. *J. Chin. Med. Assoc.* 92 (32), 2288–2291.

Heo, S.-Y., Ko, S.-C., Nam, S. Y., Oh, J., Kim, Y.-M., Kim, J.-I., et al. (2018). Fish Bone Peptide Promotes Osteogenic Differentiation of MC3T3-E1 Pre-osteoblasts through Upregulation of MAPKs and Smad Pathways Activated BMP-2 Receptor. *Cell Biochem Funct.* 36 (3), 137–146. doi:10.1002/cbf.3325

Huang, Y., Zhang, X., Du, K., Yang, F., Shi, Y., Huang, J., et al. (2014). Inhibition of β-catenin Signaling in Chondrocytes Induces Delayed Fracture Healing in Mice. *J. Orthop. Res.* 30 (2), 304–310. doi:10.1002/jor.21505

Jeong, J.-C., Kang, S.-K., Youn, C.-H., Jeong, C.-W., Kim, H.-M., Lee, Y.-C., et al. (2003). Inhibition of Drynariae Rhizoma Extracts on Bone Resorption Mediated by Processing of Cathepsin K in Cultured Mouse Osteoclasts. *Int. Immunopharmacol.* 3 (12), 1685–1697. doi:10.1016/j.intimp.2003.08.003

Jiang, B., Zhu, H., Tang, L., Gao, T., Zhou, Y., Gong, F., et al. (2021). Apatinib Inhibits Stem Properties and Malignant Biological Behaviors of Breast Cancer Stem Cells by Blocking Wnt/β-Catenin Signal Pathway through Down-Regulating LncRNA ROR. *Anticancer Agents Med. Chem.* 11, 1–10.

Jiang, Z. W., Zeng, J. Q., and Huang, F. (2018). Effects of Flavonoids of Rhizoma Drynariae on Tibia Distraction Osteogenesis Efficacy in Rat Model. *China J. Traditional Chin. Med. Pharm.* 33 (2), 661–663.

Kawano, Y., and Kypta, R. (2003). Secreted Antagonists of the Wnt Signalling Pathway. *J. Cel. Sci.* 116 (13), 2627–2634. doi:10.1242/jcs.00623

Krishnan, V. (2006). Regulation of Bone Mass by Wnt Signaling. *J. Clin. Invest.* 116 (5), 1202–1209. doi:10.1172/jci28551

Kuo, H.-C., Chang, H.-C., Lan, W.-C., Tsai, F.-H., Liao, J.-C., and Wu, C.-R. (2014). Protective Effects of Drynaria Fortunei against 6-Hydroxydopamine-Induced Oxidative Damage in B35 Cells via the PI3K/AKT Pathway. *Food Funct.* 5 (8), 1956–1965. doi:10.1039/c4fo00219a

Lasanianos, N. G., Kanakaris, N. K., and Giannoudis, P. V. (2010). Current Management of Long Bone Large Segmental Defects. *Orthopaedics and Trauma* 24 (2), 149–163. doi:10.1016/j.mporth.2009.10.003

Li, L.-J., Lyu, F., Song, Y.-W., Wang, O., Jiang, Y., Xia, W.-B., et al. (2019). Genotype-phenotype Relationship in a Large Cohort of Osteogenesis Imperfecta Patients with COL1A1 Mutations Revealed by a New Scoring System. *Chin. Med. J. (Engl).* 132 (2), 145–153. doi:10.1097/cm9.0000000000000013

Liu, H. X., Zhou, J., and Xu, J. (2010). β-Catenin Activator Enhances Bone Regeneration during Premaxillary Suture Expansion in Rat. *Zhongguo Kouqiang Hemian Waike Zazhi* 8 (6), 531–535.

Liu, X., Su, K., Sun, X., Jiang, Y., Wang, L., Hu, C., et al. (2021). Sec62 Promotes Stemness and Chemoresistance of Human Colorectal Cancer through Activating Wnt/β-Catenin Pathway. *J. Exp. Clin. Cancer Res.* 40, 132. doi:10.1186/s13046-021-01934-6

Luyten, F. P., Tylzanowski, P., and Lories, R. J. (2009). Wnt Signaling and Osteoarthritis. *Bone* 44 (4), 522–527. doi:10.1016/j.bone.2008.12.006

Lv, W., Yu, M., Yang, Q., Kong, P., and Yan, B. (2021). Rhizoma Drynariae Total Flavonoids of Ameliorate Steroid-Induced Avascular Necrosis of the Femoral Head via the PI3K/AKT Pathway. *Mol. Med. Rep.* 23 (5), 345. doi:10.3892/mmr.2021.11984

Masquelet, A. C., Fitoussi, F., Begue, T., and Muller, G. P. (2000). Reconstruction of the Long Bones by the Induced Membrane and Spongy Autograft. *Ann. Chir Plast. Esthet* 45, 346–353.

Masquelet, A. C., and Bégué, T. (2010). The Concept of Induced Membrane for Reconstruction of Long Bone Defects. *Orthop. Clin. North America* 41 (1), 27–37. doi:10.1016/j.ocl.2009.07.011

Milat, F., and Ng, K. W. (2009). Is Wnt Signalling the Final Common Pathway Leading to Bone Formation? *Mol. Cell Endocrinol.* 310 (1), 52–62. doi:10.1016/j.mce.2009.06.002

Monroe, D. G., McGee-Lawrence, M. E., Oursler, M. J., and Westendorf, J. J. (2012). Update on Wnt Signaling in Bone Cell Biology and Bone Disease. *Gene* 492 (1), 1–18. doi:10.1016/j.gene.2011.10.044

Nguyen, H., Qian, J. J., Bhatnagar, R. S., and Li, S. (2003). Enhanced Cell Attachment and Osteoblastic Activity by P-15 Peptide-Coated Matrix in Hydrogels. *Biochem. Biophysical Res. Commun.* 311 (1), 179–186. doi:10.1016/j.bbrc.2003.09.192

Nishimura, R., Hata, K., Matsubara, T., Wakabayashi, M., and Yoneda, T. (2012). Regulation of Bone and Cartilage Development by Network between BMP Signalling and Transcription Factors. *J. Biochem.* 151 (3), 247–254. doi:10.1093/jb/mvs004

Osterman, A. L., and Bora, F. W. (1984). Free Vascularized Bone Grafting for Large-gap Nonunion of Long Bones. *Orthop. Clin. North America* 15 (1), 131–142. doi:10.1016/s0030-5898(20)31284-0

Palomo, T., Vilaça, T., and Lazaretti-Castro, M. (2017). Osteogenesis Imperfecta. *Curr. Opin. Endocrinol. Diabetes Obes.* 24 (6), 381–388. doi:10.1097/med.0000000000000367

Pelissier, P. H., Masquelet, A. C., Bareille, R., Pelissier, S. M., and Amedee, J. (2004). Induced Membranes Secrete Growth Factors Including Vascular and Osteoinductive Factors and Could Stimulate Bone Regeneration. *J. Orthop. Res.* 22 (1), 73–79. doi:10.1016/s0736-0266(03)00165-7

Pinzone, J. J., Hall, B. M., Thudi, N. K., Vonau, M., Qiang, Y.-W., Rosol, T. J., et al. (2009). The Role of Dickkopf-1 in Bone Development, Homeostasis, and Disease. *Blood* 113 (3), 517–525. doi:10.1182/blood-2008-03-145169

Qian, Q. (2015). Reaserch Advances in Chemical Constituents and Pharmacological Activities of Rhizoma Drynariaed. *Chin. J. Biochem. Pharm.* 35, 186–188.

Raggatt, L. J., and Partridge, N. C. (2010). Cellular and Molecular Mechanisms of Bone Remodeling. *J. Biol. Chem.* 285 (33), 25103–25108. doi:10.1074/jbc.r109.041087

Shen, Z., Chen, Z., Li, Z., Zhang, Y., Jiang, T., Lin, H., et al. (2020). Total Flavonoids of Rhizoma Drynariae Enhances Angiogenic- Osteogenic Coupling during Distraction Osteogenesis by Promoting Type H Vessel Formation through PDGF-BB/PDGFR-β Instead of HIF-1α/VEGF Axis. *Front. Pharmacol.* 11, 503524. doi:10.3389/fphar.2020.503524

Song, S.-h., Zhai, Y.-k., Li, C.-q., Yu, Q., Lu, Y., Zhang, Y., et al. (2016). Effects of Total Flavonoids from Drynariae Rhizoma Prevent Bone Loss *In Vivo* and *In Vitro*. *Bone Rep.* 5, 262–273. doi:10.1016/j.bonr.2016.09.001

Song, S., Gao, Z., Lei, X., Niu, Y., Zhang, Y., Li, C., et al. (2017). Total Flavonoids of Drynariae Rhizoma Prevent Bone Loss Induced by Hindlimb Unloading in Rats. *Molecules* 22 (7), 1033. doi:10.3390/molecules22071033

Sun, Z., Xu, C., and Wang, J. (2016). Observation on the Therapeutic Effect of Total Flavonoids of Rhizoma Drynaria and Alfacalcidol in Treating Osteoporosis. *Chron.Pathematol. J.* 17, 327–330.

Susperregui, A. R. G., Viñals, F., Ho, P. W. M., Gillespie, M. T., Martin, T. J., and Ventura, F. (2008). BMP-2 Regulation of PTHrP and Osteoclastogenic Factors during Osteoblast Differentiation of C2C12 Cells. *J. Cel. Physiol.* 216 (1), 144–152. doi:10.1002/jcp.21389

Wiese, A., and Pape, H. C. (2010). Bone Defects Caused by High-Energy Injuries, Bone Loss, Infected Nonunions, and Nonunions. *Orthop. Clin. North America* 41, 1–4. doi:10.1016/j.ocl.2009.07.003

Wong, R. W., Rabie, B., Bendeus, M., and Hägg, U. (2007). The Effects of Rhizoma Curculiginis and Rhizoma Drynariae Extracts on Bones. *Chin. Med.* 2 (1), 1–17. doi:10.1186/1749-8546-2-13

Yang, F., Sun, Y. H., and Lv, Z. J. (2017). Effect of Rhizoma Drynariae Flavones on Alveolar Bone Quality in Patients with Osteoporosis. *China J. Oral Maxillofacial Surg.* 16 (1), 34–40. doi:10.19438/j.cjoms.2018.01.007

Yao, W., Zhang, H., Jiang, X., Mehmood, K., Iqbal, M., Li, A., et al. (2018). Effect of Total Flavonoids of Rhizoma Drynariae on Tibial Dyschondroplasia by Regulating BMP-2 and Runx2 Expression in Chickens. *Front. Pharmacol.* 9, 1251. doi:10.3389/fphar.2018.01251

Zhang, J., Li, H. P., Yang, P. L., Liu, Y. H., and Yang, B. H. (2009). Effects of Total Flavonoids from Rhizoma Drynariae Medicated Serum on Proliferation, Differentiation, Cell Cycle and Apoptosis of Osteoblasts *In Vitro*. *Zhong Yao Cai.* 32 (7), 1090–1093.

Zhao, J., Wang, Y., Han, M., Lu, H., Chen, X., Liu, S., et al. (2020). P7TP3 Inhibits Tumor Development, Migration, Invasion and Adhesion of Liver Cancer through the Wnt/β-catenin Signaling Pathway. *Cancer Sci.* 111 (3), 994–1007. doi:10.1111/cas.14243

Zhao, Y.-X., Liu, J.-F., Sun, W.-J., Zeng, R.-F., Li, T., and Ma, R.-M. (2018). Long Non-coding RNA-Enst00000434223 Suppresses Tumor Progression in Gastric Cancer Cells through the Wnt/β-Catenin Signaling Pathway. *Int. J. Biol. Macromolecules* 120 (Pt A), 491–501. doi:10.1016/j.ijbiomac.2018.08.079

Zheng, J., Li, X., Cai, C., Hong, C., and Zhang, B. (2021). MicroRNA-32 and MicroRNA-548a Promote the Drug Sensitivity of Non-small Cell Lung Cancer Cells to Cisplatin by Targeting ROBO1 and Inhibiting the Activation of Wnt/β-Catenin Axis. *Cancer. Manag. Res.* Vol. 13, 3005–3016. doi:10.2147/cmar.s295003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

CORRECTION
published: 16 July 2021
doi: 10.3389/fphar.2021.726831

Corrigendum: Total Flavonoids of Rhizoma Drynariae Promotes Differentiation of Osteoblasts and Growth of Bone Graft in Induced Membrane Partly by Activating Wnt/β-Catenin Signaling Pathway

Shuyuan Li[1,2], *Hongliang Zhou*[1,2], *Cheng Hu*[1,2], *Jiabao Yang*[1,2], *Jinfei Ye*[1,2], *Yuexi Zhou*[1,2], *Zige Li*[1,2], *Leilei Chen*[3]* and *Qishi Zhou*[4]*

[1]Guangzhou University of Chinese Medicine, Guangzhou, China, [2]Lingnan Medical Research Center of Guangzhou University of Chinese Medicine, Guangzhou, China, [3]Third Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, China, [4]First Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, China

Keywords: total flavonoids of rhizoma drynariae, induced membrane, wnt/β-catenin, bone defect, osteogenic efficacy

OPEN ACCESS

Edited and reviewed by:
Dieter Steinhilber,
Goethe University Frankfurt, Germany

***Correspondence:**
Leilei Chen
yutian_1010@sina.com
Qishi Zhou
zhouqishi@139.com

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *17 June 2021*
Accepted: *29 June 2021*
Published: *16 July 2021*

Citation:
Li S, Zhou H, Hu C, Yang J, Ye J, Zhou Y, Li Z, Chen L and Zhou Q (2021) Corrigendum: Total Flavonoids of Rhizoma Drynariae Promotes Differentiation of Osteoblasts and Growth of Bone Graft in Induced Membrane Partly by Activating Wnt/β-Catenin Signaling Pathway. Front. Pharmacol. 12:726831. doi: 10.3389/fphar.2021.726831

A Corrigendum on

Total Flavonoids of Rhizoma Drynariae Promotes Differentiation of Osteoblasts and Growth of Bone Graft in Induced Membrane Partly by Activating Wnt/β-Catenin Signaling Pathway
by Li S., Zhou H., Hu C., Yang J., Ye J., Zhou Y., Li Z., Chen L. and Zhou Q. (2021). Front. Pharmacol. 12:675470. doi: 10.3389/fphar.2021.675470

In the original article, there was a mistake in **Figures 1E**, **4A** as published. The carelessness in combining the images caused the repetition of the images (**Figure 1E** H-TFRD and L-TFRD; **Figure 4A** cyclinD and **Figure 6D** COL1A1).The corrected **Figures 1**, **4** appear below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

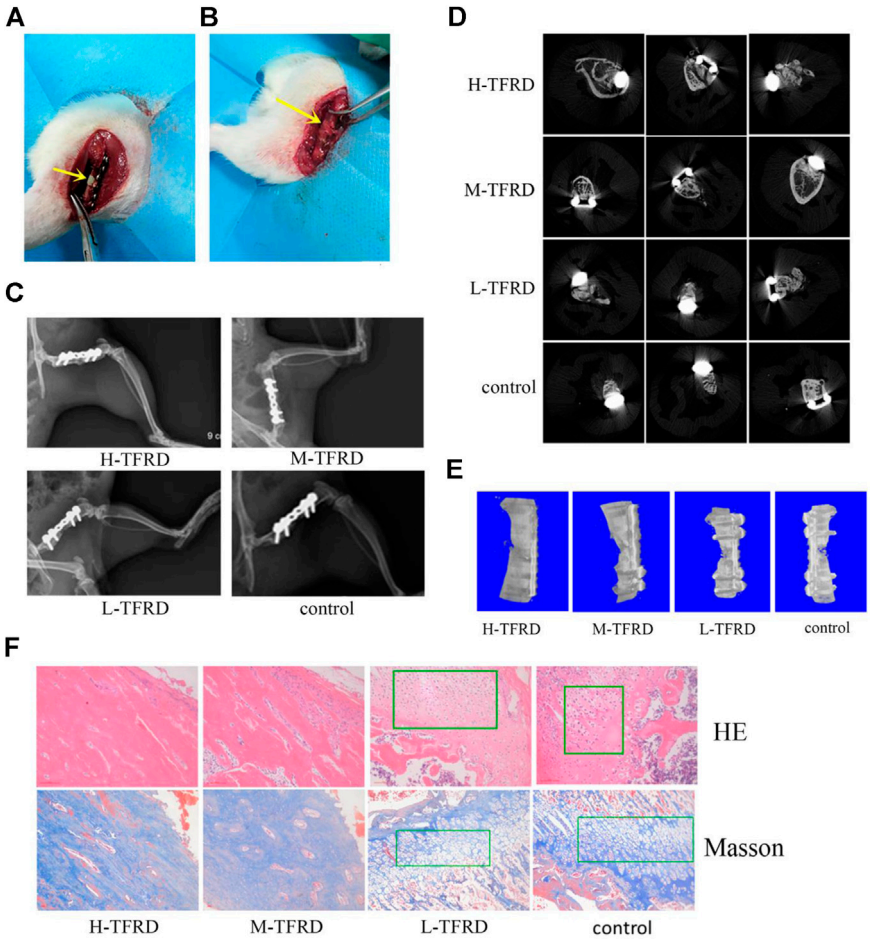


FIGURE 1 | TFRD accelerates the growth and mineralization of bone graft. **(A)** The yellow arrow in the picture refers to the 6 mm bone defect constructed in the right femur of rats during the first stage operation. PMMA spacer was implanted in this area to induce formation of biofilm. **(B)** The yellow arrow refers to the area of bone graft in the right femur of rats at the second stage operation. **(C)** X-ray was performed on the right femur of rats. Among them, the amount of callus and cortical bone shaping in the H-TFRD and M-TFRD groups were more obvious than those in the L-TFRD and control groups. **(D)** was the result of Micro-CT cross-sectional scanning of the bone graft in the right femur of rats. **(E)** was the results of three-dimensional reconstruction of the right femur of rats. **(F)** shows the histological and structural characteristics of bone graft in the right femur of rats (magnification, ×200). The green boxes show the cartilage area, and other parts in pictueres show the osteogenic area.

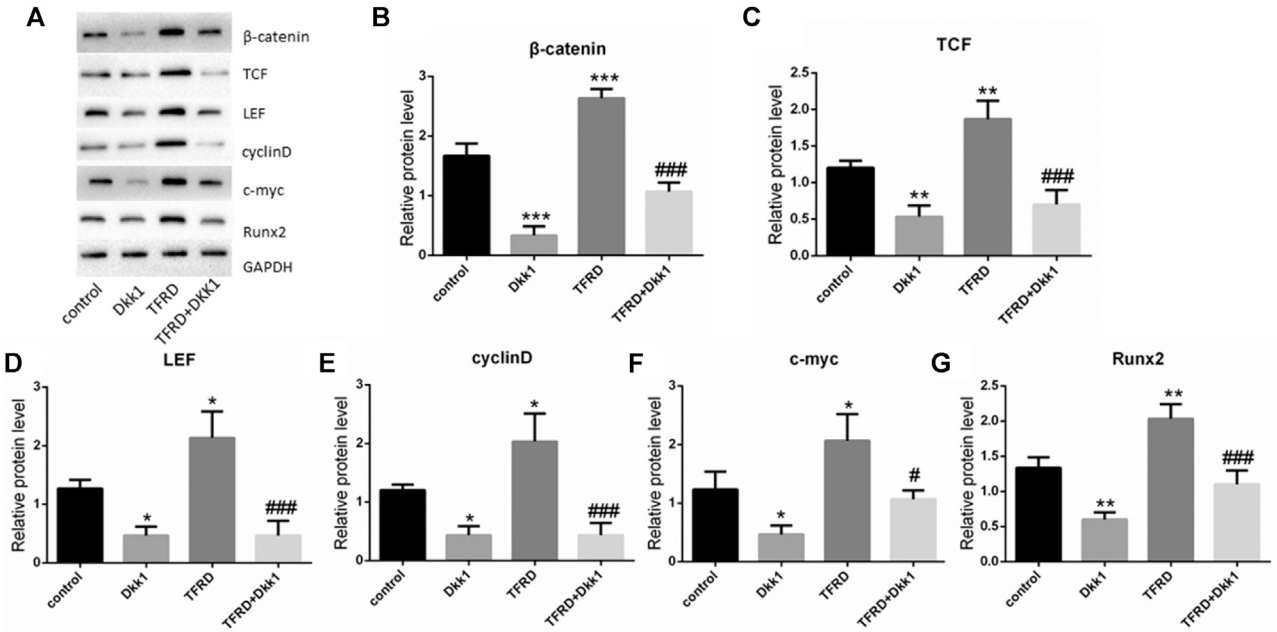


FIGURE 4 | Effect of TFRD on proteins related to Wnt/β-catenin signaling pathway in osteoblasts. **(A)** The relative protein expression levels of β-catenin, TCF, LEF, cyclinD, c-myc, and Runx2 were detected by Western blot. **(B–G)** Semi-quantitative analysis of protein expression. N = 3/group. Each value was presented as the mean ± SD. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. the control group; $^{\#\#\#}p < 0.001$, $^{\#}p < 0.05$ vs. the TFRD group.

frontiers in Pharmacology

OPINION
published: 30 June 2021
doi: 10.3389/fphar.2021.681871

Immunological Events, Emerging Pharmaceutical Treatments and Therapeutic Potential of Balneotherapy on Osteoarthritis

Anna Scanu[1,2]*, *Lucrezia Tognolo*[2], *Maria Chiara Maccarone*[2] *and Stefano Masiero*[2]

[1]Rheumatology Unit, Department of Medicine-DIMED, University of Padua, Padua, Italy, [2]Department of Neurosciences, Physical Medicine and Rehabilitation School, University of Padua, Padua, Italy

Keywords: immune system, osteoarthritis, balneotherapy, inflammation, disease-modifying OA drug

OPEN ACCESS

Edited by:
Antonella Fioravanti,
Azienda Ospedaliera Universitaria Senese, Italy

Reviewed by:
Valentina Calamia,
Institute of Biomedical Research of A Coruña (INIBIC), Spain
Valeria Tekus,
University of Pécs, Hungary
Eduardo Ortega,
University of Extremadura, Spain

***Correspondence:**
Anna Scanu
anna.scanu@unipd.it

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *17 March 2021*
Accepted: *18 June 2021*
Published: *30 June 2021*

Citation:
Scanu A, Tognolo L, Maccarone MC and Masiero S (2021) Immunological Events, Emerging Pharmaceutical Treatments and Therapeutic Potential of Balneotherapy on Osteoarthritis. Front. Pharmacol. 12:681871. doi: 10.3389/fphar.2021.681871

INTRODUCTION

Although hypotheses have been proposed, the exact pathophysiological mechanisms of osteoarthritis (OA) still remain unknown. Evidence suggests that immunological events, immune-neuroendocrine dysregulation and the presence of low-grade local and systemic inflammation play a key role in the pathogenesis and progression of this disease (Scanzello et al., 2015; Galvez et al., 2017; Chow et al., 2020; Woodell-May et al., 2020). Traditional therapies for OA focus on minimizing the symptoms, but not cure the arthritis. If none of these measures are effective, surgery is the next option. However, any medical or surgical treatment can have severe side effects. Balneotherapy is a common practice for the treatment and rehabilitation of OA patients whose role in modern medicine needs to be better defined. Studies have demonstrated that the beneficial effects of balneotherapy are mediated by regulation of inflammatory cells and mediators (Gálvez et al., 2018). This article aims to provide a standpoint on the possible involvement of immune system in these processes, and why it should be considered a target for therapy in such instances, based on published literature. Furthermore, we propose that the balneotherapy effectiveness in this context be better examined in future studies, in order to expand its employment alone or as a complement to other treatments in the OA management.

IMMUNOLOGICAL EVENTS IN OA

It is increasingly recognized that immune cells and their molecular mediators play a part in OA development. Enhanced leukocyte infiltration in the synovium and the presence of activate macrophages (M1) in synovial fluid (SF) have been identified in OA patients (Deligne et al., 2015; Kraus et al., 2016; Liu et al., 2018). Concomitantly, pro-inflammatory cytokines are produced locally by infiltrating and resident cells in early and end-stage of OA, independently or on collaboration with other mediators (Goldring et al., 2011; Punzi et al., 2016).

The main triggering of these events seems to be the activation of innate immunity by damage-associated molecular patterns, including extracellular matrix fragments, high mobility box 1, uric acid, complement system, S100 proteins, and heat shock proteins (HSPs) that are released into the joint after trauma or age-related processes (Gobezie et al., 2007; Scanzello et al., 2008; Ke et al., 2015). These molecules, generated in part by oxidative stress, are able to bind in synovial cells pathogen-recognition receptors, such as toll-like receptors, the receptor for advanced glycation end products and the NLRP3 inflammasome, and induce pro-inflammatory mediators production (Kim et al., 2006; Steenvoorden et al., 2006; McAllister et al., 2018). Indeed, the levels of several inflammatory

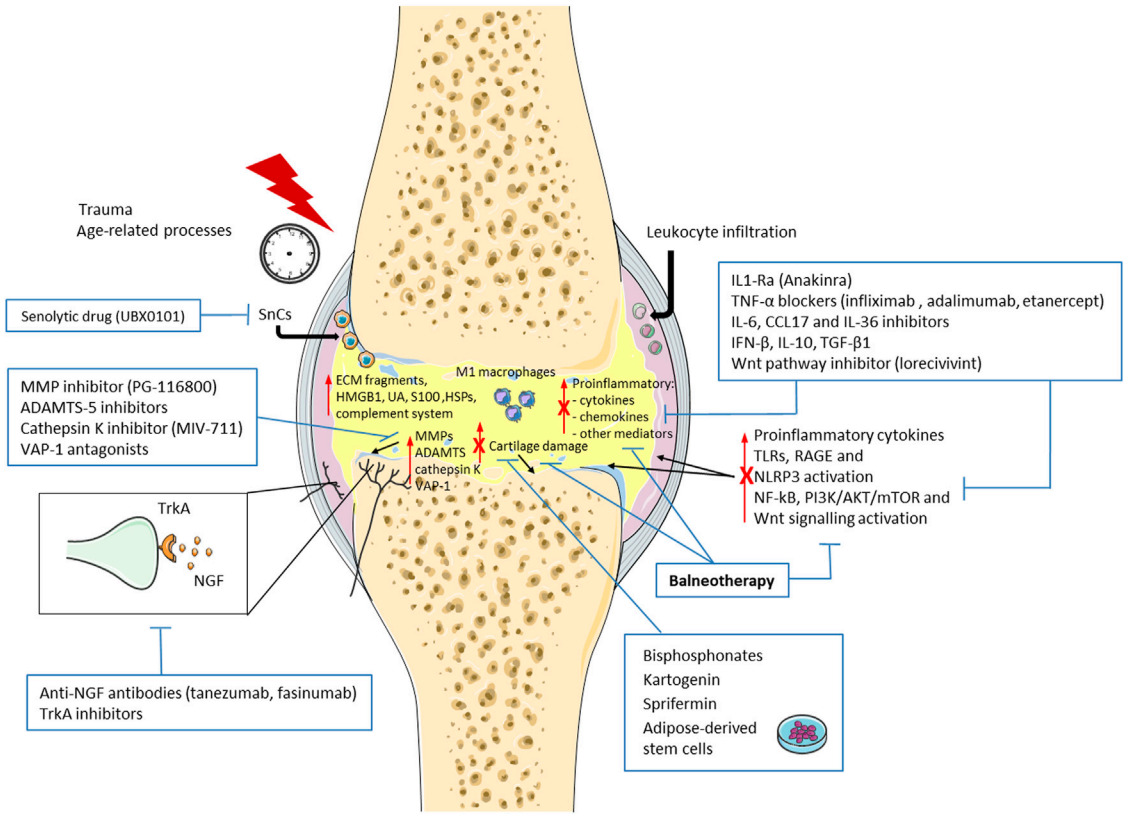


FIGURE 1 | Schematic representation of the new emerging therapies targeting immune system and immunomodulatory properties of balneotherapy in osteoarthritis (OA). Immunological events and low-grade inflammation play a key role in the pathogenesis and progression of OA. Damage-associated molecular patterns (DAMPs), including extracellular matrix (ECM) fragments, high mobility box 1 (HMGB1), uric acid (UA), complement system, S100 proteins, and heat shock proteins (HSPs) are released into the joint after trauma or age-related processes. These molecules, bind Toll-like receptors (TLRs), the receptor for advanced glycation end products (RAGE) and the intracellular NLRP3 inflammasome, and induce the production of pro-inflammatory mediators, such as cytokines and chemokines. Concomitantly, leukocyte infiltration, presence of activate macrophages (M1) and senescent cells (SnCs), levels metalloproteinases (MMPs), A Disintegrin And Metalloproteinase with Thrombospondin motif (ADAMTS), vascular adhesion protein-1 (VAP-1) and Nerve Growth factor (NGF) are enhanced, thus promoting cartilage damage and pain. Finally, NF-kB, PI3K/AKT/mTOR, and Wnt/β-Catenin signaling pathways are activated in these processes. New emerging treatment and balneotherapy have demonstrated a decrease in OA progression through immunomodulatory properties. ↑ increase; **T** inhibition; **X** reduction by balneotherapy.

cytokines, such as IL-1β and IL-6, are higher in serum from OA compared to healthy subjects (Sohn et al., 2012); whereas elevated IL-6, IL-8 and CCL2 were found in OA SF (Kaneko et al., 2000; Li et al., 2015; Oliviero et al., 2020). Increased IL-1β, IL-6, TNF-α and IL-8 concentrations are also detected in synovial tissues and articular cartilage of OA patients (Ma et al., 2015; Ni et al., 2015; Böhm et al., 2016).

Moreover, high concentrations of cytokines could be secreted from senescent cells, which accumulate in the synovium and in cartilage surface, thus predisposing the joint to OA development (Jeon et al., 2017).

In turn, these inflammatory factors, carried by the SF, can activate chondrocytes to produce metalloproteinases (MMPs) which result in further cartilage damage (Nefla et al., 2016). The activation of NF-kB, PI3K/AKT/mTOR, and Wnt/β-Catenin signaling pathways seems to play a key role in these processes (Rigoglou et al., 2013; Zhou et al., 2017; Sun et al., 2020).

Concomitantly, the upregulation of other factors, such as inducible nitric oxide synthase, nitric oxide (NO), cyclooxygenase-2, prostaglandin E2 (PGE2), A Disintegrin And Metalloproteinase with Thrombospondin motif (ADAMTS)-5, ADAMTS-4, VEGF, TGF-β and Nerve Growth factor (NGF) exert their effect influencing the OA progression (Chow et al., 2020).

Finally, microRNA can be involved in OA processes by activating different signaling pathways, and thus promoting inflammatory factor release (Wu et al., 2019).

CURRENT AND FUTURE PHARMACEUTICAL THERAPY FOR OA

Current pharmacological treatments for OA are focused on relieving symptoms and improving functional status. Paracetamol and nonsteroidal anti-inflammatory drugs (NSAIDs) are the first line medication choices for pain management, but their long-term use is associated with side effects.

Other common options include intra-articular corticosteroid or hyaluronic acid (HA) injections. Both treatments are effective at reducing pain in OA patients. However, intra-articular corticosteroid injections have shown a short duration of action, resulting in the need for repeated administration, which can lead to local and systemic side effects (Raynauld et al., 2003). The durability of pain reduction has been demonstrated longer after intra-articular HA injection when compared to corticosteroids (Colen et al., 2010). Nevertheless, due to the heterogeneity of approach, further studies should be conducted to confirm this hypothesis.

Therefore, new treatments are required to prevent OA structural changes and progression. Molecules involved in the OA pathophysiological processes, especially in immunological events, could be an interesting candidate as therapeutic target. In this context, several drugs have demonstrated disease-modifying OA effects in preclinical and clinical studies (**Figure 1**).

Cytokine inhibitors represent a putative class of these agents. As IL-1β is thought to play a key role in OA development, and intra-articular injection of IL-1 receptor antagonist (IL1-Ra) has demonstrated to improve KOOS (Knee Injury and Osteoarthritis Outcome Score) in patients with anterior cruciate ligament (ACL) tear (Krauss et al., 2012), particular attention has focused on this cytokine. However, a randomized, controlled study evaluating the clinical response, safety, and tolerability of a single intra-articular injection of IL-1Ra in patients with knee OA, revealed no improvements in symptoms when compared with placebo (Chevalier et al., 2009). Results from a phase I trial investigating the adenovirus-mediated IL-1Ra gene transfer in knee OA are pending (Latourte et al., 2020).

TNF inhibition has also been investigated using IgG monoclonal antibodies (infliximab or adalimumab) or circulating receptor fusion protein (etanercept). Treatment with anti-TNF-α blockers has demonstrated a decrease in disease progression but not in symptoms in patients with hand OA (Verbruggen et al., 2012; Kloppenburg et al., 2018; Loef et al., 2018).

Other ongoing studies are evaluating inhibition of pro-inflammatory cytokines (IL-6, CCL17 and IL-36), or intra-articular effects of anti-inflammatory cytokines (IFN-β and IL-10) (Latourte et al., 2020).

Intra-articular release of TGF-β1 by retrovirally transduced human chondrocytes has also demonstrated good results, with improvement in cartilage damage and symptoms in patients with knee OA (Ha et al., 2012; Guermazi et al., 2017; Kim et al., 2018; Lee et al., 2019).

Matrix-degrading enzyme inhibition may be another attractive approach to attenuate cartilage damage, even though musculoskeletal toxicity has been observed after PG-116800 administration to patients with knee OA (Krzeski et al., 2007). Promising results may be obtained through aggrecanase inhibition. Indeed, ADAMTS-5 small molecules inhibitors or neutralizing antibodies have shown protective effects on cartilage and safety profile *in vivo* and in clinical studies (Malfait et al., 2019).

Similar effects were observed after intra-articular treatment with UBX0101, a senolytic drug capable of removing senescent cells accumulated in the joint (Jeon et al., 2017). In addition, less cartilage loss accompanied by bone remodeling reduction was found after administration of MIV-711, a cathepsin K inhibitor (Lindstrom et al., 2018; Conaghan et al., 2019). Analogous could be confirmed for bisphosphonates in early OA (Lane, 2018).

A chondroprotective activity, with signs of cartilage repair after injury has been also reported after intra-articular injection of adipose-derived stem cells in experimental OA (ter Huurne et al., 2012), but results obtained from clinical trials were not convincing (Pers et al., 2016; Emadedin et al., 2018; Freitag et al., 2019; Kim et al., 2019; Lee et al., 2019). Evaluations on emerging drugs promoting chondrogenesis, such as kartogenin and sprifermin, are ongoing (Eckstein et al., 2020; Johnson et al., 2020).

Studies on new therapies targeting signaling have identified a small-molecule Wnt pathway inhibitor, lorecivivint, as a potential disease-modifying OA drug (DMOAD). Administration of this agent facilitated cartilage regeneration in a rodent acute OA model (Deshmukh et al., 2018), and improved pain and function, with good safety and tolerability, in subjects with unilateral symptomatic knee OA (Yazici et al., 2017; Yazici et al., 2020).

Other interesting strategies to reduce pain have been observed using anti-NGF antibodies, such as tanezumab and fasinumab, even though increased risk of rapidly progressive OA was observed after patient treatment (Lane et al., 2010; Hochberg et al., 2016; Dakin et al., 2019; Berenbaum et al., 2020). The use of high-affinity NGF receptor (TrkA) inhibitors could be a viable alternative to avoid side effects (Krupka et al., 2019). New treatments targeting pain include inhibition of vascular adhesion protein-1 (VAP-1), an amine oxidase that increases in OA cartilage. The results of a Phase II clinical trial on a VAP-1 antagonist have not yet been published (Vakal et al., 2020).

Finally, platelet-rich plasma has been recently considered as innate immune response modulator, even though administration protocols and OA phenotypes target have to be refined (Andia et al., 2021).

Despite a number of potential DMOAD molecules have been identified, currently there are no approved drugs, and further studies are needed in this area.

BALNEOTHERAPY ON OA

Besides pharmacological treatment, non-pharmacological interventions play a significant role in the OA prevention and treatment. Balneotherapy is one of the most common non-pharmacological approach for musculoskeletal complaints and rheumatic diseases that has demonstrated benefit for disease symptoms, and has been recently recommended by OARSI guidelines as a strategy for patients with multi-joint OA and comorbidities (Masiero, 2008; McAlindon et al., 2014; Cozzi et al., 2018; Masiero et al., 2018). Although the biological mechanisms underlying balneotherapy are not completely understood, it has been reported that it exerts some beneficial effects on the immune system due likely to its chemical, thermal and mechanical properties (Fioravanti et al., 2011; Tenti et al., 2015) (**Figure 1**).

In vitro researches have highlighted the immunomodulatory properties of mineral waters using animal or human OA cell

cultures, or experimental models able to reproduce OA conditions in articular tissues. Treatment with H2S donors have showed anti-inflammatory and anti-oxidant activities limiting the MAPK/ERK and NF-kB pathway activation and reducing the production of several factors implicated in OA, including IL-6, IL-8, NO, PGE2 and MMP-13 (Kloesch et al., 2011; Burguera et al., 2014; Cheleschi et al., 2020). However, the applicability of cell culture models in this context remains a subject of debate since, under non-experimental conditions, the joint cells are never in direct contact with thermal water. The use of animal models may be more appropriate to identify the mechanisms involved, but so far only four studies have been conducted. In experimental OA murine models, balneotherapy decreased the levels of pro-inflammatory mediators in articular tissues, and systemic IL-1β and NO (Caraglia et al., 2005; Tékus et al., 2018; Kim et al., 2020; Vaamonde-García et al., 2020).

Also the number of studies in patients is limited: currently only three active projects are listed in clinical trial registry (ClinicalTrials.gov, 2020). However these confirm preclinical evaluations. Indeed, decreased serum levels of pro-inflammatory mediators (PGE2, leukotriene B4, IL-1β, TNF-α, IL-8, IL-6, and eHsp72) and expression of microRNA related to cartilage degradation were observed after mud pack treatments in OA patients (Bellometti et al., 1998; Giannitti et al., 2017; Ortega et al., 2017; Maccarone et al., 2020). Interestingly, balneotherapy increased circulating cortisol concentration that enhanced monocyte chemotaxis to damaged tissue and their switch to an anti-inflammatory phenotype (Galvez et al., 2020).

Of note, the association of mud-bath and glucosamine sulfate or ultrasound therapy has indicated improvement in pain, function and life quality in OA patients; thus suggesting combined treatment as an important therapeutic approach (Peluso et al., 2016; Király et al., 2021).

CONCLUSION

On these bases, we believe that therapeutic intervention to modulate immunological events could be an innovative strategy to contain OA progression, and balneotherapy may represent an interesting candidate to support pharmacological therapy. Strategies to improve research in this field need yet to be further refined and implemented. In this context, to identify the molecules and mechanisms associated with this pathological condition and spa treatment, alone or combined with other therapies, with *in vivo* and clinical studies, is crucial to validate the effectiveness and importance of pharmacological and non-pharmacological approaches.

AUTHOR CONTRIBUTIONS

AS, LT, MM, and SM wrote and revised this manuscript.

REFERENCES

Andia, I., Atilano, L., and Maffulli, N. (2021). Moving toward Targeting the Right Phenotype with the Right Platelet-Rich Plasma (PRP) Formulation for Knee Osteoarthritis. *Ther. Adv. Musculoskelet. Dis.* 13, 1–9. doi:10.1177/1759720X211004336

Bellometti, S., and Galzigna, L. (1998). Serum Levels of a Prostaglandin and a Leukotriene after thermal Mud Pack Therapy. *J. Investig. Med.* 46, 140–145.

Berenbaum, F., Blanco, F. J., Guermazi, A., Miki, K., Yamabe, T., Viktrup, L., et al. (2020). Subcutaneous Tanezumab for Osteoarthritis of the Hip or Knee: Efficacy and Safety Results from a 24-week Randomised Phase III Study with a 24-week Follow-Up Period. *Ann. Rheum. Dis.* 79, 800–810. doi:10.1136/annrheumdis-2019-216296

Böhm, M., Apel, M., Lowin, T., Lorenz, J., Jenei-Lanzl, Z., Capellino, S., et al. (2016). α-MSH Modulates Cell Adhesion and Inflammatory Responses of Synovial Fibroblasts from Osteoarthritis Patients. *Biochem. Pharmacol.* 116, 89–99. doi:10.1016/j.bcp.2016.07.003

Burguera, E. F., Vela-Anero, A., Magalhães, J., Meijide-Faílde, R., and Blanco, F. J. (2014). Effect of Hydrogen Sulfide Sources on Inflammation and Catabolic Markers on Interleukin 1β-Stimulated Human Articular Chondrocytes. *Osteoarthritis Cartilage* 22, 1026–1035. doi:10.1016/j.joca.2014.04.031

Caraglia, M., Beninati, S., Giuberti, G., D'Alessandro, A. M., Lentini, A., Abbruzzese, A., et al. (2005). Alternative Therapy of Earth Elements Increases the Chondroprotective Effects of Chondroitin Sulfate in Mice. *Exp. Mol. Med.* 37, 476–481. doi:10.1038/emm.2005.58

Cheleschi, S., Gallo, I., and Tenti, S. (2020). A Comprehensive Analysis to Understand the Mechanism of Action of Balneotherapy: Why, How, and where They Can Be Used? Evidence from *In Vitro* Studies Performed on Human and Animal Samples. *Int. J. Biometeorol.* 64, 1247–1261. doi:10.1007/s00484-020-01890-4

Chevalier, X., Goupille, P., Beaulieu, A. D., Burch, F. X., Bensen, W. G., Conrozier, T., et al. (2009). Intraarticular Injection of Anakinra in Osteoarthritis of the Knee: A Multicenter, Randomized, Double-Blind, Placebo-Controlled Study. *Arthritis Rheum.* 61, 344–352. doi:10.1002/art.24096

Chow, Y. Y., and Chin, K. Y. (2020). The Role of Inflammation in the Pathogenesis of Osteoarthritis. *Mediators Inflamm.* 2020, 8293921. doi:10.1155/2020/8293921

ClinicalTrials.gov. (2020). *Balneotherapy & Osteoarthritis*. Available at: https://clinicaltrials.gov/ct2/results?cond=Osteoarthritis&term=balneotherapy [Accessed April 29, 2021].

Colen, S., van den Bekerom, M. P. J., Bellemans, J., and Mulier, M. (2010). Comparison of Intra-articular Injections of Hyaluronic Acid and Corticosteroid in the Treatment of Osteoarthritis of the Hip in Comparison with Intra-articular Injections of Bupivacaine. Design of a Prospective, Randomized, Controlled Study with Blinding of the Patients and Outcome Assessors. *BMC Musculoskelet. Disord.* 11, 264. doi:10.1186/1471-2474-11-264

Conaghan, P., Bowes, M. A., Kingsbury, S. R., Brett, A., Guillard, G., Rizoska, B., et al. (2019). Disease-Modifying Effects of a Novel Cathepsin K Inhibitor in Osteoarthritis: A Randomized Controlled Trial. *Ann. Intern. Med.* 172, 86–95. doi:10.7326/M19-0675

Cozzi, F., Ciprian, L., Carrara, M., Galozzi, P., Zanatta, E., Scanu, A., et al. (2018). Balneotherapy in Chronic Inflammatory Rheumatic Diseases-A Narrative Review. *Int. J. Biometeorol.* 62, 2065–2071. doi:10.1007/s00484-018-1618-z

Dakin, P., DiMartino, S. J., Gao, H., Maloney, J., Kivitz, A. J., Schnitzer, T. J., et al. (2019). The Efficacy, Tolerability, and Joint Safety of Fasinumab in Osteoarthritis Pain: A Phase IIb/III Double-Blind, Placebo-Controlled, Randomized Clinical Trial. *Arthritis Rheumatol.* 71, 1824–1834. doi:10.1002/art.41012

Deligne, C., Casulli, S., Pigenet, A., Bougault, C., Campillo-Gimenez, L., Nourissat, G., et al. (2015). Differential Expression of Interleukin-17 and Interleukin-22 in Inflamed and Non-inflamed Synovium from Osteoarthritis Patients. *Osteoarthritis Cartilage* 23, 1843–1852. doi:10.1016/j.joca.2014.12.007

Deshmukh, V., Hu, H., Barroga, C., Bossard, C., Kc, S., Dellamary, L., et al. (2018). A Small-Molecule Inhibitor of the Wnt Pathway (SM04690) as a Potential Disease Modifying Agent for the Treatment of Osteoarthritis of the Knee. *Osteoarthritis Cartilage* 26, 18–27. doi:10.1016/j.joca.2017.08.015

Eckstein, F., Kraines, J. L., Aydemir, A., Wirth, W., Maschek, S., and Hochberg, M. C. (2020). Intra-articular Sprifermin Reduces Cartilage Loss in Addition to Increasing Cartilage Gain Independent of Location in the Femorotibial Joint: post-hoc Analysis of a Randomised, Placebo-Controlled Phase II Clinical Trial. *Ann. Rheum. Dis.* 79, 525–528. doi:10.1136/annrheumdis-2019-216453

Emadedin, M., Labibzadeh, N., Liastani, M. G., Karimi, A., Jaroughi, N., Bolurieh, T., et al. (2018). Intra-articular Implantation of Autologous Bone Marrow-Derived Mesenchymal Stromal Cells to Treat Knee Osteoarthritis: a Randomized, Triple-Blind, Placebo-Controlled Phase 1/2 Clinical Trial. *Cytotherapy* 20, 1238–1246. doi:10.1016/j.jcyt.2018.08.005

Fioravanti, A., Cantarini, L., Guidelli, G. M., and Galeazzi, M. (2011). Mechanisms of Action of Spa Therapies in Rheumatic Diseases: what Scientific Evidence Is There? *Rheumatol. Int.* 31, 1–8. doi:10.1007/s00296-010-1628-6

Freitag, J., Bates, D., Wickham, J., Shah, K., Huguenin, L., Tenen, A., et al. (2019). Adipose-derived Mesenchymal Stem Cell Therapy in the Treatment of Knee Osteoarthritis: a Randomized Controlled Trial. *Regen. Med.* 14, 213–230. doi:10.2217/rme-2018-0161

Galvez, I., Torres-Piles, S., Hinchado, M. D., Alvarez-Barrientos, A., Torralbo-Jimenez, P., and Guerrero, J. (2017). Immune-neuroendocrine Dysregulation in Patients with Osteoarthritis: a Revision and a Pilot Study. *Endocr. Metab. Immune Disord. Drug Targets* 17, 78–85. doi:10.2174/1871530317666170320113613

Galvez, I., Torres-Piles, S., and Ortega, E. (2020). Effect of Mud-bath Therapy on the Innate/inflammatory Responses in Elderly Patients with Osteoarthritis: a Discussion of Recent Results and a Pilot Study on the Role of the Innate Function of Monocytes. *Int. J. Biometeorol.* 64, 927–935. doi:10.1007/s00484-019-01748-4

Gálvez, I., Torres-Piles, S., and Ortega-Rincón, E. (2018). Balneotherapy, Immune System, and Stress Response: A Hormetic Strategy? *Int. J. Mol. Sci.* 19, 1687. doi:10.3390/ijms19061687

Giannitti, C., De Palma, A., Pascarelli, N. A., Cheleschi, S., Giordano, N., Galeazzi, M., et al. (2017). Can Balneotherapy Modify microRNA Expression Levels in Osteoarthritis? A Comparative Study in Patients with Knee Osteoarthritis. *Int. J. Biometeorol.* 61, 2153–2158. doi:10.1007/s00484-017-1420-3

Gobezie, R., Kho, A., Krastins, B., Sarracino, D. A., Thornhill, T. S., Chase, M., et al. (2007). High Abundance Synovial Fluid Proteome: Distinct Profiles in Health and Osteoarthritis. *Arthritis Res. Ther.* 9, R36. doi:10.1186/ar2172

Goldring, M. B., and Otero, M. (2011). Inflammation in Osteoarthritis. *Curr. Opin. Rheumatol.* 23 (5), 471–478. doi:10.1097/BOR.0b013e328349c2b1

Guermazi, A., Kalsi, G., Niu, J., Crema, M. D., Copeland, R. O., Orlando, A., et al. (2017). Structural Effects of Intra-articular TGF-B1 in Moderate to Advanced Knee Osteoarthritis: MRI-Based Assessment in a Randomized Controlled Trial. *BMC Musculoskelet. Disord.* 18, 461. doi:10.1186/s12891-017-1830-8

Ha, C., Noh, M. J., Choi, K. B., and Lee, K. H. (2012). Initial Phase I Safety of Retrovirally Transduced Human Chondrocytes Expressing Transforming Growth Factor-Beta-1 in Degenerative Arthritis Patients. *Cytotherapy* 14, 247–256. doi:10.3109/14653249.2011.629645

Hochberg, M. C., Tive, L. A., Abramson, S. B., Vignon, E., Verburg, K. M., West, C. R., et al. (2016). When Is Osteonecrosis Not Osteonecrosis?: Adjudication of Reported Serious Adverse Joint Events in the Tanezumab Clinical Development Program. *Arthritis Rheumatol.* 68, 382–391. doi:10.1002/art.39492

Jeon, O. H., Kim, C., Laberge, R. M., Demaria, M., Rathod, S., Vasserot, A. P., et al. (2017). Local Clearance of Senescent Cells Attenuates the Development of post-traumatic Osteoarthritis and Creates a Pro-regenerative Environment. *Nat. Med.* 23, 775–781. doi:10.1038/nm.4324

Johnson, K. A., Woods, A. K., Joseph, S. B., Chatterjee, A. K., Zhu, S., Wisler, J., et al. (2020). Development of KA34 as a Cartilage Regenerative Therapy for Osteoarthritis. *Osteoarthritis Cartilage* 28 (Suppl. 1), S518. doi:10.1016/j.joca.2020.02.814

Kaneko, S., Satoh, T., Chiba, J., Ju, C., Inoue, K., Kagawaet, J., et al. (2000). Interleukin-6 and Interleukin-8 Levels in Serum and Synovial Fluid of Patients with Osteoarthritis. *Cytokines Cel. Mol. Ther.* 6, 71–79. doi:10.1080/13684730050515796

Ke, X., Jin, G., Yang, Y., Cao, X., Fang, R., Feng, X., et al. (2015). Synovial Fluid HMGB-1 Levels Are Associated with Osteoarthritis Severity. *Clin. Lab.* 61, 809–818. doi:10.7754/clin.lab.2015.141205

Kim, C. G., Lee, D. G., Oh, J., Lee, Y. H., Lee, Y. J., Song, P. H., et al. (2020). Effects of Balneotherapy in Jeju Magma-Seawater on Knee Osteoarthritis Model. *Sci. Rep.* 10, 6620. doi:10.1038/s41598-020-62867-2

Kim, H. A., Cho, M. L., Choi, H. Y., Yoon, C. S., Jhun, J. Y., Oh, H. J., et al. (2006). The Catabolic Pathway Mediated by Toll-like Receptors in Human Osteoarthritic Chondrocytes. *Arthritis Rheum.* 54, 2152–2163. doi:10.1002/art.21951

Kim, M. K., Ha, C. W., In, Y., Cho, S. D., Choi, E. S., Ha, J. K., et al. (2018). A Multicenter, Double-Blind, Phase III Clinical Trial to Evaluate the Efficacy and Safety of a Cell and Gene Therapy in Knee Osteoarthritis Patients. *Hum. Gene Ther. Clin. Dev.* 29, 48–59. doi:10.1089/humc.2017.249

Kim, S. H., Ha, C. W., Park, Y. B., Nam, E., Lee, J. E., and Lee, H. J. (2019). Intra-articular Injection of Mesenchymal Stem Cells for Clinical Outcomes and Cartilage Repair in Osteoarthritis of the Knee: a Meta-Analysis of Randomized Controlled Trials. *Arch. Orthop. Trauma Surg.* 139, 971–980. doi:10.1007/s00402-019-03140-8

Király, M., Gömöri, E., Kiss, R., Nógrádi, N., Nusser, N., Hodosi, K., et al. (2021). Effects of Various Types of Ultrasound Therapy in Hip Osteoarthritis - a Double-Blind, Randomized, Controlled, Follow-Up Study. *Physiother. Theor. Pract.* 10, 1–11. doi:10.1080/09593985.2021.1895386

Kloesch, B., Liszt, M., Broell, J., and Steiner, G. (2011). Dimethyl Sulphoxide and Dimethyl Sulphone Are Potent Inhibitors of IL-6 and IL-8 Expression in the Human Chondrocyte Cell Line C-28/I2. *Life Sci.* 89, 473–478. doi:10.1016/j.lfs.2011.07.015

Kloppenburg, M., Ramonda, R., Bobacz, K., Kwok, W. Y., Elewaut, D., Huizinga, T. W. J., et al. (2018). Etanercept in Patients with Inflammatory Hand Osteoarthritis (EHOA): a Multicentre, Randomised, Double-Blind, Placebo-Controlled Trial. *Ann. Rheum. Dis.* 77, 1757–1764. doi:10.1136/annrheumdis-2018-213202

Kraus, V. B., McDaniel, G., Huebner, J. L., Stabler, T. V., Pieper, C. F., Shipes, S. W., et al. (2016). Direct *In Vivo* Evidence of Activated Macrophages in Human Osteoarthritis. *Osteoarthritis Cartilage* 24, 1613–1621. doi:10.1016/j.joca.2016.04.010

Krauss, V. B., Birmingham, J., Stabler, T. V., Feng, S., Taylor, D. C., Moorman, C. T., et al. (2012). Effects of Intraarticular IL1-Ra for Acute Anterior Cruciate Ligament Knee Injury: a Randomized Controlled Pilot Trial (NCT00332254). *Osteoarthritis Cartilage* 20, 271–278. doi:10.1016/j.joca.2011.12.009

Krupka, E., Jiang, G. L., and Jan, C. (2019). Efficacy and Safety of Intra-articular Injection of Tropomyosin Receptor Kinase A Inhibitor in Painful Knee Osteoarthritis: a Randomized, Double-Blind and Placebo-Controlled Study. *Osteoarthritis Cartilage* 27, 1599–1607. doi:10.1016/j.joca.2019.05.028

Krzeski, P., Buckland-Wright, C., Bálint, G., Cline, G. A., Stoner, K., Lyon, R., et al. (2007). Development of Musculoskeletal Toxicity without clear Benefit after Administration of PG-116800, a Matrix Metalloproteinase Inhibitor, to Patients with Knee Osteoarthritis: a Randomized, 12-month, Double-Blind, Placebo-Controlled Study. *Arthritis Res. Ther.* 9, R109. doi:10.1186/ar2315

Lane, N. E. (2018). Osteoarthritis: Bisphosphonates and OA - Is There a Bone and Joint Connection? *Nat. Rev. Rheumatol.* 14, 185–186. doi:10.1038/nrrheum.2018.18

Lane, N. E., Schnitzer, T. J., Birbara, C. A., Mokhtarani, M., Shelton, D. L., Smith, M. D., et al. (2010). Tanezumab for the Treatment of Pain from Osteoarthritis of the Knee. *N. Engl. J. Med.* 363, 1521–1531. doi:10.1056/NEJMoa0901510

Latourte, A., Kloppenburg, M., and Richette, P. (2020). Emerging Pharmaceutical Therapies for Osteoarthritis. *Nat. Rev. Rheumatol.* 16, 673–688. doi:10.1038/s41584-020-00518-6

Lee, B., Parvizi, J., Bramlet, D., Romness, D. W., Guermazi, A., Noh, M., et al. (2019a). Results of a Phase II Study to Determine the Efficacy and Safety of Genetically Engineered Allogeneic Human Chondrocytes Expressing TGF-B1. *J. Knee Surg.* 33, 167–172. doi:10.1055/s-0038-1676803

Lee, W. S., Kim, H. J., Kim, K. I., Kim, G. B., and Jin, W. (2019b). Intra-Articular Injection of Autologous Adipose Tissue-Derived Mesenchymal Stem Cells for the Treatment of Knee Osteoarthritis: A Phase IIb, Randomized, Placebo-Controlled Clinical Trial. *Stem Cel Transl. Med.* 8, 504–511. doi:10.1002/sctm.18-0122

Li, L., and Jiang, B. E. (2015). Serum and Synovial Fluid Chemokine Ligand 2/monocyte Chemoattractant Protein 1 Concentrations Correlates with Symptomatic Severity in Patients with Knee Osteoarthritis. *Ann. Clin. Biochem.* 52, 276–282. doi:10.1177/0004563214545117

Lindstrom, E., Rizoska, B., Tunblad, K., Edenius, C., Bendele, A. M., Maul, D., et al. (2018). The Selective Cathepsin K Inhibitor MIV-711 Attenuates Joint Pathology in Experimental Animal Models of Osteoarthritis. *J. Transl. Med.* 16, 56. doi:10.1186/s12967-018-1425-7

Liu, B., Zhang, M., Zhao, J., Zheng, M., and Yang, H. (2018). Imbalance of M1/M2 Macrophages Is Linked to Severity Level of Knee Osteoarthritis. *Exp. Ther. Med.* 16, 5009–5014. doi:10.3892/etm.2018.6852

Loef, M., Kroon, F. P. B., Bergstra, S. A., van der Pol, J. A., Lems, W. F., Kerstens, P. J. S. M., et al. (2018). TNF Inhibitor Treatment Is Associated with a Lower Risk of Hand Osteoarthritis Progression in Rheumatoid Arthritis Patients after 10 Years. *Rheumatology* 57, 1917–1924. doi:10.1093/rheumatology/key016

Ma, C., Zhang, Y., Li, Y. Q., Chen, C., Cai, W., and Zeng, Y. L. (2015). The Role of PPARγ in Advanced Glycation End Products-Induced Inflammatory Response in Human Chondrocytes. *PLoS One* 10, e0125776. doi:10.1371/journal.pone.0125776

Maccarone, M. C., Magro, G., Solimene, U., and ScanuMasiero, A. S. (2020). From *In Vitro* Research to Real Life Studies: an Extensive Narrative Review of the Effects of Balneotherapy on Human Immune Response. *Sport Sci. Health* 20, 1–19. doi:10.1007/s11332-021-00778-z

Malfait, A. M., and Tortorella, M. D. (2019). The "elusive DMOAD": Aggrecanase Inhibition from Laboratory to Clinic. *Clin. Exp. Rheumatol.* 37 (Suppl. 120), 130–134.

Masiero, S. (2008). Thermal Rehabilitation and Osteoarticular Diseases of the Elderly. *Aging Clin. Exp. Res.* 20, 189–194. doi:10.1007/BF03324772

Masiero, S., Vittadini, F., Ferroni, C., Bosco, A., Serra, R., Frigo, A. C., et al. (2018). The Role of thermal Balneotherapy in the Treatment of Obese Patient with Knee Osteoarthritis. *Int. J. Biometereol.* 62, 243–252. doi:10.1007/s00484-017-1445-7

McAlindon, T. E., Bannuru, R. R., Sullivan, M. C., Arden, N. K., Berenbaum, F., Bierma-Zeinstra, S. M., et al. (2014). OARSI Guidelines for the Non-surgical Management of Knee Osteoarthritis. *Osteoarthritis Cartilage* 22, 363–388. doi:10.1016/j.joca.2014.01.003

McAllister, M. J., Chemaly, M., Eakin, A. J., Gibson, D. S., and McGilligan, V. E. (2018). NLRP3 as a Potentially Novel Biomarker for the Management of Osteoarthritis. *Osteoarthritis Cartilage* 26, 612–619. doi:10.1016/j.joca.2018.02.901

Nefla, M., Holzinger, D., Berenbaum, F., and Jacques, C. (2016). The Danger from within: Alarmins in Arthritis. *Nat. Rev. Rheumatol.* 12, 669–683. doi:10.1038/nrrheum.2016.162

Ni, S., Miao, K., Zhou, X., Xu, N., Li, C., Zhu, R., et al. (2015). The Involvement of Follistatin-like Protein 1 in Osteoarthritis by Elevating NF-Kb-Mediated Inflammatory Cytokines and Enhancing Fibroblast like Synoviocyte Proliferation. *Arthritis Res. Ther.* 17, 91. doi:10.1186/s13075-015-0605-6

Oliviero, F., Ramonda, R., Scanu, A., Galozzi, P., Favero, M., and Punzi, L. (2020). Levels of Inflammatory Cytokines and Metalloproteinases Are Increased in Knee Synovial Fluid of Patients with Concomitant Erosive Hand Osteoarthritis. *Clin. Exp. Rheumatol.* 38, 800.

Ortega, E., Gálvez, I., Hinchado, M. D., Guerrero, J., Martín-Cordero, L., and Torres-Piles, S. (2017). Anti-inflammatory Effect as a Mechanism of Effectiveness Underlying the Clinical Benefits of Pelotherapy in Osteoarthritis Patients: Regulation of the Altered Inflammatory and Stress Feedback Response. *Int. J. Biometeorol.* 61, 1777–1785. doi:10.1007/s00484-017-1361-x

Peluso, R., Caso, F., Costa, L., Sorbo, D., Carraturo, N., Di Minno, M. N., et al. (2021). Mud-bath Therapy and Oral Glucosamine Sulfate in Patients with Knee Osteoarthritis: a Randomized, Controlled, Crossover Study. *Clin. Exp. Rheumatol.* 34, 618–624.

Pers, Y. M., Rackwitz, L., Ferreira, R., Pullig, O., Delfour, C., Barry, F., et al. (2016). Adipose Mesenchymal Stromal Cell-Based Therapy for Severe Osteoarthritis of the Knee: A Phase I Dose-Escalation Trial. *Stem Cell Transl. Med.* 5, 847–856. doi:10.5966/sctm.2015-0245

Punzi, L., Galozzi, P., Luisetto, R., Favero, M., Ramonda, R., Oliviero, F., et al. (2016). Post-traumatic Arthritis: Overview on Pathogenic Mechanisms and Role of Inflammation. *RMD Open* 2, e000279. doi:10.1136/rmdopen-2016-000279

Raynauld, J. P., Buckland-Wright, C., Ward, R., Choquette, D., Haraoui, B., Martel-Pelletier, J., et al. (2003). Safety and Efficacy of Long-Term Intraarticular Steroid Injections in Osteoarthritis of the Knee: a Randomized, Double-Blind, Placebo-Controlled Trial. *Arthritis Rheum.* 48, 370–377. doi:10.1002/art.10777

Rigoglou, S., and Papavassiliou, A. G. (2013). The NF-Kb Signalling Pathway in Osteoarthritis. *Int. J. Biochem. Cel. Biol.* 45, 2580–2584. doi:10.1016/j.biocel.2013.08.018

Scanzello, C. R., and Loeser, R. F. (2015). Editorial: Inflammatory Activity in Symptomatic Knee Osteoarthritis: Not All Inflammation Is Local. *Arthritis Rheumatol.* 67, 2797–2800. doi:10.1002/art.39304

Scanzello, C. R., Plaas, A., and Crow, M. K. (2008). Innate Immune System Activation in Osteoarthritis: Is Osteoarthritis a Chronic Wound? *Curr. Opin. Rheumatol.* 20, 565–572. doi:10.1097/BOR.0b013e32830aba34

Sohn, D. H., Sokolove, J., Sharpe, O., Erhart, J. C., Chandra, P. E., Lahey, L. J., et al. (2012). Plasma Proteins Present in Osteoarthritic Synovial Fluid Can Stimulate Cytokine Production via Toll-like Receptor 4. *Arthritis Res. Ther.* 14, R7. doi:10.1186/ar3555

Steenvoorden, M. M. C., Huizinga, T. W. J., Verzijl, N., Bank, R. A., Ronday, H. K., Luning, H. A. F., et al. (2006). Activation of Receptor for Advanced Glycation End Products in Osteoarthritis Leads to Increased Stimulation of Chondrocytes and Synoviocytes. *Arthritis Rheum.* 54, 253–263. doi:10.1002/art.21523

Sun, K., Luo, J., Guo, J., Yao, X., Jing, X., and Guo, F. (2020). The PI3K/AKT/mTOR Signaling Pathway in Osteoarthritis: a Narrative Review. *Osteoarthritis Cartilage* 28, 400–409. doi:10.1016/j.joca.2020.02.027

Tékus, V., Borbély, É., Kiss, T., Perkecz, A., Kemény, Á., Horváth, J., et al. (2018). Investigation of Lake Hévíz mineral Water Balneotherapy and Hévíz Mud Treatment in Murine Osteoarthritis and Rheumatoid Arthritis Models. *Evid. Based Complement. Alternat. Med.* 2018, 4816905. doi:10.1155/2018/4816905

Tenti, S., Cheleschi, S., Galeazzi, M., and Fioravanti, A. (2015). Spa Therapy: Can Be a Valid Option for Treating Knee Osteoarthritis? *Int. J. Biometeorol.* 59, 1133–1143. doi:10.1007/s00484-014-0913-6

ter Huurne, M., Schelbergen, R., Blattes, R., Blom, A., de Munter, W., Grevers, L. C., et al. (2012). Antiinflammatory and Chondroprotective Effects of Intraarticular Injection of Adipose-Derived Stem Cells in Experimental Osteoarthritis. *Arthritis Rheum.* 64, 3604–3613. doi:10.1002/art.34626

Vaamonde-García, C., Vela-Anero, Á., Hermida-Gómez, T., Fernández-Burguera, E., Filgueira-Fernández, P., Goyanes, N., et al. (2020). Effect of Balneotherapy in Sulfurous Water on an *In Vivo* Murine Model of Osteoarthritis. *Int. J. Biometeorol.* 64, 307–318. doi:10.1007/s00484-019-01807-w

Vakal, S., Jalkanen, S., Dahlström, K. M., and Salminen, T. A. (2020). Human Copper-Containing Amine Oxidases in Drug Design and Development. *Molecules* 25, 1293. doi:10.3390/molecules25061293

Verbruggen, G., Wittoek, R., Vander Cruyssen, B., and Elewaut, D. (2012). Tumour Necrosis Factor Blockade for the Treatment of Erosive Osteoarthritis of the Interphalangeal finger Joints: a Double Blind, Randomised Trial on Structure Modification. *Ann. Rheum. Dis.* 71, 891–898. doi:10.1136/ard.2011.149849

Woodell-May, J. E., and Sommerfeld, S. D. (2020). Role of Inflammation and the Immune System in the Progression of Osteoarthritis. *J. Orthop. Res.* 38, 253–257. doi:10.1002/jor.24457

Wu, Y., Lu, X., Shen, B., and Zeng, Y. (2019). The Therapeutic Potential and Role of miRNA, lncRNA, and circRNA in Osteoarthritis. *Curr. Gene Ther.* 19, 255–263. doi:10.2174/1566523219666190716092203

Yazici, Y., McAlindon, T. E., Fleischmann, R., Gibofsky, A., Lane, N. E., Kivitz, A. J., et al. (2017). A Novel Wnt Pathway Inhibitor, SM04690, for the Treatment of Moderate to Severe Osteoarthritis of the Knee: Results of a 24-week, Randomized, Controlled, Phase 1 Study. *Osteoarthritis Cartilage* 25, 1598–1606. doi:10.1016/j.joca.2017.07.006

Yazici, Y., McAlindon, T. E., Gibofsky, A., Lane, N. E., Clauw, D., Jones, M., et al. (2020). Lorecivivint, a Novel Intraarticular CDC-like Kinase 2 and Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A Inhibitor and Wnt Pathway Modulator for the Treatment of Knee Osteoarthritis: A Phase II Randomized Trial. *Arthritis Rheumatol.* 72, 1694–1706. doi:10.1002/art.41315

Zhou, Y., Wang, T., Hamilton, J. L., and Chen, D. (2017). Wnt/β-catenin Signaling in Osteoarthritis and in Other Forms of Arthritis. *Curr. Rheumatol. Rep.* 19, 53. doi:10.1007/s11926-017-0679-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 12 July 2021
doi: 10.3389/fphar.2021.687033

Selonsertib Alleviates the Progression of Rat Osteoarthritis: An *in vitro* and *in vivo* Study

Jiyuan Yan[1,2], Yingchi Zhang[3], Gaohong Sheng[2], Bowei Ni[2], Yifan Xiao[4], Shanxi Wang[2], Tianqi Wang[2], Yongzhuang Ma[5], Huaixi Wang[6], Hua Wu[2] and Chang Tu[1]**

[1]Department of Orthopedics, Renmin Hospital of Wuhan University, Wuhan, China, [2]Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [3]Department of Traumatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [4]Department of Pathology and Pathophysiology, Medical College, Jianghan University, Wuhan, China, [5]Department of Orthopedics, Shanxi Bethune Hospital, Taiyuan, China, [6]Department of Spine and Spinal Cord Surgery, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, Zhengzhou, China

Osteoarthritis (OA) is a prevalent degenerative joint disease. Its development is highly associated with inflammatory response and apoptosis in chondrocytes. Selonsertib (Ser), the inhibitor of Apoptosis Signal-regulated kinase-1 (ASK1), has exhibited multiple therapeutic effects in several diseases. However, the exact role of Ser in OA remains unclear. Herein, we investigated the anti-arthritic effects as well as the potential mechanism of Ser on rat OA. Our results showed that Ser could markedly prevent the IL-1β-induced inflammatory reaction, cartilage degradation and cell apoptosis in rat chondrocytes. Meanwhile, the ASK1/P38/JNK and NFκB pathways were involved in the protective roles of Ser. Furthermore, intra-articular injection of Ser could significantly alleviate the surgery induced cartilage damage in rat OA model. In conclusion, our work provided insights into the therapeutic potential of Ser in OA, indicating that Ser might serve as a new avenue in OA treatment.

Keywords: selonsertib, osteoarthritis, apotosis, ASK1, p38, JNK, NFκB

OPEN ACCESS

Edited by:
Sara Cheleschi,
University Hospital of Siena, Italy

Reviewed by:
Pier Maria Fornasari,
Regen Health Solutions, Italy
Ranjan KC,
Children's Hospital of Philadelphia, United States

****Correspondence:***
Hua Wu
wuhua360@aliyun.com
Chang Tu
tuchang@whu.edu.cn

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *28 March 2021*
Accepted: *30 June 2021*
Published: *12 July 2021*

Citation:
Yan J, Zhang Y, Sheng G, Ni B, Xiao Y, Wang S, Wang T, Ma Y, Wang H, Wu H and Tu C (2021) Selonsertib Alleviates the Progression of Rat Osteoarthritis: An in vitro *and* in vivo *Study. Front. Pharmacol. 12:687033. doi: 10.3389/fphar.2021.687033*

INTRODUCTION

Osteoarthritis (OA), featured with joint pain, stiffness and dysfunction, is the most frequently seen degenerative arthritis in the elderly (Glyn-Jones et al., 2015). Over the past decades, the prevalence of OA continues to rise globally. It is the leading cause of chronic pain and impaired physical mobility in aged population (Losina et al., 2013; Saberi Hosnijeh et al., 2019). Until now, most therapeutic strategies targeting OA work on the symptoms relief rather than disease progression reversal. Therefore, joint replacement surgery is the only choice for patients in end-stage OA (Bellamy et al., 2006). This undoubtedly brings huge burden to the society and individuals.

Despite OA is a complex process which pathogenesis involves aging, mechanical injury, inflammatory response and metabolic dysfunction, inflammation plays a critical role in OA course (Hunter and Bierma-Zeinstra, 2019). Reportedly, local inflammatory stimulation and cartilage metabolism disorders contribute to the progression of OA (de Lange-Brokaar et al., 2012; van den Bosch, 2021). Accumulating evidence has showed that excessive pro-inflammatory cytokine, especially interleukin-1β (IL-1β) was detected in the synovial fluid from OA patients (Kellesarian et al., 2016). Previous study confirmed that IL-1β treatment could increase the expression of metallo-proteinases (MMPs) and aggrecanase-2 (ADAMTS5) in chondrocytes,

thus resulting in the cartilage matrix damage (Tu et al., 2019b). Meanwhile, elevated dose of IL-1β could trigger the severer inflammatory reaction due to overproduction of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase2 (COX2) (Tu et al., 2019a). Programmed cell death apoptosis plays important roles in maintaining cartilage homeostasis (Carames et al., 2015). Excessive apoptosis due to local inflammatory microenvironment is a great challenge in OA therapy (Dai et al., 2018). It is proved that IL-1β could markedly induce the mitochondrial dysfunction-related apoptosis in chondrocytes (Wang et al., 2020). Moreover, oral gavage of apoptosis inhibitors administration could effectively alleviate the cartilage damage in mice OA model (Wang et al., 2019). Therefore, regents against IL-1β may provide breakthrough in OA therapy.

Selonsertib (Ser), a selective inhibitor of Apoptosis Signal-regulated kinase-1 (ASK1), has earned its reputation for its anti-inflammatory and anti-apoptotic properties in nonalcoholic steatohepatitis therapy (Loomba et al., 2018). In addition, it is reported that Ser could effectively slow diabetic kidney disease progression (Chertow et al., 2019; Cuarental et al., 2019). However, the detailed role of Ser in OA treatment remains to explore. Previous study reported that Ser could block ASK1/MAPK pathway in hepatic stellate cells and down-regulate ASK1-JNK-DRP1 pathway in macrophage, suggesting the possible mechanism of its action (Yoon et al., 2020; Lou et al., 2021). In this study, we aim to elucidate the therapeutic effects and molecular mechanisms of Ser in OA. We expect to excavate promising strategies in OA treatment.

MATERIALS AND METHODS

Ethics Approval

This study was performed in strict accordance with the Guidelines of Animal Care and Use Committee for Teaching and Research, Tongji Medical College, Huazhong University of Science and Technology. All experimental protocols were confirmed by the Institutional Animal Care and Use Committee, Tongji Medical College, Huazhong University of Science and Technology. All efforts were conducted to minimize animal suffering.

Regents

Selonsertib (purity: 99.4%) was purchased from Selleck Chemicals (United States). DMSO was used to dissolve selonsertib, and equal volume of DMSO was added to all experiment groups. Dulbecco's modified Eagle's medium F12 (DMEM/F12) and Fetal bovine serum (FBS) were procured from Gibco (NY, United States). Recombinant rat IL-1β was acquired from R&D systems (MN, United States). Antibodies specific for P-P65 (#3003), COX2 (#12882), P-ASK1 (#3764), ASK1 (#8662), P-P38 (#4511), P38 (#8690), P-JNK (#9255), JNK (#9258), P-IκBα (#5209) were purchased from Cell Signaling Technology (MA, United States). Antibodies against C-caspase3 (#PB0183) MMP3 (#BM4074), ADAMTS5 (#BA3020), GAPDH (#BM3876), β-ACTIN (#BM0627) were obtained from Boster (Wuhan, China). Antibodies specific for P65 (#10745-1-AP), P53 (#60283-2-Ig), Collagen II (#15943-1-AP), Bcl-XL (#10783-1-AP), BAX (#50599-2-Ig) were purchased from Proteintech Group (Wuhan, China). Antibody against C-caspase 9 (#A0281) was procured from ABclonal (Wuhan, China). Antibody against MMP13 (ab84594) was obtained from Abcam (MA, United States). Antibody against iNOS (#sc-7271) was purchased from Santa Cruz Biotechnology (CA, United States).

Chondrocytes Isolation and Culture

Primary rat chondrocytes were isolated from 1-week old Sprague-Dawley (SD) male rat as described before (Ma et al., 2019). Concisely, cartilage was obtained from the bilateral knee joints and minced into small pieces. Then the pieces were firstly digested with 0.25% trypsin for 30 min at room temperature. Subsequently, 0.25% collagenase II was added to fully digest the cartilage fragments overnight. Afterwards, the cell suspension was collected and centrifuged at a speed of 1,500 rpm. The precipitated cell pallet was taken after centrifugation. Finally, cells were cultured in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin solution. Chondrocytes at passage two or three were used in subsequent experiment.

Cell Viability

Effects of Ser on rat chondrocyte viability were detected with a Cell Counting Kit-8 (CCK-8) assay. Briefly, cells were seeded into 96-well plates at a density of 10,000 cells/well. After adhesion, cells were treated with various doses of Ser (0, 5, 10, 25, 50 μM) alone or in combination with IL-1β (10 ng/ml) for 24 h. Then, cells in each well were treated with 10 μL CCK-8 solutions and incubated for 1 h in dark. After incubation, the absorbance of each well was measured at 450 nm using a microplate reader (Bio-Rad, CA, United States).

Western Blotting

Chodrocytes were seeded onto 6-well plates at a density of 2×10^5 cells/well for further research. Cells were washed with phosphate buffered saline (PBS) twice and lysed in RIPA solutions supplemented with 1% protease/phosphatase inhibitor cocktail. Then, a bicinchoninic acid (BCA) kit was used to determine the protein concentration. Subsequently, each amount of protein samples (30 μg) were separated on 8–12% SDS-PAGE gels, transferred onto PVDF membranes and blocked in 5% BSA for 1 h at 37°C. Afterwards, the membranes were incubated with the corresponding primary antibodies at 4°C overnight. After washing with TBST, the membranes were next incubated with indicated secondary antibodies for 1 h at room temperature. Finally, the bounded protein was visualized using enhanced ECL kit. All bands were photographed using a Bio-Rad scanner system and quantified with the Image-J software.

Measurement of Cell Apoptosis

Cells were seeded onto 6-well plates at a density of 2×10^5 cells/well for futher research. An Annexin V-FITC/PI Apoptosis Detection kit (Beyotime, China) was introduced to measure the chondrocytes apoptosis. According to the manufacturer's

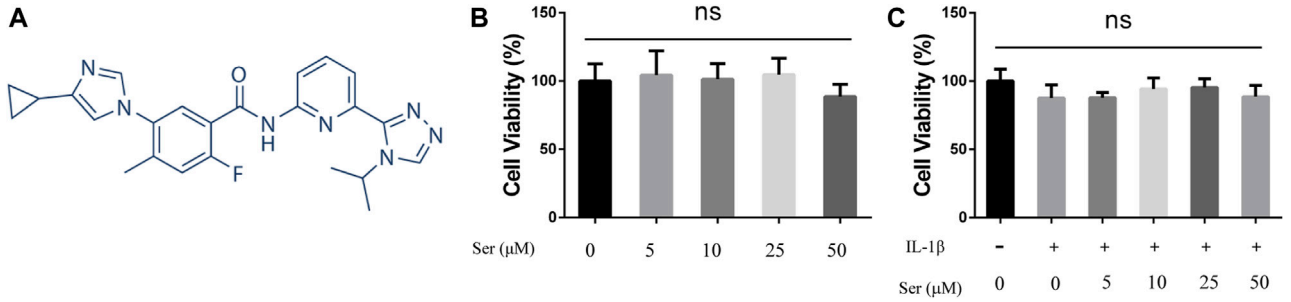


FIGURE 1 | Effects of Ser on Cell Viability. **(A)** Chemical structure of Ser. **(B)** Rat chondrocytes were treated with different concentrations of Ser in the absence or **(C)** presence of IL-1β (10 ng/ml) for 24 h. Cell viability was evaluated using CCK-8 assay. NS indicated no significance.

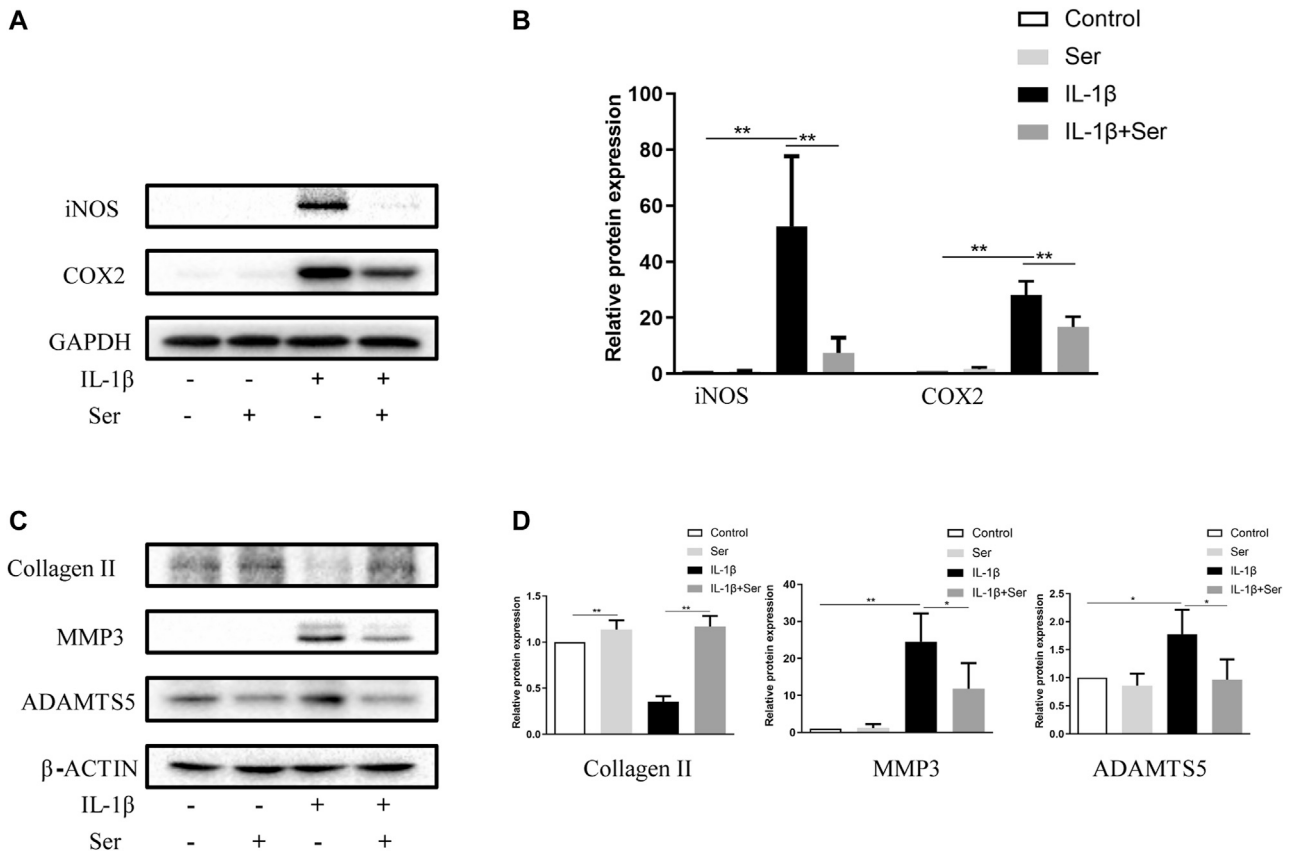


FIGURE 2 | Ser attenuates IL-1β-induced inflammatory responses and cartilage matrix degradation in chondrocytes. Chondrocytes were treated with Ser (25 μM) in the absence or presence of IL-1β (10 ng/ml) for 24 h. **(A)** Western blot and **(B)** quantitative analysis of iNOS and COX2. **(C)** Western blot and **(D)** quantitative analysis of collagen II, MMP3 and ADAMTS5. GAPDH and β-ACTIN were used as the external control respectively. $^{*}p < 0.05$,$^{**}p < 0.01$, $n = 3$.

instruction, rat chondrocytes were collected and washed with cold PBS twice. Then, cells were resuspended in binding buffer and transferred to centrifuge tubes. Subsequently, cells were stained with Annexin V-FITC/PI for 15 min in dark. The apoptotic chondrocytes were analyzed with a FACScan flow cytometer (United States). The sum of Annexin V+/PI+ and Annexin V+/PI- chondrocytes were considered as apoptotic cells.

Immunofluorescence

Chondrocytes were seeded into 24 well plates for P65 immunofluorescence staining at a density of 10,000 cells/well. After reaching 50% confluence, cells were treated with 10 ng/ml IL-1β alone or in combination of 25 μM Ser. Then, cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS three times. Subsequently, cells were

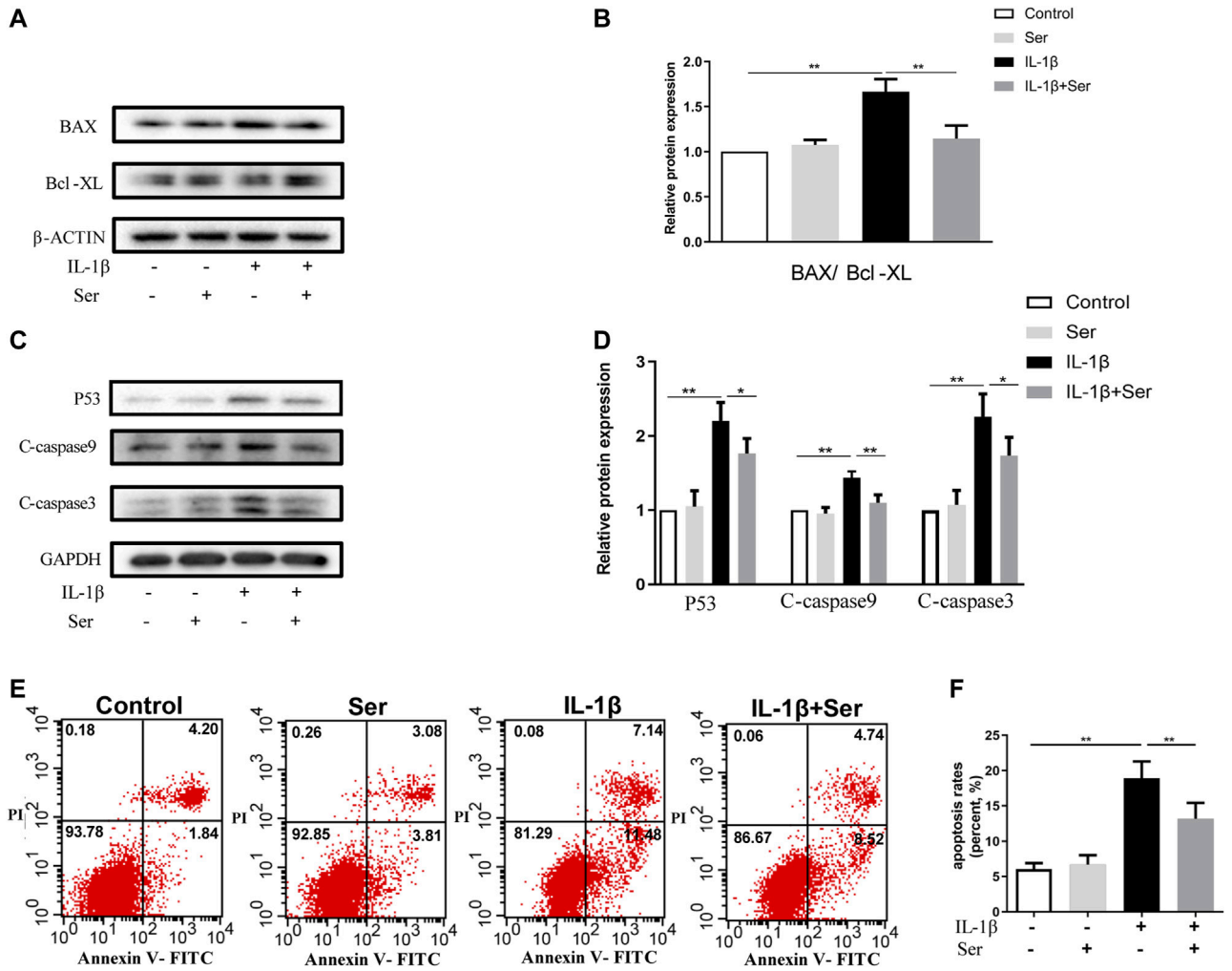


FIGURE 3 | Ser ameliorates IL-1β-induced apoptosis in rat chondrocytes. Cells were treated with Ser (25 μM) alone or in combination with IL-1β (10 ng/ml) for 24 h. **(A)** Western blot of the expression of BAX, Bcl-XL **(B)** quantitative analysis BAX/Bcl-XL. **(C)** Western blot and **(D)** quantitative analysis of P53, C-caspase9 and C-caspase3. GAPDH was used as the loading control. **(E)** Apoptotic cells were detected with Annexin V-FITC/PI staining and flow cytometry. **(F)** Quantitative of the apoptosis rates of each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$.

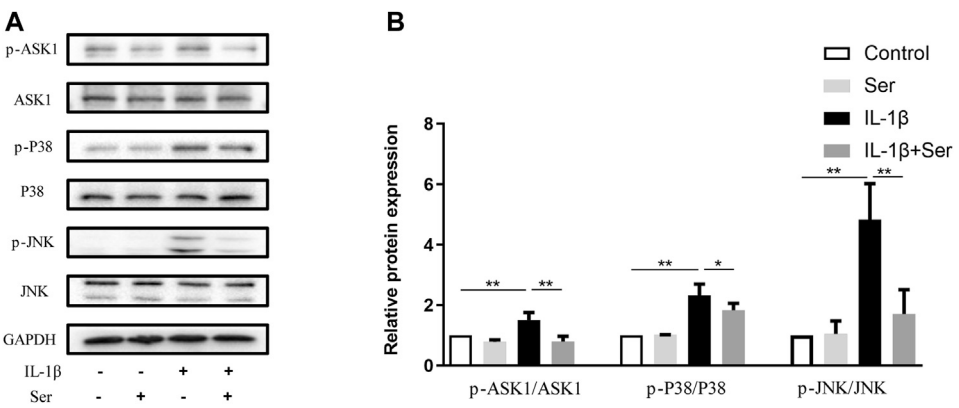


FIGURE 4 | Ser blocks the IL-1β-induced ASK1/P38/JNK activation in chondrocyte. Cells were exposed to L-1β (10 ng/ml) with or without 25 μM Ser for 30 min. **(A)** Western blot and **(B)** quantitative analysis of ASK1/P38/JNK pathway in each group as above. ASK1, P38 and JNK were used as the external control. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$.

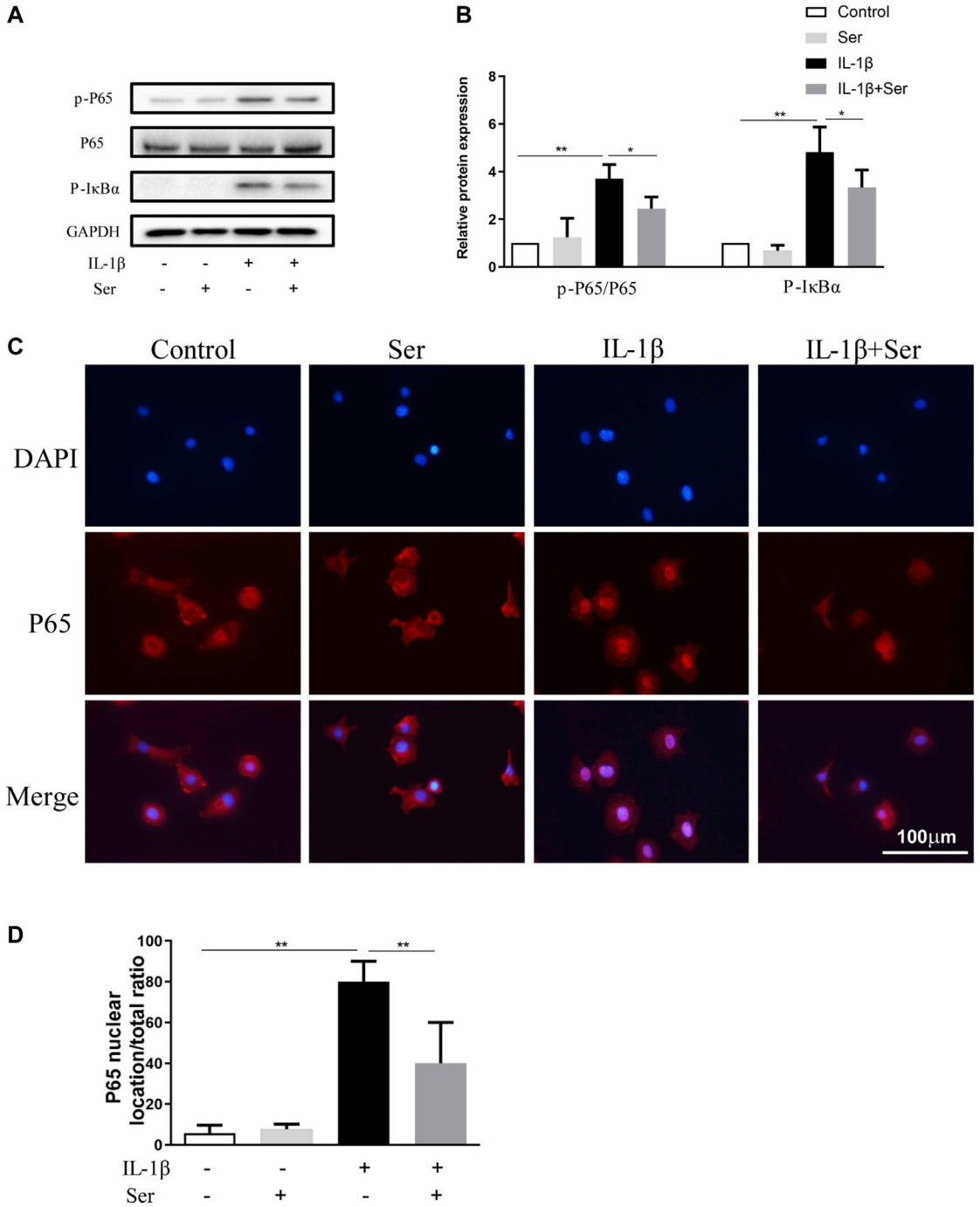


FIGURE 5 | Ser blocks the activation of NFκB pathway in IL-1β-treated chondrocyte. Cells were exposed to L-1β (10 ng/ml) with or without 25 μM Ser for 30 min. **(A)** Western blot and **(B)** quantitative analysis of P-P65 and P-IκBα. P65 and GAPDH were used as the loading control respectively. **(C)** P65 nuclear translocation was detected using immunofluorescence. **(D)** Quantitative analysis of P65 nuclear location ratio of each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$.

permeabilized by 0.2% Triton X-100 for 10 min and blocked by 5% BSA for 1 h. Next, the chondrocytes were incubated with antibody against P65 at 4°C overnight. Afterwards, cells were washed with PBS and incubated with Cy3-conjugated secondary antibody for 1 h at room temperature in dark. Finally, cells were mounted with DAPI for 10 min and observed using a fluoresecence microscope (Evos FL auto, United States).

Animal Osteoarthritis Model

Eighteen male 2-month-old SD rats were supplied from the Laboratory Animal Center of Tongji Hospital. Anterior

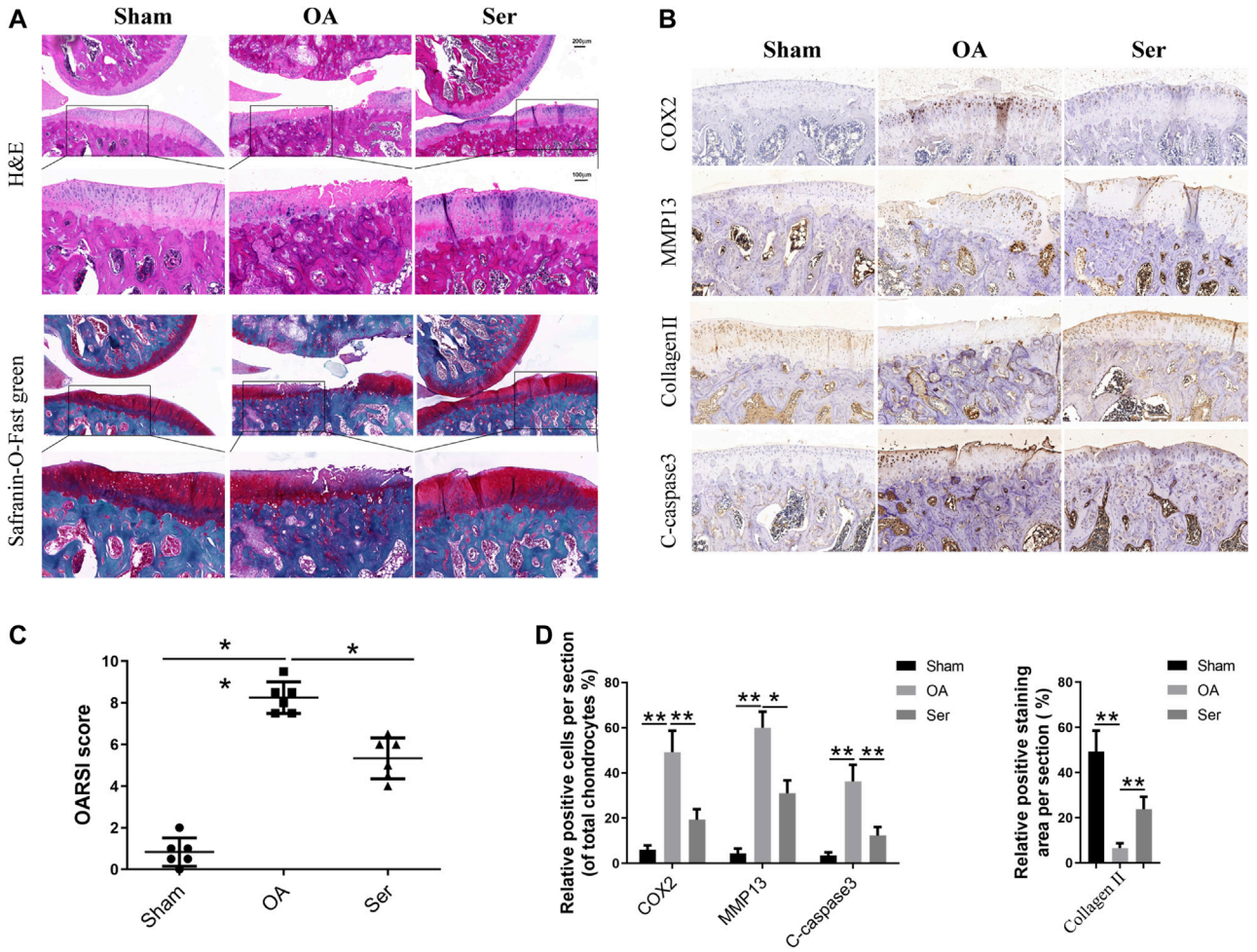


FIGURE 6 | Ser prevents cartilage damage in rat OA model. **(A)** H&E and Safranin-O-Fast green staining of cartilage samples from each group 8 weeks post sugery. **(B)** OARIS scores of each group. **(C)** Immunohistochemical staining and **(D)** quantitative of COX2, MMP13, collagen II and C-caspase3 in the cartilage samples from each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

cruciate ligament transection (ACL-T) and partial medial meniscus removal was performed on the right knee to built OA model as reported previously (Chu et al., 2013). All animals were randomly divided into sham, Ser and OA groups. For sham group (n = 6), rats accepted sham operation without ACL-T or medical meniscus removal. For Ser group (n = 6), rats accepted surgery and intra-articular injection of 25 μM Ser once a week. For OA group (n = 6). Rats accepted surgery and intra-articular injection of equal volume of sterile saline once a week. All animals were sacrificed at 2 months post surgery. The knee joints samples were fixed in 4% paraformaldehyde for next analysis.

Histological Assessment

Fixed joints were decalcidied using 10% EDTA solution for 2 weeks, embedded in paraffin and cut into 5 μm thickness sections coronally. Then sections were stained with Safranin-O-Fast green and H&E. The Osteoarthritis Research Society International (OARST) grading system was introduced to assess the OA changes in a blinded manner. Immunohistological staining was further conduted using antibodies specific for COX2, MMP13, Collagen II, and Cleaved-caspase 3.

Statistical Analysis

Values are expressed as mean ± standard deviation (SD). All of the data analysis were performed using one-way analysis of variance (ANOVA) followed by a Turkey's post hoc test. p-values ˂ 0.05 were considered statistically significant. All *in vitro* experiments were conducted at least in triplicate.

RESULTS

Effects of Ser on Cell Viability

The cytotoxic effects of different doses of Ser with or without IL-1β (10 ng/ml) on cultured rat chondrocytes were determined via CCK-8 assay. As shown in **Figure 1**, Ser (concentrations of 5, 10,

25, 50 μM) alone or Ser (concentrations of 5, 10, 25 μM) combined with 10 ng/ml IL-1β had no toxic effects on cultured cells. Ser at the dose of 25 μM was used in subsequent study.

Ser Attenuates IL-1β-Induced Inflammatory Responses and Cartilage Matrix Degradation in Chondrocytes

To investigate whether Ser could ameliorate inflammatory responses and cartilage matrix degradation induced by IL-1β, western blotting was conducted. As shown in **Figures 2A,B**, administration of 25 μM Ser could notably attenuate the overexpression of INOS and COX2 induced by IL-1β treatment in cultured chondrocytes. Moreover, as shown in **Figures 2C,D**, administration of IL-1β significantly induce the upregulation of MMP13, ADMTS5 and the downregulation of collagen II. However, 25 μM Ser treatment could partly reverse this change.

Ser Ameliorates IL-1β-Induced Apoptosis in Chondrocyte

Apoptosis acts as an important role in maintaining cartilage homeostasis. To observe the effects of Ser on cultured chondrocyte apoptosis induced by IL-1β, we first detected apoptosis related proteins using western blotting. As shown in **Figures 3A,B**, Ser (25 μM) could effectively reverse the elevated ratio of BAX/Bcl-XL induced by IL-1β. Besides, 25 μM Ser could attenuate the IL-1β-induced upregulation of P53, C-caspase9 and C-caspase3 (**Figures 3C,D**). The Annexin V-FITC/PI Apoptosis Detection kit was performed to measure apoptosis levels in cultured chondrocytes. As exhibited in **Figures 3E,F**, administration of Ser caused a notable decrease of apoptotic cells induced by IL-1β.

Effects of Ser on IL-1β-Induced ASK1/P38/JNK Pathway Activation in Chondrocytes

ASK1/P38/JNK pathways play important roles in modulating cell proliferation and inflammation response. In present study, we investigated the exact role of Ser on IL-1β-induced ASK1/P38/JNK pathway activation in cultured chondrocytes. As shown in **Figure 4 A, B**, 25 μM Ser could significantly block the phosphorylation of ASK1/P38/JNK in IL-1β-treated rat chondrocytes.

Effects of Ser on IL-1β-Induced NFκB Pathway Activation in Chondrocytes

NFκB pathway is highly involved in the progression of OA. In present study, western blotting was performed to detect the activation of NFκB P65, IκBα and immunofluorescence staining was employed to evaluate P65 nuclear translocation. As shown in **Figures 5A,B**, IL-1β (10 ng/ml) could dramatically induce the phosphorylation of P65 and IκBα in cultured chondrocytes, while Ser could partly reverse this change. Furthermore, 25 μM Ser could notably inhibit the IL-1β-induced P65 nuclear translocation in cultured rat chondrocytes (**Figures 5C,D**).

Ser Prevents Cartilage Damage in Rat Osteoarthritis Model

To manifest the effects of Ser on the pathogenesis of rat OA *in vivo*, we established rat OA model via anterior cruciate ligament transection and partial medial meniscus removal. As shown in **Figure 6A**, extensive cartilage erosions, proteoglycan loss and chondrocytes disorganization were observed in OA group, while cartilage in Ser group exhibited a smoother and more intact structure. Moreover, a lower OARSI score was seen in Ser group compared to OA group (**Figure 6B**). The immunohistochemistry staining analysis indicated the consistent trend with the vitro study (**Figures 6C,D**). Intra-articular injection of 25 μM Ser could markedly attenuate inflammatory reaction, cartilage matrix degradation and chondrocyte apoptosis *in vivo*.

DISCUSSION

Osteoarthritis (OA) is a degenerative joint disorder with high incidence in the elderly. Due to shortage of effective treatments for revsersing the progression of OA, symptomatic relief such as killing pain is routinely provided for early stage patient (Mandl, 2019). Recently, selonsertib (Ser), a selective inhibitor of Apoptosis Signal-regulated kinase-1 (ASK1), has attracted wide close attention for its anti-apoptosis and anti-inflammation effects in nonalcoholic steatohepatitis (NASH) treatment (Younossi et al., 2018). In present study, we firstly report the therapeutic potential and related molecular mechanisms of Ser in rat OA.

IL-1β is a key pro-inflammatroy cytokine involved in the pathogenesis of OA (Kapoor et al., 2011). Massive IL-1β is secreted in the joint of OA patients (Kellesarian et al., 2016). Large amount of evidence suggested that IL-1β could induce severe inflammatory reaction and cartilage matrix degradation in OA (Tu et al., 2019a; Jenei-Lanzl et al., 2019). Moreover, *in vitro*, IL-1β exhibited the strongest reaction effects in rat chondrocytes at a dose of 10 ng/ml (Wang et al., 2018). Therefore, IL-1β treatment (10 ng/ml) was introduced as a stimulus in our vitro study. Our data revealed that Ser could antagonize IL-1β induced production of inflammatory mediators including INOS and COX2. Besides, the IL-1β induced downregulation of collagen II and upregulation of MMP13 and ADAMTS5 were also blocked by Ser. Summarily, we have proved the anti-inflammatory as well as the anti-degenerative effects of Ser on IL-1β-treated rat chodrocytes.

Apoptosis plays a crucial role in pathogenesis of OA. Elevated level of apoptosis results in the compromising of chondrocyte surval and function (Hwang and Kim, 2015). Previous study confirmed that apoptosis was promoted by caspases, which was regulated by Bcl-2 family (Boise et al., 1995). In the Bcl-2 family, Bcl-XL represents the anti-apoptotic subfamily while BAX represents the pro-apoptotic one (Elmore, 2007). Activated P53 transcriptionally promotes the apoptosis, and acts as a key regulator in this process (Hafner et al., 2019). In this study, we showed that Ser could effectively reduce the

amount of apoptotic chondrocytes induced by IL-1β. Moreover, Ser had been shown to alleviate the IL-1β-induced increase of P53, C-caspase9, C-caspase3 and BAX/Bcl-XL ratio. Taken together, the vitro anti-apoptotic effects of Ser on rat OA was confirmed.

Attempts to prevent P38 or JNK mediated disorders using specific inhibitors of P38 or JNK have been proved unsuccessful. ASK1, the upstream regulator of P38 and JNK, has been proposed as the promising drug target in multiple inflammatory diseases including OA (Ogier et al., 2020). As a selective inhibitor of ASK1, Ser might work via regulating ASK1/P38/JNK pathways in OA. Besides, activation of NFκB is ubiquitous in OA progression. Proinflmmatory cytokines like IL-1β could trigger the phosphorylation of IκBα, thus contributing to the production of inflammatory factors and metallo-proteinases (MMPs) in OA (Baldwin, 2001; Roman-Blas and Jimenez, 2006). Therefore, in our study, we focused on these two pathways. Our work showed Ser could significantly block the activation of ASK1/P38/JNK and NFκB signal pathways induced by IL-1β in rat chondrocytes. The observations partly mimic results of Yoon et al. study, which demonstrated that selonsertib alleviates liver fibrosis via blocking ASK1/MAPK pathway in hepatic stellate cells (Yoon et al., 2020). Collectively, the two pathways were involved in the protective roles of Ser in OA. To illustrate the therapeutic potential of Ser in OA, vitro testing is far from enough. We further built rat OA model and assessed the protective effects of Ser *in vivo*. Data proved intra-articular injection of Ser could effectively alleviate the progression of rat OA.

Some limitations should be pointed out. OA is a complex disease caused by mutilple pathological factors (Hunter and Bierma-Zeinstra, 2019). We mainly focused on the role of Ser on the inflammation during OA in this study, and the effects of Ser on other pathological changes in OA remains further investigation. Besides, it has been reported that Ser failed in the phase III stellar clinical trials (Harrison et al., 2020). Although the pathogenesis of OA and NASH is quite different and the protective effects of Ser on rat OA is promising, it is necessary to assess the safety and efficacy of Ser in lager animals and human OA model. Moreover, in this study, we failed to conduct the serological test and observe the subchondral bone change. Furthermore, the direct target of Ser in OA remains unknown. In view of the achievement we have completed, further exploration is needed.

CONCLUSION

In conlusion, we are the first to report the anti-inflammatroy, anti-degenerative and anti-apoptotic effects of Ser in rat OA. We also revealed the related pathways involved in this process, indicating the underlying mechanisms. Our vivo study proved that Ser may serve as a promising candidate in OA therapy via delaying cartilage damage and degradation. We believe our study will provide insight into the OA therapy as well as supplement to the pharmacology of Ser.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

CT worked on conception and design. *In vivo* experiments were performed by JY. YZ and YX performed the *in vitro* experiments. CT wrote the paper. Other authors contribute to the experimentation. YX and HW supervised and financed the study. All authors have read and approved the final manuscript.

FUNDING

This work was financially sponsored by the Fundamental Research Funds for the Central Universities (Grant No. 2042021kf0088) to CT, The National Natural Science Foundation for Young Scientists of China (Grant No. 82001281) to YX and the National Natural Science Foundation of China (Grant No.51537004) to HW.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.687033/full#supplementary-material

REFERENCES

Baldwin, A. S., Jr. (2001). Series Introduction: The Transcription Factor NF-Kb and Human Disease. *J. Clin. Invest.* 107 (1), 3–6. doi:10.1172/jci11891

Bellamy, N., Campbell, J., Robinson, V., Gee, T., Bourne, R., and Wells, G. (2006). Intraarticular Corticosteroid for Treatment of Osteoarthritis of the Knee. *Cochrane Database Syst. Rev.* 2, CD005328. doi:10.1002/14651858.CD005328.pub2

Boise, L. H., Gottschalk, A. R., Quintáns, J., and Thompson, C. B. (1995). Bcl-2 and Bcl-2-Related Proteins in Apoptosis Regulation. *Curr. Top. Microbiol. Immunol.* 200, 107–121. doi:10.1007/978-3-642-79437-7_8

Caramés, B., Olmer, M., Kiosses, W. B., and Lotz, M. K. (2015). The Relationship of Autophagy Defects to Cartilage Damage during Joint Aging in a Mouse Model. *Arthritis Rheumatol.* 67 (6), 1568–1576. doi:10.1002/art.39073

Chertow, G. M., Pergola, P. E., Chen, F., Kirby, B. J., Sundy, J. S., Patel, U. D., et al. (2019). Effects of Selonsertib in Patients with Diabetic Kidney Disease. *J. Am. Soc. Nephrol.* 30 (10), 1980–1990. doi:10.1681/asn.2018121231

Chu, X., You, H., Yuan, X., Zhao, W., Li, W., and Guo, X. (2013). Protective Effect of Lentivirus-Mediated siRNA Targeting ADAMTS-5 on Cartilage Degradation in a Rat Model of Osteoarthritis. *Int. J. Mol. Med.* 31 (5), 1222–1228. doi:10.3892/ijmm.2013.1318

Cuarental, L., Sucunza-Sáenz, D., Valiño-Rivas, L., Fernandez-Fernandez, B., Sanz, A. B., Ortiz, A., et al. (2019). MAP3K Kinases and Kidney Injury. *Nefrología* 39 (6), 568–580. doi:10.1016/j.nefro.2019.03.004

Dai, M., Sui, B., Xue, Y., Liu, X., and Sun, J. (2018). Cartilage Repair in Degenerative Osteoarthritis Mediated by Squid Type II Collagen via Immunomodulating Activation of M2 Macrophages, Inhibiting Apoptosis and Hypertrophy of Chondrocytes. *Biomaterials* 180, 91–103. doi:10.1016/j.biomaterials.2018.07.011

de Lange-Brokaar, B. J. E., Ioan-Facsinay, A., van Osch, G. J. V. M., Zuurmond, A.-M., Schoones, J., Toes, R. E. M., et al. (2012). Synovial Inflammation, Immune Cells and Their Cytokines in Osteoarthritis: a Review. *Osteoarthritis and Cartilage* 20 (12), 1484–1499. doi:10.1016/j.joca.2012.08.027

Elmore, S. (2007). Apoptosis: a Review of Programmed Cell Death. *Toxicol. Pathol.* 35 (4), 495–516. doi:10.1080/01926230701320337

Glyn-Jones, S., Palmer, A. J. R., Agricola, R., Price, A. J., Vincent, T. L., Weinans, H., et al. (2015). Osteoarthritis. *The Lancet* 386 (9991), 376–387. doi:10.1016/s0140-6736(14)60802-3

Hafner, A., Bulyk, M. L., Jambhekar, A., and Lahav, G. (2019). The Multiple Mechanisms that Regulate P53 Activity and Cell Fate. *Nat. Rev. Mol. Cel Biol.* 20 (4), 199–210. doi:10.1038/s41580-019-0110-x

Harrison, S. A., Wong, V. W.-S., Okanoue, T., Bzowej, N., Vuppalanchi, R., Younes, Z., et al. (2020). Selonsertib for Patients with Bridging Fibrosis or Compensated Cirrhosis Due to NASH: Results from Randomized Phase III STELLAR Trials. *J. Hepatol.* 73 (1), 26–39. doi:10.1016/j.jhep.2020.02.027

Hunter, D. J., and Bierma-Zeinstra, S. (2019). Osteoarthritis. *The Lancet* 393 (10182), 1745–1759. doi:10.1016/s0140-6736(19)30417-9

Hwang, H., and Kim, H. (2015). Chondrocyte Apoptosis in the Pathogenesis of Osteoarthritis. *Int. J. Mol. Sci.* 16 (11), 26035–26054. doi:10.3390/ijms161125943

Jenei-Lanzl, Z., Meurer, A., and Zaucke, F. (2019). Interleukin-1β Signaling in Osteoarthritis - Chondrocytes in Focus. *Cell Signal.* 53, 212–223. doi:10.1016/j.cellsig.2018.10.005

Kapoor, M., Martel-Pelletier, J., Lajeunesse, D., Pelletier, J.-P., and Fahmi, H. (2011). Role of Proinflammatory Cytokines in the Pathophysiology of Osteoarthritis. *Nat. Rev. Rheumatol.* 7 (1), 33–42. doi:10.1038/nrrheum.2010.196

Kellesarian, S. V., Al-Kheraif, A. A., Vohra, F., Ghanem, A., Malmstrom, H., Romanos, G. E., et al. (2016). Cytokine Profile in the Synovial Fluid of Patients with Temporomandibular Joint Disorders: A Systematic Review. *Cytokine* 77, 98–106. doi:10.1016/j.cyto.2015.11.005

Loomba, R., Lawitz, E., Mantry, P. S., Jayakumar, S., Caldwell, S. H., Arnold, H., et al. (2018). The ASK1 Inhibitor Selonsertib in Patients with Nonalcoholic Steatohepatitis: A Randomized, Phase 2 Trial. *Hepatology* 67 (2), 549–559. doi:10.1002/hep.29514

Losina, E., Weinstein, A. M., Reichmann, W. M., Burbine, S. A., Solomon, D. H., Daigle, M. E., et al. (2013). Lifetime Risk and Age at Diagnosis of Symptomatic Knee Osteoarthritis in the US. *Arthritis Care Res.* 65 (5), 703–711. doi:10.1002/acr.21898

Lou, G., Li, A., Cen, Y., Yang, Q., Zhang, T., Qi, J., et al. (2021). Selonsertib, a Potential Drug for Liver Failure Therapy by Rescuing the Mitochondrial Dysfunction of Macrophage via ASK1-JNK-DRP1 Pathway. *Cell Biosci* 11 (1), 9. doi:10.1186/s13578-020-00525-w

Ma, Y., Tu, C., Liu, W., Xiao, Y., and Wu, H. (2019). Isorhapontigenin Suppresses Interleukin-1β-Induced Inflammation and Cartilage Matrix Damage in Rat Chondrocytes. *Inflammation* 42 (6), 2278–2285. doi:10.1007/s10753-019-01092-0

Mandl, L. A. (2019). Osteoarthritis Year in Review 2018: Clinical. *Osteoarthritis and Cartilage.* 27 (3), 359–364. doi:10.1016/j.joca.2018.11.001

Ogier, J. M., Nayagam, B. A., and Lockhart, P. J. (2020). ASK1 Inhibition: a Therapeutic Strategy with Multi-System Benefits. *J. Mol. Med.* 98 (3), 335–348. doi:10.1007/s00109-020-01878-y

Roman-Blas, J. A., and Jimenez, S. A. (2006). NF-κB as a Potential Therapeutic Target in Osteoarthritis and Rheumatoid Arthritis. *Osteoarthritis and Cartilage.* 14 (9), 839–848. doi:10.1016/j.joca.2006.04.008

Saberi Hosnijeh, F., Bierma-Zeinstra, S. M., and Bay-Jensen, A. C. (2019). Osteoarthritis Year in Review 2018: Biomarkers (Biochemical Markers). *Osteoarthritis and Cartilage* 27 (3), 412–423. doi:10.1016/j.joca.2018.12.002

Tu, C., Huang, X., Xiao, Y., Song, M., Ma, Y., Yan, J., et al. (2019a). Schisandrin A Inhibits the IL-1beta-Induced Inflammation and Cartilage Degradation via Suppression of MAPK and NF-kappaB Signal Pathways in Rat Chondrocytes. *Front. Pharmacol.* 10, 41. doi:10.3389/fphar.2019.00041

Tu, C., Ma, Y., Song, M., Yan, J., Xiao, Y., and Wu, H. (2019b). Liquiritigenin Inhibits IL-1β-induced Inflammation and Cartilage Matrix Degradation in Rat Chondrocytes. *Eur. J. Pharmacol.* 858, 172445. doi:10.1016/j.ejphar.2019.172445

van den Bosch, M. H. J. (2021). Osteoarthritis Year in Review 2020: Biology. *Osteoarthritis and Cartilage* 29 (2), 143–150. doi:10.1016/j.joca.2020.10.006

Wang, B.-W., Jiang, Y., Yao, Z.-l., Chen, P.-S., Yu, B., and Wang, S.-N. (2019). Aucubin Protects Chondrocytes against IL-1β-Induced Apoptosis *In Vitro* and Inhibits Osteoarthritis in Mice Model. *Drug Des Devel Ther.* 13, 3529–3538. doi:10.2147/dddt.s210220

Wang, B., Shao, Z., Gu, M., Ni, L., Shi, Y., Yan, Y., et al. (2020). Hydrogen Sulfide Protects against IL-1beta-induced Inflammation and Mitochondrial Dysfunction-Related Apoptosis in Chondrocytes and Ameliorates Osteoarthritis. *J. Cel Physiol.* 236 (6), 4369–4386. doi:10.1002/jcp.30154

Wang, P., Mao, Z., Pan, Q., Lu, R., Huang, X., Shang, X., et al. (2018). Histone Deacetylase-4 and Histone Deacetylase-8 Regulate Interleukin-1β-Induced Cartilage Catabolic Degradation through MAPK/JNK and ERK Pathways. *Int. J. Mol. Med.* 41 (4), 2117–2127. doi:10.3892/ijmm.2018.3410

Yoon, Y.-C., Fang, Z., Lee, J. E., Park, J. H., Ryu, J.-K., Jung, K. H., et al. (2020). Selonsertib Inhibits Liver Fibrosis via Downregulation of ASK1/MAPK Pathway of Hepatic Stellate Cells. *Biomolecules Ther.* 28 (6), 527–536. doi:10.4062/biomolther.2020.016

Younossi, Z. M., Stepanova, M., Lawitz, E., Charlton, M., Loomba, R., Myers, R. P., et al. (2018). Improvement of Hepatic Fibrosis and Patient-Reported Outcomes in Non-alcoholic Steatohepatitis Treated with Selonsertib. *Liver Int.* 38 (10), 1849–1859. doi:10.1111/liv.13706

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

CASE REPORT
published: 23 August 2021
doi: 10.3389/fphar.2021.718060

Case Report: Rehabilitation After Platelet-Rich Growth Factors' Intra-Articular Injections for Knee Osteoarthritis: Two Case Reports of a Home-Based Protocol

Francesco Negrini[1]†, Francesco De Lucia[2]†, Stefano Negrini[1,3], Davide Tornese[4], Francesca Facchini[1], Michele Vecchio[2,5] and Laura de Girolamo[1]*

[1]IRCCS Istituto Ortopedico Galeazzi, Milan, Italy, [2]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy, [3]Department of Biomedical, Surgical, and Dental Sciences, University La Statale, Milan, Italy, [4]Servizio di Fisioterapia, Synlab CAM, Monza, Italy, [5]Rehabilitation Unit, A.O.U. Policlinico S. Marco, Catania, Italy

OPEN ACCESS

Edited by:
Andrea Bernetti,
Sapienza University of Rome, Italy

Reviewed by:
Massimiliano Mangone,
Sapienza University of Rome, Italy
Lucrezia Tognolo,
University Hospital of Padua, Italy

***Correspondence:**
Francesco Negrini
francesco.negrini@gmail.com

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 31 May 2021
Accepted: 05 July 2021
Published: 23 August 2021

Citation:
Negrini F, De Lucia F, Negrini S, Tornese D, Facchini F, Vecchio M and de Girolamo L (2021) Case Report: Rehabilitation After Platelet-Rich Growth Factors' Intra-Articular Injections for Knee Osteoarthritis: Two Case Reports of a Home-Based Protocol. Front. Pharmacol. 12:718060. doi: 10.3389/fphar.2021.718060

Knee osteoarthritis (KOA) is a chronic progressive disease that can cause pain, functional impairment, and ultimately disability. A novel and promising therapeutic approach to KOA is the so-called regenerative medicine, a set of procedures designed to harness tissue regenerative capacity and optimize functional recovery. Increasing evidence points out that platelet-rich plasma (PRP) intra-articular injections can decrease pain and improve functional abilities in KOA patients. In the present case reports, we analyze two patients who were treated with PRP injections coupled with a posttreatment home-based rehabilitation program. The two patients were selected to represent two different populations: patient 1 was an 85-year-old with severe impairment of functional abilities, while patient 2 was a younger (59 years old) and more active patient. The protocol consisted in a series of exercise to be performed at home, during the five days following PRP injection for two consecutive weeks (10 days in total). The exercises were designed to reduce the inflammation after the injection, enhance the proprioceptive control of the treated lower limb, and strengthen hip and knee flexors and extensors, mainly by isometric work. Results were evaluated at two time points: before and 2 months after the first PRP injection. The outcomes considered were as follows: visual analog scale for pain, EuroQol 5 dimensions questionnaire, Tegner Activity Scale for functioning, and Knee Injury and Osteoarthritis Outcome Score (KOOS). Both patients did not report any side effects from the treatment. Improvement in patient 1 was drastic at the two months follow-up as far as pain and functional abilities are concerned. Patient 2's improvement was less evident, probably due to the higher starting point in both pain and functionality. Overall, the developed program seemed safe and was tolerated by the patients analyzed in the study, who performed it with good compliance.

Keywords: knee OA, exercise, physiotherapy, functionality, platelet-rich plasma

INTRODUCTION

Osteoarthritis (OA) is a chronic progressive disease that mainly affects the articular cartilage, causing pain, stiffness, and limitation of articular range of motion (ROM) (Haq et al., 2003). It consists of degeneration of the cartilage, which can also extend to the other tissues forming the joint, including synovial membrane and subchondral bone (Man and Mologhianu, 2014). While OA can affect every human joint, the knee is one of the most involved (knee osteoarthritis, KOA). Alone, it accounts for 83% of the total osteoarthritis burden (Vos et al., 2012). OA is significant in terms of epidemiologic, clinical, and socioeconomic features; it involves about 250 million individuals all over the world (Hunter et al., 2011), and about 14 million people in the United States suffer from symptomatic KOA (Deshpande et al., 2016), with an increasing trend in the last 25 years (Spitaels et al., 2020). KOA especially involves older adults; it occurs in 13% of people after age 60 years, with a high risk of mobility restriction (Hunter et al., 2011).

Multiple therapeutic approaches are currently available for KOA, including pharmacological treatments, rehabilitation (Kolasinski et al., 2020), and surgical solutions such as unicompartmental or total knee replacement (Kennedy et al., 2020). A widely used approach includes intra-articular injections, particularly with hyaluronic acid (HA), because of its relative security profile and effectiveness (Xing et al., 2016). An emerging therapeutic option is the so-called regenerative medicine, that is, a set of procedures designed to harness tissue regenerative capacity and optimize functional recovery after injury or during diseases. Regenerative medicine procedures are applied in several medical and surgical branches, like dentistry, dermatology, spine and orthopedic surgery, and rehabilitation medicine. The goal of regenerative medicine is to restore tissue homeostasis mainly by regulating anti-inflammatory processes that are responsible for most of the patients' symptoms (Ambrosio and Rando, 2018).

In particular, blood-derived products are among the most used therapeutic options in regenerative medicine because of their efficacy together with ease of preparation and use. They consist of fluid phase plasma, with platelets, white cells, and red cells suspended in different concentrations, depending on the specific preparation protocol and indication of use (DeLong et al., 2012). Platelet-rich plasma (PRP) is the most common term used to indicate a wide category of blood-derived products containing hyperphysiological concentration of growth factors, cytokines, and bioactive factors stored in platelet α-granules (Nguyen et al., 2011; Flaumenhaft and Koseoglu, 2017).

The rationale of PRP use in patients with KOA is the high and easily accessible content of these critical growth factors and other signaling molecules, which are involved in both healing processes and immune- and inflammatory modulation, therefore potentially leading to the restoration of joint tissue homeostasis (Cole et al., 2010; Woodell-May et al., 2021). Nevertheless, the term PRP indicates a wide diversity of products, differing in preparation, composition, and application protocols. The main differences that also affect the classification of blood-derived products are platelet concentration (range 1.5–9 times above baseline) and leukocyte concentration, calling the products with low white blood cell (WBC) concentrations leukocyte-poor platelet-rich plasma (LP-PRP) and those with higher WBC concentrations as leukocyte-rich platelet-rich plasma (LR-PRP). Among them, plasma rich in growth factors (PRGF) is an autologous blood-derived product with a standardized composition and dosage. It is characterized by moderated platelet concentration (2–2.5 times compared with peripheral blood) and no WBCs. PRGF must be activated before being injected with calcium chloride to release all the growth factors.

An important classification distinguishes between leukocyte-rich PRP (LR-PRP) and leukocyte-poor PRP (LP-PRP) preparations, depending on a higher or lower concentration of leukocytes (neutrophils particularly) from the baseline (Le et al., 2018). LR-PRP could be able to increase inflammatory response: Dragoo et al. observed a clinical inflammatory pattern (peak in 5th day, resolution after 2 weeks) and an increased release of MMP-3, MMP-13, IL-1b, IL-6, and TNF-α in rabbits treated with LR-PRP injections in patellar tendon (Dragoo et al., 2012). On the other hand, Yan et al. described a lower inflammatory response in terms of clinical symptoms and released inflammatory cytokines in LP-PRP intra-tendon injections in rabbit, associated to a relative better regenerative process (Yan et al., 2017). Although clinical practice is oriented in using poor formulations of leukocytes, more studies in humans are needed for a definitive choice (Lana et al., 2019).

There is increasing evidence on the efficacy of intra-articular injections of blood-derived products in patients with KOA in terms of pain reduction and improvement of function and activities of daily living (Shen et al., 2017; Hohmann et al., 2020). Rehabilitation seems to be among the best option for KOA patients. Fransen et al. described the effects of individually supervised or class exercise programs of muscle strengthening, functional training, and aerobic fitness. They found reduced knee pain and a relative improvement in physical function up to 6 months after the end of treatment (Fransen et al., 2015). Hurley et al. highlighted the positive role of land- or water-based exercise programs on pain (an absolute reduction of 6%), function (reduction of Western Ontario and McMaster Universities Osteoarthritis—WOMAC Index, from 49.9 to 44.3%), and health-related quality of life (improvement of 36-item Short Form—SF-36, by 7.9%) (Hurley et al., 2018). An interesting perspective in KOA treatment is the combination of HA intra-articular injections and rehabilitation. Block and Miller proposed an eight-week exercise program of low-impact aerobics and muscle flexibility exercises, joint mobilization, and physical therapy modalities after HA injection for symptomatic KOA patients. The authors observed increased WOMAC scores in the pain, functional, and stiffness domains in 79, 75, and 76% of patients, respectively (Block and Miller, 2013).

Currently, we are not aware of validated guidelines or trials on the association of a rehabilitation exercise-based program with PRP intra-articular injection for pain, function, and quality of life on human patients with KOA. Therefore, these case reports aim to check the feasibility and safety of a rehabilitation program following PRP intra-articular injection.

SUGGESTED POSTTREATMENT AND REHABILITATION PROGRAM

During the first 48 h after PRP (PRGF) injection, activities of daily living were allowed. In case of pain, 1,000 mg acetaminophen were prescribed for max three times a day, and the local application of ice for 20 min, three times a day. Instructions were given both orally and in written on a pamphlet by the clinician performing the injection (Appendix n.1).

The rehabilitation program was designed to work in synergy with the injection. It aimed to reduce the inflammation after the injection, enhance the proprioceptive control of the treated lower limb, and strengthen hip and knee flexors and extensors, mainly by isometric work. The program included three exercises, which are as follows:

- Isometric co-contractions of quadriceps and hamstrings in the supine position, maintaining knee extended: 5 repetitions lasting 10 s for three series, twice a day.
- Straight leg raises (SLR) in the supine position: 10 repetitions for three series, twice a day.
- "Alphabet," in the supine position, maintaining hip flexed at about 45° and knee extended. In this exercise, the patient has to "draw" with his foot some letters of the alphabet, maintaining the lower limb's described position: ten letters for three series, twice a day.

After an accurate explanation, both patients performed the program during the 5 days following PRP injection for two consecutive weeks (10 days in total). We recommended not practicing sports for the entire duration of the rehabilitation program.

CASE REPORTS

We tested the program on two consecutive female patients recruited at RE.GA.IN (Regenerative Galeazzi Institute) Centre of IRCCS Galeazzi, Milan, Italy, between January and March 2020. The following case report adheres to the CARE (CAse REporting) structure and reporting guidelines (Gagnier et al., 2013).

Case 1

A woman, 85 years old, with a BMI of 28.1, presented a diagnosis of left KOA, with pain onset around six months before. She had no knee traumas in the last year. She was a nonsmoker with hypertension and hypercholesterolemia, in treatment with NSAIDs, pain killers (including acetaminophen and opioid drugs), antithrombotics/antiplatelets, anticholesteremic agents, proton-pump inhibitors, and vitamin D plus calcium supplements. She already underwent a complete KOA rehabilitation program, intra-articular injections of corticosteroids and hyaluronic acid with no results.

The PRGF-Endoret® was prepared using the method described by Sanchez et al. (Sánchez et al., 2012) and administered twice intra-articularly one week apart accordingly to our personal protocol. Before each injection, the patients underwent a venous blood withdrawal of 18 ml, which was put into two extraction tubes containing the 3.8% sodium citrate anticoagulant. The blood was centrifuged at 2000 rpm (580 g) for 8 min in a BTI Biotechnology Institute system centrifuge at room temperature. After the centrifugation, the 2 ml of PRGF between the red series and the "buffy coat," the layer rich in leukocytes, was extracted by mechanical pipetting of each tube. The 2 ml of PRGF were injected into a single tube (4 ml in total). Before a knee intra-articular injection, 400 μL of calcium chloride were added for activation. Every step was conducted respecting strictly sterile conditions.

The outcome measures were as follows: visual analog scale (VAS) for pain (0–10, with 0 no pain and 10 maximum pain perceivable) (Wewers and Lowe, 1990); EuroQol 5 dimensions (EQ-5D) questionnaire, a standardized instrument that studies the quality of life (QoL) (van Reenen et al., 2020) of patients rating 5 dimensions (mobility, self-care, usual activities, pain, and anxiety/depression) with 3 possible levels of problems (1: none, 2: mild to moderate, and 3: severe); the Tegner Activity Scale for functioning (Tegner and Lysholm, 1985; Collins et al., 2011), a scale that ranges from 0, that means sick leave or disability, to 10, participation in national or international competitive sport; and Knee Injury and Osteoarthritis Outcome Score (KOOS) for evaluating function and ability to perform activities of daily living (ADL) (Roos et al., 1998; Collins et al., 2011), a test with 5 subscales (pain, symptoms, activities of daily living, function in sports and play, and knee-related quality of life) scored from 0 to 100, where 0 means bigger issues and 100 no issues. Starting from the raw values of EQ-5D, we calculated the utility value needed to estimate quality-adjusted life-years (QALYs), using the country-specific correction proposed by Scalone et al. (Scalone et al., 2013). The utility value can range from 0.00 (death) to 1.00 (perfect health). We evaluated all the outcomes at two different time points: before (T0) and two months after the first PRP injection (T1).

The pain-related score showed improvement, in terms of VAS, EQ VAS, the pain-related domain of EQ-5D, and the KOOS pain domain (**Table 1**). The Tegner Activity score 1) did not change after the treatment time, whereas KOOS passed from 6.6 to 60.1 points, with bigger changes in the ADL domain (from 5.88 to 60.29) and sport domain (from 0 to 80). The utility values calculated from EQ-5D scores were 0.55 at T0 and 0.77 at T1 (**Table 2**).

Case 2

A woman, 59 years old, with a BMI of 20.3, presented a diagnosed with right KOA, lasting for over a year before PRP injection. She had no knee traumas in the last year. She reported recent smoking cessation, hypertension, and consumption of antithrombotic/antiplatelet drugs and vitamin D plus calcium supplements. She previously received intra-articular injections of hyaluronic acid for the same knee with no results. She received two intra-articular injections of PRGF-Endoret® one week apart, following the same procedure as reported for patient 1. Likewise patient 1, she was evaluated by using VAS, EQ-5D, Tegner Activity Scale, and KOOS-ADL.

TABLE 1 | Results of VAS for pain, EQ-5D, Tegner Scale at T0 (before injection) and T1 (two months after the first PRP injection).

	PRP type	VAS T0	VAS T1	EQ-5D T0	EQ-5D T1	EQ VAS T0	EQ VAS T1	Tegner T0	Tegner T1
Patient 1	PRGF-Endoret	10	8	22,231	22,221	20	70	1	1
Patient 2	PRGF-Endoret	4.2	4.2	11,122	11,122	53	61	4	2

TABLE 2 | Results of the KOOS score and its domains at T0 and T1.

	KOOS Activity Scale					
	TOT	**Pain**	**Symptoms**	**Function**	**Sports**	**Q.O.L**
	T0	**T0**	**T0**	**T0**	**T0**	**T0**
Patient 1	6.55	2.77	17.86	5.88	0	6.25
Patient 2	79.76	86.11	75	92.65	60	43.75
	T1	**T1**	**T1**	**T1**	**T1**	**T1**
Patient 1	60.12	55.55	75	60.29	80	18.75
Patient 2	80.95	88.88	64.28	97.06	70	37.5

All the pain-related scores did not change during the follow-up, with persisting mild-to-severe symptoms (**Table 1**). Concerning outcomes related to function and activity, Tegner showed a decrease, whereas KOOS remained stable. The utility value calculated from EQ-5D was 0.85 at both T0 and T1.

DISCUSSION

Injections of blood-derived products are a widely diffused treatment for KOA, and increasing evidence shows their safety and efficacy (Shen et al., 2017; Hohmann et al., 2020; Nie et al., 2021). However, while they can often improve symptoms, in many cases, pain, functional impairment, and reduced quality of life persist even after the injections. For these reasons, introducing this treatment into an overall rehabilitation approach whose efficacy has already been shown could improve the patient outcomes. In this perspective, blood-derived products would be a facilitator of patients' recovery and find a better role in supporting more active treatments. While rehabilitation, mainly including therapeutic exercises, is considered an optimal ally for the conservative KOA treatment (Fransen et al., 2015), there is no current evidence that intra-articular injections of blood-derived products could enhance a specific exercise program's efficacy. It has not yet been defined which kind of exercises could be proposed safely after injections of blood-derived products. However, it is reasonable that an exercise program specifically tailored for these kinds of treatment could enhance their effect on pain and functional impairment in KOA patients. In our study, we propose a new treatment with the ambition to be adjuvant to PRGF injections and potentially even be synergic to it. We developed the program with two essential concepts in our mind: feasibility and safety. During the COVID-19 pandemic, hospitals should be visited as little as possible, and the home-based rehabilitation program is safer (DE Sire et al., 2021). Furthermore, many of our KOA patients (like patient 2 of our study) are young and active, and often do not have the time to go to the hospital. For these reasons, a 15- to 20-min home-based program performed twice a day seemed the right solution to maintain patients' compliance while ensuring a valid number of repetitions. An article described the results of 4 patients undergoing StroMed and PRP injections and participating in a four-month rehabilitative program, including many outpatient sessions (Gibbs et al., 2015). After the program, patients' pain and functional abilities improved; however, a 4-month outpatient rehabilitative program is not always feasible, especially in the current sanitary and socioeconomic situation, and different strategies should be considered. During the testing of our program, we realized that incorporating concepts of telemedicine and tele-rehabilitation could probably maintain the safety and feasibility of the program while possibly improving compliance and efficacy. Future studies should explore the potential of telemedicine, especially in young, active patients. The exercises prescribed were carefully chosen to be easy and feasible while maintaining efficacy. All the exercises should be executed while lying supine, eliminating the risk of falling. Isometric exercises do not need wide spaces to be performed, and they have been found beneficial in patients suffering from KOA (Anwer and Alghadir, 2014). While exercise #1 focuses mainly on strength, exercises #2 and #3 are designed to work also on proprioception and control of the affected limb, which is crucial for pain control in KOA (Jeong et al., 2019). Another important concept that led to the choice of these exercise was the possibility of a synergic effect with intra-articular injections. Actually, while PRGF injections could lead eventually to a partial restoration of damaged tissues, they cannot act directly on functional capabilities, an aspect of crucial important in osteoarthritis treatment. Improvement of tissues can theoretically enhance the improvement of functional capabilities due to the prescribed exercises. On the other hand, improvement of functional capabilities can potentially enhance tissue regeneration providing more physiological biomechanical stimuli (e.g., correct biomechanical load) (Bokaeian et al., 2021), thus creating a "virtuous circle."

Due to the limited sample size, we can only evaluate the feasibility and safety of the program. However, the treatment of the two patients obtained the results we expected. The patients did not report any side effects due to the exercise program. The two cases studied were carefully selected in order to represent two different populations that can benefit from adding a short exercise program to PRP injections. While both patients suffered from KOA, patient 1 is an 85-year-old with severe

KOA and reduced functional capabilities, as underlined by a baseline total KOOS score of 6.55, with a function sub-score of 5.88. On the other hand, patient 2 is a 59-year-old with better baseline functional performance with a total KOOS score of 60.12 and a function sub-score of 60.29. Indeed, the two patients represent two different populations that can potentially have different responses to therapy as well as different expectations. The difference in the results reflects the difference in baseline condition. Patient 1 dramatically improved at T2 in almost every outcome. It is interesting to notice that KOOS improved significantly. While exercises are often beneficial in KOA also for pain relief, especially in the long run, they are inherently linked to improving strength and proprioception needed to improve function and independence on ADL. Patient 2 showed less improvement. However, outcomes manifested a ceiling effect due to the relatively good clinical condition pretreatment. Furthermore, we prescribed the same set of exercises for both patients. However, the two patients had very different clinical conditions. It is possible that the intensity and degree of difficulty of exercises were enough to warrant a bettering in condition in a less performing patient such as patient 1, but were not enough for patient 2. Indeed, for future studies including young and active patients, functional tests with no ceiling effect, such as 6-minute walking test, 2-minute step-test, and 10-m walking test at maximum speed, should be implemented. Furthermore, exercise intensity should be tailored on functional capabilities of the individual patient.

Our study limitation is represented by the short follow-up. Unfortunately, the patients were treated right before the first pandemic lockdown in our country, and it was impossible to reach them out for longer time points. Certainly, future patients will have to be monitored at least until 12 months from the treatment.

Overall, the developed program seemed safe and was tolerated by the patients analyzed in the study, who performed it with good compliance. The results obtained motivated us to plan another study based on the same program, with the implementation of telemedicine and biomechanical evaluation to enhance compliance, efficacy, and outcomes that do not reach a ceiling effect in high-performance patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FN, FD, DT, MV, and LG contributed to conception and design of the study. FD, FF, and LG recruited patients and collected data. FN and FD wrote the first draft of the manuscript. LG and SN wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

REFERENCES

Ambrosio, F., and Rando, T. A. (2018). The Regenerative Rehabilitation Collection: a Forum for an Emerging Field. *Npj Regen. Med.* 3, 1–2. doi:10.1038/s41536-018-0058-z

Anwer, S., and Alghadir, A. (2014). Effect of Isometric Quadriceps Exercise on Muscle Strength, Pain, and Function in Patients with Knee Osteoarthritis: a Randomized Controlled Study. *J. Phys. Ther. Sci.* 26, 745–748. doi:10.1589/jpts.26.745

Block, J., and Miller, L. (2013). An 8-week Multimodal Treatment Program Improves Symptoms of Knee Osteoarthritis: a Real-World Multicenter Experience. *Por* 4, 39. doi:10.2147/por.s53608

Bokaeian, H. R., Esfandiarpour, F., Zahednejad, S., Mohammadi, H. K., and Farahmand, F. (2021). Effects of an Exercise Therapy Targeting Knee Kinetics on Pain, Function, and Gait Kinetics in Patients with Knee Osteoarthritis: A Randomized Clinical Trial. *Adapt. Phys. Act. Q. APAQ* 38, 377–395. doi:10.1123/apaq.2020-0144

Cole, B. J., Seroyer, S. T., Filardo, G., Bajaj, S., and Fortier, L. A. (2010). Platelet-rich Plasma: Where Are We Now and where Are We Going? *Sports Health* 2, 203–210. doi:10.1177/1941738110366385

Collins, N. J., Misra, D., Felson, D. T., Crossley, K. M., and Roos, E. M. (2011). Measures of Knee Function: International Knee Documentation Committee (IKDC) Subjective Knee Evaluation Form, Knee Injury and Osteoarthritis Outcome Score (KOOS), Knee Injury and Osteoarthritis Outcome Score Physical Function Short Form (KOOS-PS), Knee Ou. *Arthritis Care Res.* 63, S208–S228. doi:10.1002/acr.20632

DE Sire, A., Andrenelli, E., Negrini, F., Patrini, M., Lazzarini, S. G., Ceravolo, M. G., et al. (2021). Rehabilitation and COVID-19: a Rapid Living Systematic Review by Cochrane Rehabilitation Field Updated as of December 31st, 2020 and Synthesis of the Scientific Literature of 2020. *Eur. J. Phys. Rehabil. Med.* 57, 181-188. doi:10.23736/S1973-9087.21.06870-2

DeLong, J. M., Russell, R. P., and Mazzocca, A. D. (2012). Platelet-rich Plasma: the PAW Classification System. *Arthrosc. J. Arthroscopic Relat. Surg.* 28, 998–1009. doi:10.1016/j.arthro.2012.04.148

Deshpande, B. R., Katz, J. N., Solomon, D. H., Yelin, E. H., Hunter, D. J., Messier, S. P., et al. (2016). Number of Persons with Symptomatic Knee Osteoarthritis in the US: Impact of Race and Ethnicity, Age, Sex, and Obesity. *Arthritis Care Res.* 68, 1743–1750. doi:10.1002/acr.22897

Dragoo, J. L., Braun, H. J., Durham, J. L., Ridley, B. A., Odegaard, J. I., Luong, R., et al. (2012). Comparison of the Acute Inflammatory Response of Two Commercial Platelet-Rich Plasma Systems in Healthy Rabbit Tendons. *Am. J. Sports Med.* 40, 1274–1281. doi:10.1177/0363546512442334

Flaumenhaft, R., and Koseoglu, S. (2017). Platelet Contents in *Molecular and Cellular Biology of Platelet Formation: Implications in Health and Disease*. (Springer International Publishing), 133–152. doi:10.1007/978-3-319-39562-3_6

Fransen, M., Mcconnell, S., Harmer, A. R., Van der Esch, M., Simic, M., and Bennell, K. L. (2015). Exercise for Osteoarthritis of the Knee. *Cochrane Database Syst. Rev.* 1, CD004376. doi:10.1002/14651858.CD004376.pub3

Gagnier, J. J., Kienle, G., Altman, D. G., Moher, D., Sox, H., and Riley, D. (2013). The CARE Guidelines: Consensus-Based Clinical Case Reporting Guideline Development. *Glob. Adv. Health Med.* 2, 38–43. doi:10.7453/gahmj.2013.008

Gibbs, N., Diamond, R., Sekyere, E., and Thomas, W. (2015). Management of Knee Osteoarthritis by Combined Stromal Vascular Fraction Cell Therapy, Platelet-Rich Plasma, and Musculoskeletal Exercises: a Case Series. *Jpr* 8, 799–806. doi:10.2147/JPR.S92090

Haq, I., Murphy, E., and Dacre, J. (2003). Osteoarthritis. *Postgrad. Med. J.* 79, 377–383. doi:10.1136/pmj.79.933.377

Hohmann, E., Tetsworth, K., and Glatt, V. (2020). Is Platelet-Rich Plasma Effective for the Treatment of Knee Osteoarthritis? A Systematic Review and Meta-Analysis of Level 1 and 2 Randomized Controlled Trials. *Eur. J. Orthop. Surg. Traumatol.* 30, 955–967. doi:10.1007/s00590-020-02623-4

Hunter, D. J., Neogi, T., and Hochberg, M. C. (2011). Quality of Osteoarthritis Management and the Need for Reform in the US. *Arthritis Care Res.* 63, 31–38. doi:10.1002/acr.20278

Hurley, M., Dickson, K., Hallett, R., Grant, R., Hauari, H., Walsh, N., et al. (2018). Exercise Interventions and Patient Beliefs for People with Hip, Knee or Hip and Knee Osteoarthritis: A Mixed Methods Review. *Cochrane Database Syst. Rev.* 4 (4), CD010842. doi:10.1002/14651858.CD010842.pub2

Jeong, H. S., Lee, S.-C., Jee, H., Song, J. B., Chang, H. S., and Lee, S. Y. (2019). Proprioceptive Training and Outcomes of Patients with Knee Osteoarthritis: A Meta-Analysis of Randomized Controlled Trials. *J. Athl. Train.* 54, 418–428. doi:10.4085/1062-6050-329-17

Kennedy, J. A., Mohammad, H. R., Mellon, S. J., Dodd, C. A. F., and Murray, D. W. (2020). Age Stratified, Matched Comparison of Unicompartmental and Total Knee Replacement. *The Knee* 27, 1332–1342. doi:10.1016/j.knee.2020.06.004

Kolasinski, S. L., Neogi, T., Hochberg, M. C., Oatis, C., Guyatt, G., Block, J., et al. (2020). 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis Care Res.* 72, 149–162. doi:10.1002/acr.24131

Lana, J. F., Huber, S. C., Purita, J., Tambeli, C. H., Santos, G. S., Paulus, C., et al. (2019). Leukocyte-rich PRP versus Leukocyte-Poor PRP - the Role of Monocyte/macrophage Function in the Healing cascade. *J. Clin. Orthopaedics Trauma* 10, S7–S12. doi:10.1016/j.jcot.2019.05.008

Le, A. D. K., Enweze, L., DeBaun, M. R., and Dragoo, J. L. (2018). Current Clinical Recommendations for Use of Platelet-Rich Plasma. *Curr. Rev. Musculoskelet. Med.* 11, 624–634. doi:10.1007/s12178-018-9527-7

Man, G. S., and Mologhianu, G. (2014). Osteoarthritis Pathogenesis - a Complex Process that Involves the Entire Joint. *J. Med. Life* 7, 37–41.

Nguyen, R. T., Borg-Stein, J., and McInnis, K. (2011). Applications of Platelet-Rich Plasma in Musculoskeletal and Sports Medicine: An Evidence-Based Approach. *PM&R* 3, 226–250. doi:10.1016/j.pmrj.2010.11.007

Nie, L.-Y., Zhao, K., Ruan, J., and Xue, J. (2021). Effectiveness of Platelet-Rich Plasma in the Treatment of Knee Osteoarthritis: A Meta-Analysis of Randomized Controlled Clinical Trials. *Orthopaedic J. Sports Med.* 9, 232596712097328. doi:10.1177/2325967120973284

Roos, E. M., Roos, H. P., Lohmander, L. S., Ekdahl, C., and Beynnon, B. D. (1998). Knee Injury and Osteoarthritis Outcome Score (KOOS)-Development of a Self-Administered Outcome Measure. *J. Orthop. Sports Phys. Ther.* 28, 88–96. doi:10.2519/jospt.1998.28.2.88

Sánchez, M., Fiz, N., Azofra, J., Usabiaga, J., Aduriz Recalde, E., Garcia Gutierrez, A., et al. (2012). A Randomized Clinical Trial Evaluating Plasma Rich in Growth Factors (PRGF-Endoret) versus Hyaluronic Acid in the Short-Term Treatment of Symptomatic Knee Osteoarthritis. *Arthrosc. J. Arthroscopic Relat. Surg.* 28, 1070–1078. doi:10.1016/j.arthro.2012.05.011

Scalone, L., Cortesi, P. A., Ciampichini, R., Belisari, A., D'Angiolella, L. S., Cesana, G., et al. (2013). Italian Population-Based Values of EQ-5D Health States. *Value in Health* 16, 814–822. doi:10.1016/j.jval.2013.04.008

Shen, L., Yuan, T., Chen, S., Xie, X., and Zhang, C. (2017). The Temporal Effect of Platelet-Rich Plasma on Pain and Physical Function in the Treatment of Knee Osteoarthritis: Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J. Orthop. Surg. Res.* 12. doi:10.1186/s13018-017-0521-3

Spitaels, D., Mamouris, P., Vaes, B., Smeets, M., Luyten, F., Hermens, R., et al. (2020). Epidemiology of Knee Osteoarthritis in General Practice: a Registry-Based Study. *BMJ Open* 10, e031734. doi:10.1136/bmjopen-2019-031734

Tegner, Y., and Lysholm, J. (1985). Rating Systems in the Evaluation of Knee Ligament Injuries. *Clin. Orthop. Relat. Res.*, 43–49. doi:10.1097/00003086-198509000-00007

van Reenen, M., Janssen, B., Oppe, M., Kreimeier, S., Greiner, W., Stolk, E., et al. (2020). *EuroQol Research Foundation. EQ-5d-Y User Guide*. Available from: https://euroqol.org/publications/user-guides.

Vos, T., Flaxman, A. D., Naghavi, M., Lozano, R., Michaud, C., Ezzati, M., et al. (2012). Years Lived with Disability (YLDs) for 1160 Sequelae of 289 Diseases and Injuries 1990-2010: A Systematic Analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2163–2196. doi:10.1016/S0140-6736(12)61729-2

Wewers, M. E., and Lowe, N. K. (1990). A Critical Review of Visual Analogue Scales in the Measurement of Clinical Phenomena. *Res. Nurs. Health* 13, 227–236. doi:10.1002/nur.4770130405

Woodell-May, J., Steckbeck, K., and King, W. (2021). Potential Mechanism of Action of Current Point-of-Care Autologous Therapy Treatments for Osteoarthritis of the Knee-A Narrative Review. *Ijms* 22, 2726. doi:10.3390/ijms22052726

Xing, D., Wang, B., Liu, Q., Ke, Y., Xu, Y., Li, Z., et al. (2016). Intra-articular Hyaluronic Acid in Treating Knee Osteoarthritis: A PRISMA-Compliant Systematic Review of Overlapping Meta-Analysis. *Sci. Rep.* 6. doi:10.1038/srep32790

Yan, R., Gu, Y., Ran, J., Hu, Y., Zheng, Z., Zeng, M., et al. (2017). Intratendon Delivery of Leukocyte-Poor Platelet-Rich Plasma Improves Healing Compared with Leukocyte-Rich Platelet-Rich Plasma in a Rabbit Achilles Tendinopathy Model. *Am. J. Sports Med.* 45, 1909–1920. doi:10.1177/0363546517694357

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

SYSTEMATIC REVIEW
published: 17 September 2021
doi: 10.3389/fphar.2021.714974

Melatonin Maintains Anabolic-Catabolic Equilibrium and Regulates Circadian Rhythm During Osteoarthritis Development in Animal Models: A Systematic Review and Meta-analysis

Dewan Md. Sumsuzzman[1,2,3,4], *Jeonghyun Choi*[2,3,4†], *Zeeshan Ahmad Khan*[2,3,4†], *George Kamenos*[1,2,3] *and Yonggeun Hong*[1,2,3,4,5*]

[1]*Department of Rehabilitation Science, Graduate School of Inje University, Gimhae, Korea,* [2]*Biohealth Products Research Center (BPRC), Inje University, Gimhae, Korea,* [3]*Ubiquitous Healthcare and Anti-aging Research Center (u-HARC), Inje University, Gimhae, Korea,* [4]*Department of Physical Therapy, College of Healthcare Medical Science and Engineering, Gimhae, Korea,* [5]*Department of Medicine, Division of Hematology/Oncology, Harvard Medical School and Beth Israel Deaconess Medical Center, Boston, MA United States*

Background: The driving force behind osteoarthritis (OA) pathogenesis is an anabolic-catabolic (a/c) imbalance. Melatonin (MT) is a key player in maintaining a/c stability and mitigates OA pathogenesis, but mechanisms underlying its effects remain poorly understood.

Objectives: We performed a systematic review analyzing the experimental data that support the clinical applicability of MT in the treatment of OA pathogenesis, placing particular emphasis on the regulation of circadian rhythms and a/c balance.

Methods: Major electronic databases and grey literature were used to identify related original articles. Methodological quality of all selected studies was evaluated using the SYRCLE risk of bias tool. Pooled mean differences (MDs)/standardized mean differences (SMDs) with 95% confidence intervals (CIs) were calculated to estimate the effect size.

Results: Eleven trials were included in this systematic review. Compared with the control group, MT significantly decreased the levels of interleukin-1β (IL-1β; SMD = –5.45; 95% CI [–6.78, –4.12]; $p < 0.00001$, and histological grading scale (SMD = –3.46; 95% CI, [–5.24, –1.68]; $p < 0.0001$). MT significantly increased the transforming growth factor-β1 (TGF-β1; SMD = 1.17; 95% CI [0.31, 2.03]; $p < 0.0007$). Furthermore, core circadian clock genes *Per2* and *Cry1* mRNA levels were regulated by MT treatment in OA progression.

Conclusion: MT may maintain a/c balance and regulate circadian rhythms during OA development. MT could be used in as adjunct with other interventions to manage pain and OA severity.

Keywords: melatonin, osteoarthritis, anabolic, catabolic, circadian rhythms, systematic review, meta-analysis

OPEN ACCESS

Edited by:
Raquel Largo,
Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain

Reviewed by:
Nguan Soon TAN,
Nanyang Technological University, Singapore
Jing Cao,
China Agricultural University, China

***Correspondence:**
Yonggeun Hong
yonghong@inje.ac.kr

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *26 May 2021*
Accepted: *01 September 2021*
Published: *17 September 2021*

Citation:
Sumsuzzman DM, Choi J, Khan ZA, Kamenos G and Hong Y (2021) Melatonin Maintains Anabolic-Catabolic Equilibrium and Regulates Circadian Rhythm During Osteoarthritis Development in Animal Models: A Systematic Review and Meta-analysis. Front. Pharmacol. 12:714974. doi: 10.3389/fphar.2021.714974

INTRODUCTION

Osteoarthritis (OA) a commonly diagnosed degenerative joint disease predominantly characterized by progressive degradation of cartilage components, eventually escalating to structural damage and functional failure of the cartilage (Pap and Korb-Pap, 2015; Loeser et al., 2016; Chen et al., 2017). Knee OA (KOA) accounts for about 80% of the burden of OA globally (Global Burden of Disease Study, 2016). In 2020, there were almost 654.1 million individuals (≥40 years) with KOA worldwide (Cui et al., 2020). Aging has always been considered a significant etiological factor for OA (Rahmati et al., 2017), however, the underlying mechanism between age and OA pathogenesis is still not completely understood. Aging contributes to the disruption of anabolic and catabolic cell signaling, leading eventually to osteoarthritic cartilage destruction (Lotz and Loeser, 2012; Loeser, 2017). Understanding the aging process responsible for metabolic dysregulation is critical to identifying and developing effective drugs to treat the pathobiological symptoms of OA. In addition, despite significant efforts over the past several decades to develop KOA therapies, only controversial analgesics (gastrointestinal complications and cardiovascular adverse events) are widely used to treat OA (Pelletier et al., 2016). Hence, targeting a/c balance during aging may provide a novel strategy to prevent/treat cartilage damage in OA.

Imbalance between catabolic and anabolic factors accelerates catabolic activity as a result chondrocytes (major regulators of matrix anabolism-catabolism) completely fail to compensate for the depletion of extracellular matrix (ECM) molecules (Kim et al., 2014; Nummenmaa et al., 2015; Kang et al., 2019). The catabolic phenotype is regulated by several proinflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor α (TNF-α), and IL-6 (Mueller and Tuan, 2011). IL-1β, a well-known marker for OA progression found in synovial fluid and expressed by both synovial tissue and chondrocytes in osteoarthritic joints (Melchiorri et al., 1998; Mueller and Tuan, 2011). IL-1β triggers the matrix-degrading enzymes such as matrix metalloproteinase (MMP)-13 and suppresses the production of ECM proteins such as collagen type II by chondrocytes (Mobasheri et al., 2017; Wang et al., 2019). Likewise, TNF-α synergistically increases MMPs and reduces ECM proteins (Nambi, 2021). On the other hand, the anabolic phenotype is maintained by a number of growth factors, including transforming growth factor-β1 (TGF-β1), bone morphogenetic protein-2 (BMP-2), and fibroblast growth factors (FGF) (Mueller and Tuan, 2011). TGF-β1 involved in synthesis of collagen type II and downregulates MMPs (Li et al., 2016; Hwang et al., 2020), thereby counteracting IL-1β-mediated cartilage matrix degradation. Interestingly, TGF-β1 gradually declines with aging (van der Kraan, 2017), which may be responsible for cartilage damage in aged cartilage. Recent evidence demonstrated that the misalignment of circadian rhythms is associated with a/c imbalance in various animal models (Gossan et al., 2015; Kc et al., 2015; Hossain et al., 2019). Recent work by Kc et al. (Kc et al., 2015) has shown that chronic disruption of circadian rhythms (weekly 12-h phase shift in the light-dark cycle for 22 weeks) induced OA pathology, including upregulation of matrix-degrading enzymes and downregulation of anabolic mediators in the mouse knee joint. Furthermore, the circadian core clock transcription factor *Bmal1* (brain and muscle ARNT-like 1) was downregulated both in human OA and aged mouse cartilage and contributes to disruption in cartilage homeostasis (Dudek et al., 2016). Contrastly, overexpression of *Bmal1* equilibrated the metabolic imbalance of chondrocytes (Yang et al., 2016). Therefore, targeting a/c homeostasis and regulating circadian rhythms by the same candidate drug would be an interesting approach for the treatment of OA.

Melatonin (MT) is a pineal hormone secreted in all vertebrates, including humans, exhibiting anti-oxidant (Sumsuzzman et al., 2020), anti-inflammatory (Ling et al., 2020), and anti-aging (Sumsuzzman et al., 2021a) functions. Endogenous MT levels are inversely proportional to age (Sumsuzzman et al., 2021b) and serum MT declines in OA patients (Oskoii et al., 2020). Additionally, circadian synchronization of MT is dysregulated with advancing age and it closely related to OA etiology (Jahanban-Esfahlan et al., 2018). Several studies showed that MT can protect chondrocyte growth and promote the expression of cartilage-related genes, which may be related to its anti-inflammatory and anti-oxidative stress effects (Pei et al., 2009; Liu et al., 2014). Although MT shows some progress in mitigating OA pathogenesis, many mechanisms underlying its effects remain poorly understood (Hong et al., 2014), and evidence synthesis is needed to justify the specific role of MT in cartilage homeostasis. Furthermore, MT exerts beneficial effects with other interventions, including antiosteoporotic agents, betamethasone, and exercise (Huang et al., 2010b; Hong et al., 2014; Paulino Silva et al., 2021). In OA animal models, however, these combined intervention strategies aimed at a/c homeostasis have not yet been systemically reviewed. Therefore, we conducted a systematic review and meta-analysis aimed at analyzing the experimental data supporting the clinical applicability of MT in the treatment of OA pathogenesis, with particular emphasis on the regulation of circadian rhythms and anabolic-catabolic balance.

MATERIALS AND METHODS

Search Strategy

We performed a comprehensive literature search using major electronic databases, including PubMed, Embase, and China National Knowledge Infrastructure (CNKI) as well as grey literature sources, including Worldcat and Mednar. We identified peer-reviewed studies published up until April 2021 that focused on assessing the impact of exogenous MT in animal models of OA. Furthermore, the reference lists of the included studies and of relevant reviews were examined for additional relevant trials. To obtain animal studies, animal search filters in PubMed and Embase were utilized (Hooijmans et al., 2010; de Vries et al., 2011). The in-depth search strategy performed in PubMed and Embase electronic databases are given in **Supplementary Table S1**. No limits (e.g., on language or publication date) were used.

Inclusion Criteria

The inclusion criteria for the selected studies were as follows: 1) the subjects were laboratory animals of any age, sex, or strain used as OA models; 2) the interventions encompassed any type of MT treatment or MT combined with other interventions that was compared with a placebo control, 3) the control intervention consisted of saline, dimethyl sulfoxide, or another vehicle. In addition, the following study outcomes were required for inclusion in the analysis. 1) Primary outcomes: catabolic (i.e., IL-1β, IL-6, TNF-α, MMP-13, ADAMTS-4; a disintegrin and metalloproteinase with thrombospondin motifs-4). and anabolic factors (i.e., TGF-β1, BMP-2, FGF, SOX-9; transcription factor SOX-9, COL2A1; Collagen type II α1) were chosen as the dependent variables to measure the effects of MT in animal models of OA. 2) Secondary outcomes: the effects of MT on circadian regulation and histological scoring in an animal model of OA.

Exclusion Criteria

The following exclusion criteria were applied for study selection as shown in **Supplementary Table S2**: 1) all clinical case reports and studies that were solely *In vitro*, 2) not original studies (e.g., editorial or literature review), 3) studies using genetically modified animals, and 4) if used interventions other than MT, studies were excluded.

Study Selection

After the removal of duplicates, all unique trials were imported into a Rayyan-a web application to allocate the references randomly (Ouzzani et al., 2016). Then, two of the authors individually screened the titles and abstracts to select relevant studies from the randomly allocated references. Finally, the full-texts of the selected articles were evaluated to identify the trials that fulfilled our eligibility criteria. Any disagreement concerning study selection was settled by consultation with the third author. It should be noted that we did not screen for the presence or absence of specific outcome measures during this phase, because often not all outcome measures were described in the abstract.

Data Extraction

Two authors (DS and ZK) individually extracted the data from each of the included studies. Information related to the authors, publication year, species, age, weight, sample size, animal model, intervention (dose, frequency, and route of administration), and outcome measures were extracted. In studies with multiple arms, only data from the control, MT, MT combined with other intervention groups were considered in this study. If the published outcome data were incomplete, we attempted contacting the authors of the study to obtain the original data. A reminder was sent by email to those who had not responded within 2 weeks. If efforts to achieve the original data failed, the article was eliminated from the meta-analysis. If the data were presented graphically only, GetData Graph Digitizer was employed to extract numerical data from graphs or figures (Sumsuzzman et al., 2021a).

Assessment of Methodological Quality

The risk of bias (RoB) in the included articles was evaluated by two independent reviewers (DS and JC) using the SYRCLE RoB tool (Hooijmans et al., 2014). Based on the Cochrane RoB tool (Higgins et al., 2011), the RoB tool was developed to evaluate the aspects of bias specifically seen in animal intervention studies. The tool contains 10 items related to six types of bias (selection, performance, detection, attrition, reporting, and other bias). The scores "yes", "no", and "unsure" indicate a low, high, and unclear RoB, respectively.

Data Analysis

The experimental and control group data from the included studies were extracted and inputted into the Review Manager Software (ver. 5.3, The Nordic Cochrane Centre, Copenhagen, Denmark). The meta-analysis was executed when a minimum of two studies were analogous and when these studies provided relevant data (Sumsuzzman et al., 2021a). In the effect size analysis, the mean difference (MD) was used when the outcome measure of all studies employed the same scale, whereas the standardized mean difference (SMD) was used when the studies assessed the same outcome but measured it in different ways (Sumsuzzman et al., 2021a). For both strategies, 95% confidence intervals (CIs) were calculated. The Cochrane Q and I^2 tests were utilized to assess heterogeneity among the studies; $p < 0.10$ in the Cochrane Q test and $I^2 > 50\%$ were considered to indicate the presence of heterogeneity among the included studies. The fixed-effects model was used for the meta-analysis when statistical heterogeneity had been found lower ($I^2 = < 50\%$), and the random-effects model was applied when statistical heterogeneity had been found higher ($I^2 = > 50\%$) (DerSimonian and Laird, 1986). Post-hoc sub-group analysis was performed to investigate the sources of heterogeneity based on wider versus narrow confidence intervals. Subgroup analyses were performed only when subgroups contained at least two independent comparisons. When at least three studies were included, a leave-one-out sensitivity analysis was performed to validate our findings and assess potential sources of heterogeneity (Patsopoulos et al., 2008). Publication bias was analyzed when one outcome variable was associated with at least 10 studies in the meta-analysis, since with <10 studies the power of the tests is too low (Sumsuzzman et al., 2020).

RESULTS

Study Search and Selection

A total of 233 trials (Pubmed = 70, Embase = 122, CNKI = 9, and grey literature databases = 32) were retrieved from the electronic database search. After the removal of duplicates, a total of 155 studies remained, 136 of which were excluded because the titles and abstracts did not meet the eligibility criteria. The remaining 19 studies were selected for full-text analysis, and of these, 11 studies fulfilled our eligibility criteria and were selected for review (**Figure 1**).

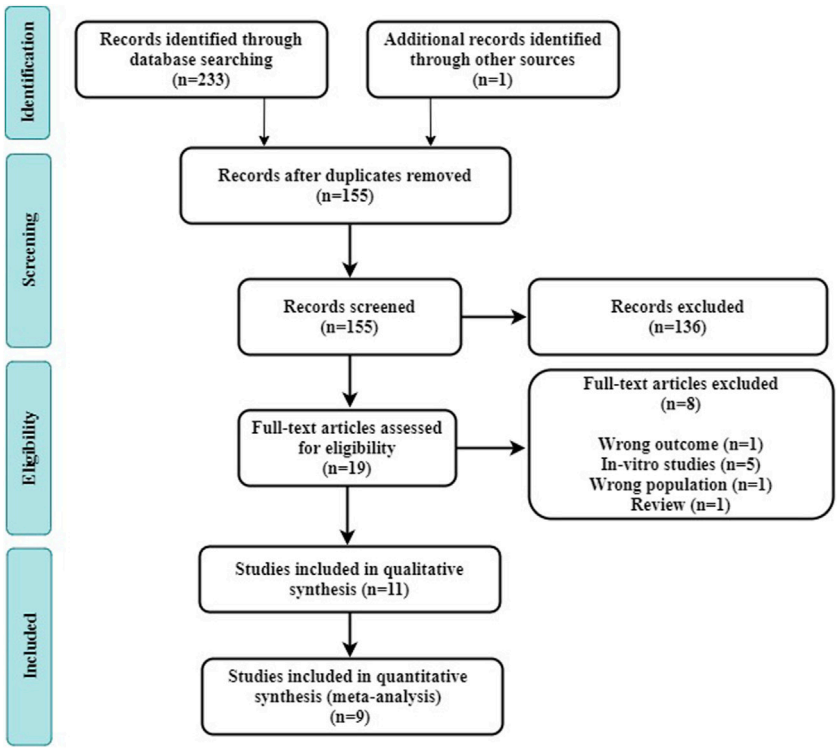


FIGURE 1 | Flow diagram of the systematic review and literature search results of the meta-analysis.

Study Characteristics

The characteristics of all included studies are reported in **Table 1**. Three different animal species were used: rats in nine studies (Huang et al., 2010a, 2010b, 2010c; Hong et al., 2014, 2017; Jiang, 2014; Guo et al., 2017; Savtekin et al., 2018; Paulino Silva et al., 2021), mice in one study (Zhang et al., 2019), and rabbits in one study (Lim et al., 2012). In addition, the OA models were created using different methods: collagenase injection in two studies (Hong et al., 2014, 2017), anterior cruciate ligament transection surgery in two (Guo et al., 2017; Paulino Silva et al., 2021), partial medial meniscectomy surgery in one (Lim et al., 2012), intra-articular injection of zymosan in one (Savtekin et al., 2018), destabilization of the medial meniscus in one (Zhang et al., 2019), and injection of papain solution in four studies (Huang et al., 2010a, 2010b, 2010c; Jiang, 2014). MT was administered *via* three different routes: subcutaneously in three studies (Hong et al., 2014, 2017; Paulino Silva et al., 2021), intraperitoneally in one (Guo et al., 2017), and intra-articularly in seven (Huang et al., 2010a, 2010b, 2010c; Lim et al., 2012; Jiang, 2014; Savtekin et al., 2018; Zhang et al., 2019). The data collection period in all studies ranged from 3 to 7 weeks.

Risk of Bias and Quality of Reporting

The abridged RoB assessment is presented in **Figure 2A**, and the individual RoB scores of each study are presented in **Figure 2B**. In all studies, although the animals were randomly allocated, insufficient details regarding the allocation procedure were reported. Baseline characteristics, including sex, age, and weight, were adequately described. Random housing, blinding of caregivers, investigators, and random outcome assessment were partially described in all studies. However, blinding of the outcome assessment showed a high risk of detection bias in nearly all studies. All animals were included in the analyses of all studies. Of the 11 studies, five did not use selective outcome reporting providing sufficient details of the pre-defined outcomes (Lim et al., 2012; Hong et al., 2014, 2017; Guo et al., 2017; Paulino Silva et al., 2021), and remaining six out of three studies had an unclear rating (Huang et al., 2010c; Jiang, 2014; Zhang et al., 2019), and three studies high risk of reporting bias owing to not explicitly reported ethical approval (Huang et al., 2010a, 2010b; Savtekin et al., 2018).

Meta-Analysis

Anticatabolic Effect of Melatonin on Osteoarthritis

The inhibitory effect of MT on the level of IL-1β over a 3–4-week period was evaluated in three studies (Huang et al., 2010c; Jiang, 2014; Guo et al., 2017). Of these, left cartilage of femoral condyle IL-1β level was measured from two studies (Huang et al., 2010c; Jiang, 2014), one study (Guo et al., 2017) from synovial fluid. For the meta-analysis, biochemical outcomes were pooled and analyzed using a fixed-effect model (**Figure 3A**). The analysis showed that MT significantly reduced the IL-1β level compared with the control (SMD = −5.45; 95% CI [−6.78, −4.12]; $I^2 = 28\%$, $p < 0.00001$). Similarly, anticatabolic effects of MT on the level of TNF-α over a 3–4-week period was evaluated in three studies (Hong et al., 2014, 2017; Guo et al., 2017). Of these, serum TNF-α

TABLE 1 | Characteristics of the included studies.

Author (Year)	Animal Characteristics			Study Characteristics			Intervention Characteristics		Outcomes
	Species (Sex)	Age (wks)	Weight (gm)	Model	Exp. (n)	Con. (n)	Dose (ROA)	Frequency	
Paulino Silva et al. (2021)	Rat (Male)	9–12	250–320	ACLT	6	6	200 µg/ 100g (s.c)	Once/daily for 4 weeks	H&E stain
Zhang et al. (2019)	Mice (Male)	9	NR	DMM surgery	10	10	10 mg/ mL (i.a)	twice/wk for 4 weeks	Histological grading: OARSI scores
Savtekin et al. (2018)	Rat (Both)	NR	200–250	Zymosan injection	8	8	10 mg/kg (i.a)	Once[a]	Catabolic factor: COX-1, COX-2
Guo et al. (2017)	Rat (Male)	9–10	NR	ACLT	6	6	10 mg/ mL (i.p)	3 weeks	Catabolic factor: TNF-α, IL-1β, MMP-13
Hong et al. (2017)	Rat (Male)	8	NR	Collagenase injection	8	8	10 mg/kg (s.c)	twice/daily for 4 weeks	1. Anabolic factor: TGF-β1, SOX-9, and COL2A1 2. Catabolic factor: TNF-α, MMP-13, VEGF 3. Circadian gene: *Bmal1, Per2* and *Cry1*
Hong et al. (2014)	Rat (Male)	8	250–285	Collagenase injection	5	5	10 mg/kg (s.c)	twice/daily for 4 weeks	1. Anabolic factor: TGF-β1 2. Catabolic factor: TNF-α, IL-6, MMP-13, ADAMTS-4
Jiang (2014)	Rat (Male)	4	130–150	Papain solution injection	10	10	20 mg/ mL (i.a)	Four/wk for 4 weeks	1. Histological grading: Mankin grading score 2. Catabolic factor: IL-1β 3. Anabolic factor: bFGF 4. Serum melatonin level
Lim et al. (2012)	Rabbit (Female)	24–18	2000–3,000	PMM surgery	5	5	20 mg/kg (i.a)	Once/wk for 4 weeks	1. Histological grading: Mankin grading score
Huang et al. (2010a)	Rat (Male)	4	120–150	Papain solution injection	10	10	20 mg/ mL (i.a)	Four/wk for 4 weeks	1. Histological grading: Mankin grading score 2. Anabolic factor: TGF-β1 3. Serum melatonin level
Huang et al. (2010b)	Rat (Male)	4	120–150	Papain solution injection	10	10	20 mg/ mL (i.a)	Four/wk for 4 weeks	1. Histological grading: Mankin grading score 2. Anabolic factor: BMP-2 3. Catabolic factor: IL-1β
Huang et al. (2010c)	Rat (Male)	4	120–150	Papain solution injection	10	10	20 mg/ mL (i.a)	Four/wk for 4 weeks	1. Histological grading: Mankin grading score 2. Serum melatonin level

[a]*intervention has given prior to sacrificing.*

Exp, experimental; Con, control; n, sample size; ROA, route of administration; wks, weeks; NR, not reported; ACLT, anterior cruciate ligament transection; DMM, destabilization of the medial meniscus; OARSI, osteoarthritis research society international; i. a, intra-articular; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; i. p, intraperitoneal; MMP-13, matrix metalloproteinase-13; IL-6, interleukin-6; s. c, subcutaneous; TGF-β1, transforming growth factor-β1; COL2A1, Collagen type II-alpha1; PMM, partial medial meniscectomy; ADAMTS-4, A disintegrin and metalloproteinase with thrombospondin motifs four; bFGF, basic fibroblast growth factor; BMP-2, bone morphogenetic protein-2.

level was measured from two studies (Hong et al., 2014, 2017), one study (Guo et al., 2017) from synovial fluid. For the meta-analysis, biochemical outcomes were pooled and analyzed using a random-effects model (**Figure 3B**). The analysis showed that MT significantly reduced the TNF-α level compared with the control (MD = −71.63; 95% CI [−127.20, −16.05]; I^2 = 99%, p = 0.01). Finally, the inhibitory effect of MT on the MMP-13 level in articular cartilage was evaluated in three studies (Hong et al., 2014, 2017; Guo et al., 2017). For the meta-analysis, biochemical outcomes were pooled and analyzed using a random-effect model (**Figure 3C**). The analysis showed that MT significantly reduced the MMP-13 level compared with the control (SMD = −5.08; 95% CI [−9.82 to −0.34]; I^2 = 90%, p = 0.04).

Pro-anabolic Effect of Melatonin on Osteoarthritis

The anabolic effect of MT on the level of TGF-β1 was evaluated in cartilage tissue from two studies (Hong et al., 2014, 2017). For the meta-analysis, biochemical outcomes were pooled and analyzed

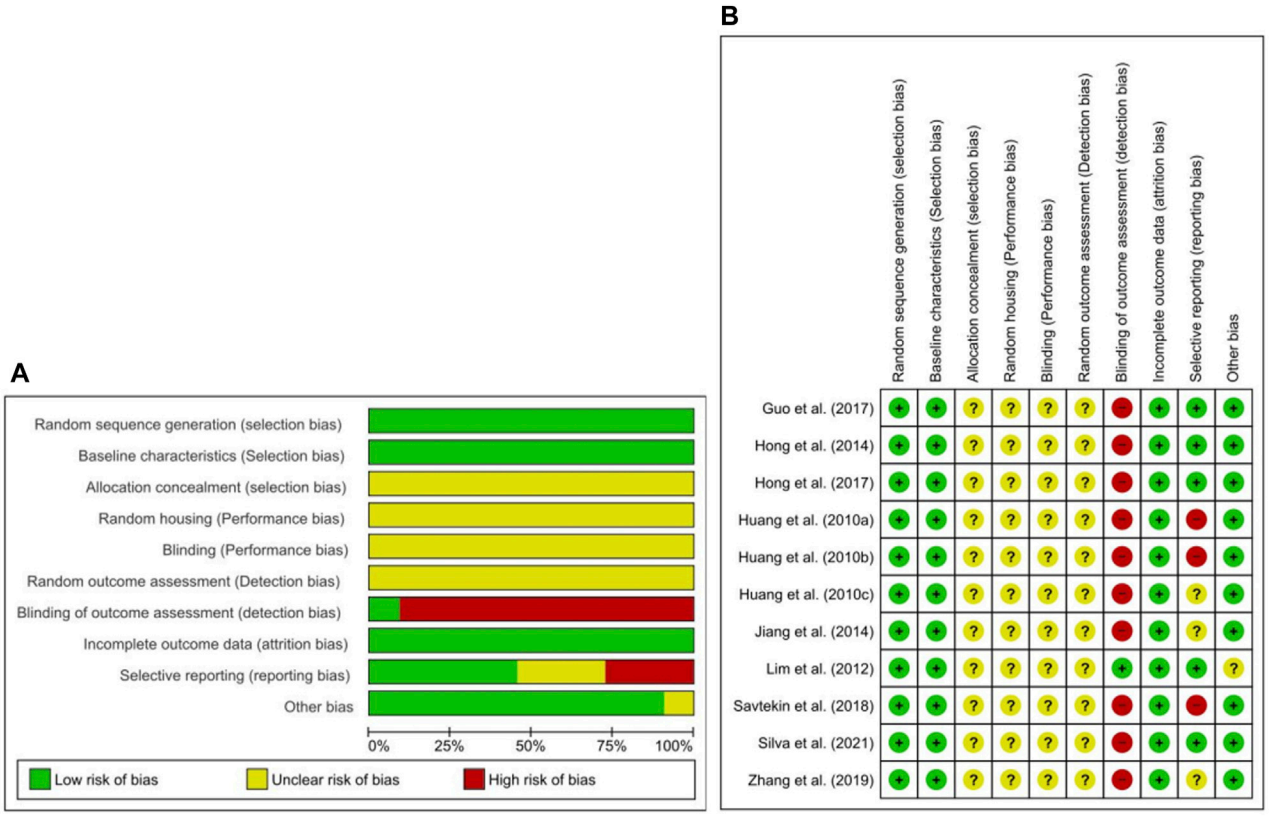


FIGURE 2 | Risk of bias. **(A)** Overall risk of bias for each item in the SYRCLE tool for all included studies. Each risk of bias item is presented as a percentage based on all included studies. **(B)** Individual risk of bias for each of the included animal studies. Each item in the SYRCLE tool was scored as "yes", "no", or "unclear".

using a fixed-effect model (**Figure 4**). The analysis showed that MT significantly increased the TGF-β1 expression compared with the control (SMD = 1.17; 95% CI [0.31, 2.03]; $I^2 = 0\%$, $p < 0.0007$).

Effect of Melatonin on Histological Grading Score

The effect of MT on the histological score was evaluated in five studies (Huang et al., 2010a, 2010c; Lim et al., 2012; Jiang, 2014; Zhang et al., 2019). For the meta-analysis, histological outcomes were pooled and analyzed using a random-effects model (**Figure 5**). The analysis showed that MT significantly reduced the histological scoring compared with the control (SMD = −3.46; 95% CI [−5.24, −1.68]; $I^2 = 84\%$, $p < 0.0001$).

Serum Melatonin Levels in Osteoarthritis

The serum MT levels in OA and sham control groups were measured at two time points (night = 2AM, day = 2PM) in three studies (Huang et al., 2010a, 2010b; Jiang, 2014). For the meta-analysis, serum MT level was pooled and analyzed using a random-effects model. The analysis showed that serum MT level significantly reduced in OA animals compared with the sham control at mid-night (SMD = −150.63; 95% CI [−186.64, −132.62,]; $I^2 = 61\%$, $p < 0.00001$, **Figure 6A**). Likewise, serum MT concentration declined in OA animals at day time (SMD = −16.91; 95% CI [−23.57, −10.25]; $I^2 = 55\%$, $p < 0.00001$, **Figure 6B**).

Subgroup Analysis

Subgroup analysis was performed to investigate heterogeneity among the studies. With regard to histological grading score, we found considerable heterogeneity ($I^2 = 84\%$). Thus, we performed post-hoc subgroup analysis based on wider-CI versus narrow-CI. The test for subgroup differences indicated a statistically significant subgroup effect ($p < 0.00001$), implying that wider-CI subgroup relatively produced a larger effect size than the narrow-CI subgroup. Importantly, there is no heterogeneity between the trials within each of these subgroups (wider-CI: $I^2 = 0\%$; narrow-CI: $I^2 = 0\%$) (**Figure 7**).

Sensitivity Analysis

Sensitivity analysis by excluding individual studies revealed that the IL-1β (**Supplementary Table S3**), and TNF-α (**Supplementary Table S4**), MMP-13 (**Supplementary Table S5**), and histological grading score (**Supplementary Table S6**) results were not modified when compared to the overall effect and their heterogeneity, indicating that our results were robust and that similar results could be obtained after excluding any of the included studies. The sensitivity analysis based on serum MT level both time points indicated that heterogeneity decreased ($I^2 = 0\%$) when reference (Huang et al., 2010b) was excluded; the adjusted effect size were MD = −159.67; 95% CI [−173.73,

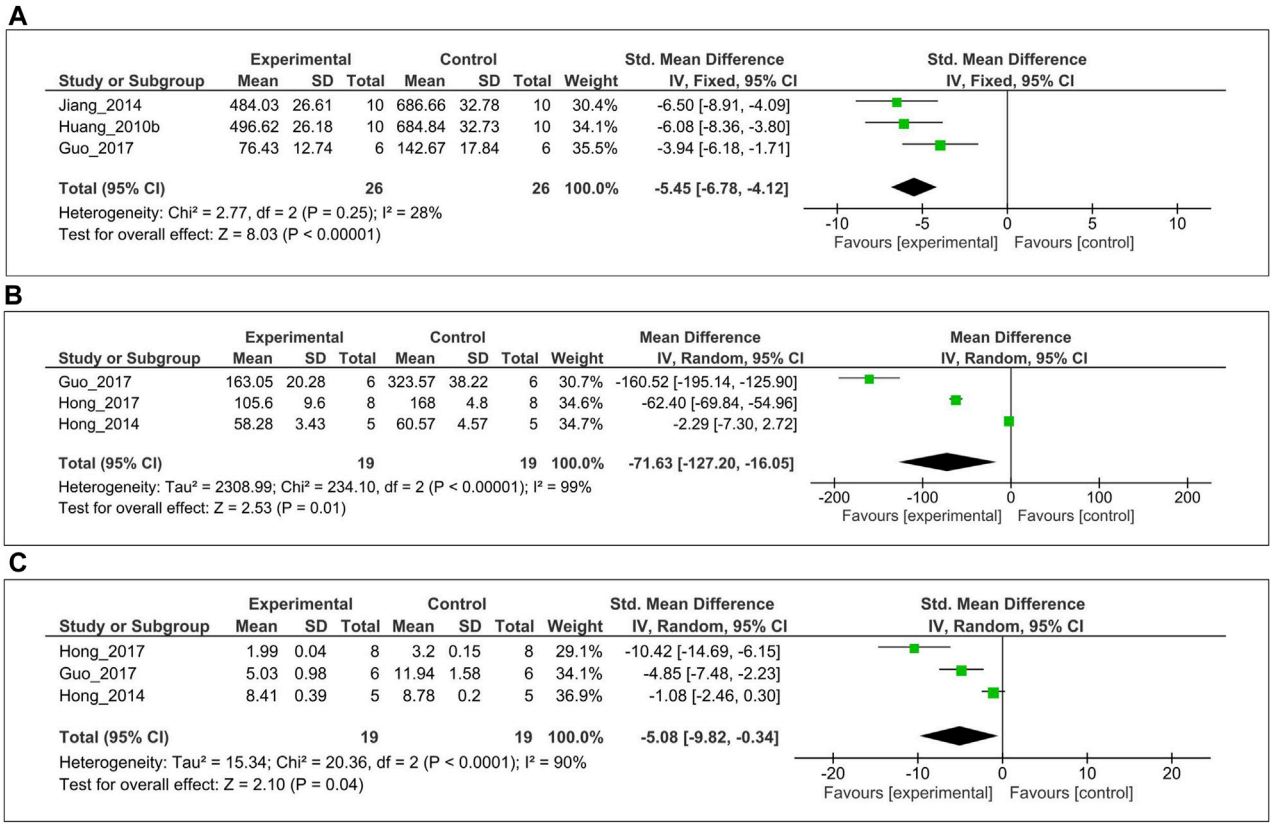


FIGURE 3 | Forest plot showing the effects of MT on IL-1β, TNF-α, and MMP-13 levels. **(A)** IL-1β **(B)** TNF-α, and **(C)** MMP-13 levels. The unit for TNF-α is pg/ml. MT, melatonin; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; MMP-13, matrix metalloproteinase-13; CI, confidence interval.

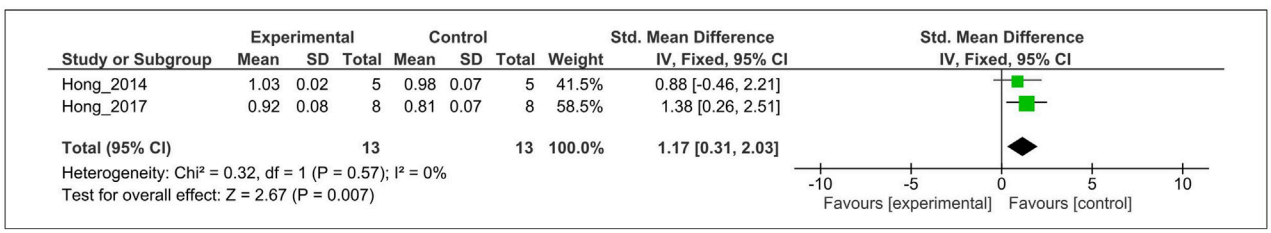


FIGURE 4 | Forest plot showing the effects of MT on TGF-β1 levels. MT, melatonin; TGF-β1, transforming growth factor-β1; CI, confidence interval; IV, independent variable.

−145.62] and MD = −20.20; 95% CI [−25.78, −14.62] at 2 AM and 2 PM, respectively (**Supplementary Table S7, S8**).

Systematic Review

Effects of Melatonin on Others Anabolic-Catabolic Factors

Inflammatory cytokines, including IL-6, appear at higher concentrations in the OA joint and are the major contributors of altered chondrocyte function and cartilage degeneration (Hong et al., 2014). Our group has previously shown that serum IL-6 concentration was higher in collagenase-induced OA rats compared with control, and MT treatment significantly attenuated IL-6 level ($p < 0.05$) (Hong et al., 2014). Increased vascular endothelial growth factor (VEGF) levels are involved in OA-specific pathologies such as cartilage damage, osteophyte formation, and pain (Hamilton et al., 2016). Recently, Hong *et al.*(Hong et al., 2017) demonstrated that treatment with MT notably decreased VEGF level in an OA animal model when compared with a vehicle-treated group ($p < 0.05$). Aggrecanase-1 (ADAMTS-4) inhibitors are considered to have potential therapeutic strategy in the treatment of OA as their expression is elevated during OA progression and they are responsible for

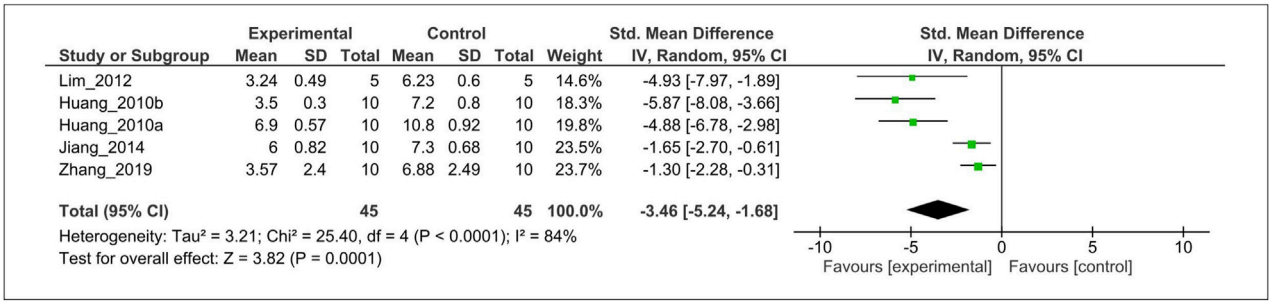


FIGURE 5 | Forest plot showing that MT treatment improved the histological score in OA models. Mankin grading score was used to assess the histological score almost all studies, except Zhang *et al.* (OARSI scores). OA, osteoarthritis; OARSI, osteoarthritis research society international; I, heterogeneity; CI, confidence interval; SD, standard deviation; IV, independent variable.

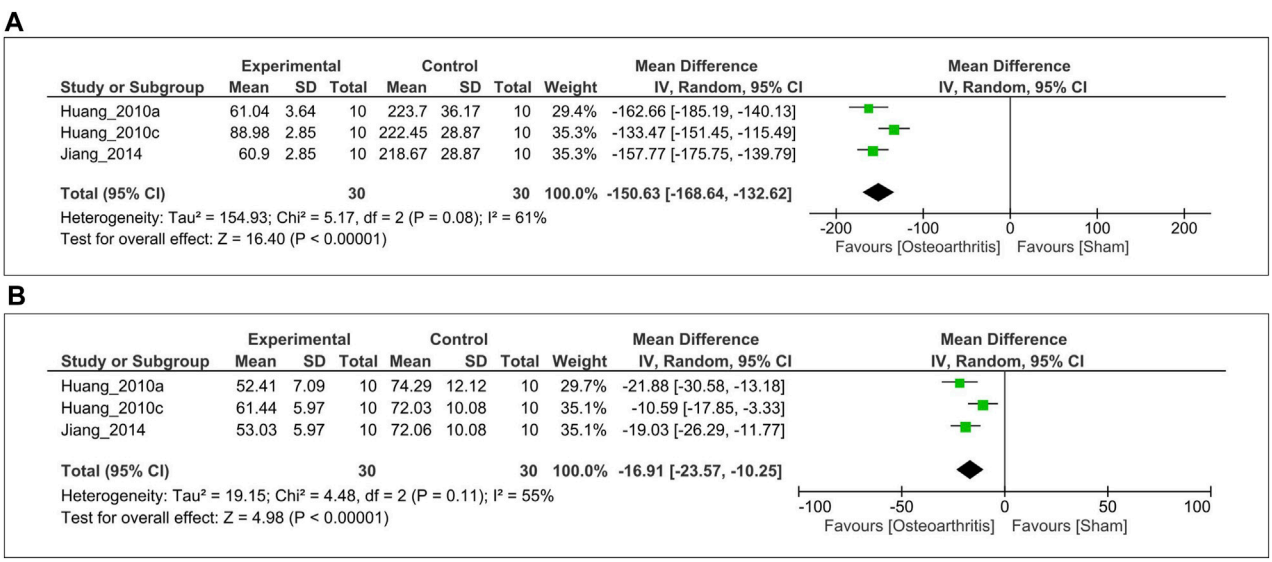


FIGURE 6 | Forest plot showing that serum MT levels. **(A)** 2 AM, and **(B)** 2 PM. The unit for MT is pg/ml. MT, melatonin; CI, confidence interval, IV, independent variable.

cartilage damage (Verma et al., 2016). Our colleagues also previously disclosed that ADAMTS-4 level 1.5 times higher in the collagenase-induced OA group than the control group, whereas MT treatment markedly attenuated ADAMTS-4 ($p < 0.05$) (Hong et al., 2014). In the past, it has been shown that the COX-2 enzyme plays a significant role in the development of OA pathogenesis. Savtekin *et al.* (Savtekin et al., 2018) corroborated with this finding and reported that MT treatment blocked the COX-2 enzymatic activity in the zymosan-induced OA animal model. However, MT treatment was not showed statistically significant in reducing of COX-1 level (Savtekin et al., 2018).

In OA, mRNA expression of many anabolic factors such as SOX-9, and COL2A1 gradually decreased, leading to cartilage damage (Zhong et al., 2016). Interestingly, Hong *et al.* (Hong et al., 2017) reported that MT treatment suppressed SOX-9, and COL2A1 mRNA expression in collagenase-induced OA cartilage compared with control. Conflicting evidence from the same group of investigators show that MT may exert it's protective effects against OA by increasing COL2A1 protein synthesis (Hong et al., 2014). Upregulation of anabolic factors, including BMP-2 and basic fibroblast growth factor (bFGF) are also associated with cartilage repair in the pathological features of OA (Li et al., 2013; Chang et al., 2015) and previous studies reported that MT treatment significantly elevated BMP-2 and bFGF in OA rats (Huang et al., 2010c; Jiang, 2014).

Melatonin Combined With Other Interventions

The anti-arthritic effects of MT combined with strontium ranelate, 5-Methoxytryptophol, exercise, and betamethasone interventions were evaluated on the articular cartilage of OA in rats. Savtekin *et al.* (Savtekin et al., 2018) reported that MT combined with 5-Methoxytryptophol notably downregulated COX-2 when compared with MT alone ($p < 0.001$). Although MT in combination with exercise reduced the level of catabolic

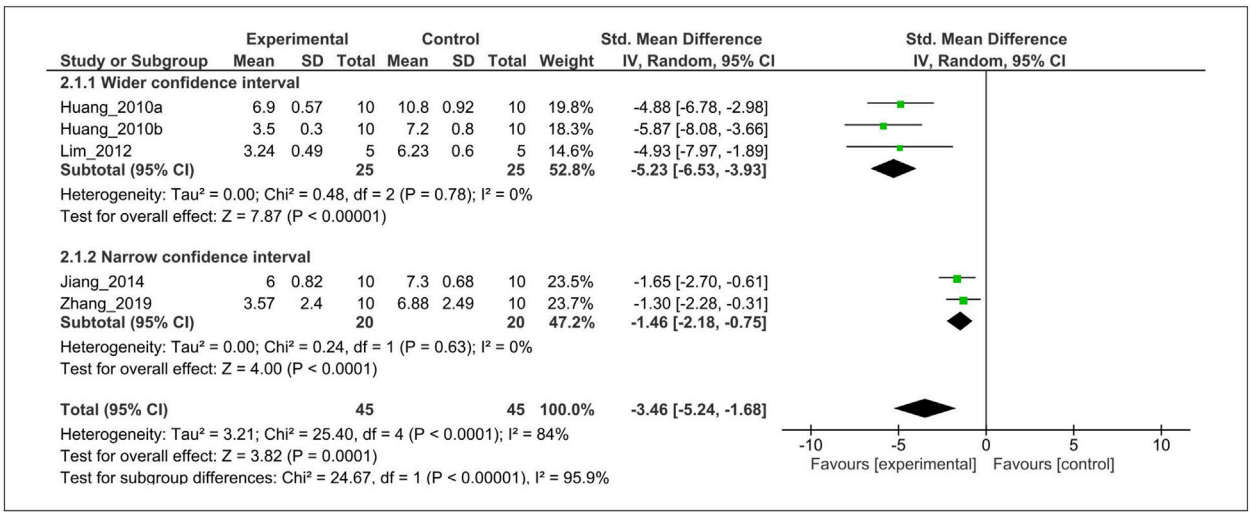


FIGURE 7 | Subgroup analysis of histological score stratified according to the wide 95% CI *versus* narrow 95% CI. The test for subgroup differences showed a statistically significant subgroup effect ($p < 0.00001$), indicating that the wide 95% CI subgroup produced larger effects. Furthermore, there is no heterogeneity between the trials within each of these subgroups (wider-CI: $I^2 = 0\%$; narrow-CI: $I^2 = 0\%$). CI, confidence interval; SD, standard deviation; IV, independent variable.

enzymes such as MMP-13, catabolic cytokines including TNF-alpha and IL-6 were present significantly higher levels (Hong et al., 2014). Besides, there was a significant difference in histological grading score between MT plus betamethasone and control groups ($p < 0.05$) (Huang et al., 2010b). Silva *et al.* (Paulino Silva et al., 2021) reported administration of the MT combined with strontium ranelate presented chondroprotective effects.

Modulatory Effects of Melatonin in Circadian Rhythms

The relationship between circadian misalignment and increase in catabolic activities commonly appear during aging, which may contribute to increasing OA susceptibility (Gossan et al., 2015). Hong et al. reported that several catabolic factors, including MMP-13, and VEGF were upregulated in OA cartilage when the core circadian clock components such as *Per2* (period circadian regulator 2) and *Cry1* (Cry1 cryptochrome circadian regulator 1) genes were decreased (Hong et al., 2017). In contrast, MT treatment not only reduced the catabolic factors in cartilage tissue but also upregulated *Per2* and mRNA levels than the vehicle-treated group (Hong et al., 2017). However, the expression of *Bmal1* was unchanged with the MT-treated group (Hong et al., 2017).

DISCUSSION

Preclinical trials are typically mandatory for developing and evaluating an intervention prior to performing clinical trials in humans. In addition, systematic reviews are crucial for translating the results from preclinical to clinical trials (Lemon and Dunnett, 2005). The principal aim of this work was to provide evidence for the therapeutic potential of MT in OA pathogenesis by the maintenance of a/c homeostasis and modulation of circadian rhythms. To our best knowledge, this is the first systematic review and meta-analysis concerning the effects of MT on a/c balance and modulation of circadian rhythms in OA. Our results suggest that MT treatment increases TGF-β1 (anabolic factor), reduces catabolic factors (IL-1β, TNF-α, and MMP-13), and reduces histological grading score in OA animals. Furthermore, serum MT level significantly declined in osteoarthritic animals than the sham control group both at midnight and middle of the day. Additionally, others anabolic factors (SOX-9, COL2A1, BMP-2, and bFGF) and catabolic factors (IL-6, VEGF, ADAMTS-4, and COX-2), combined effects of MT, and modulatory effects of MT in circadian rhythms were systematically reviewed in the OA animal models.

Accumulating evidence indicates that several proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, trigger catabolic OA development (Mueller and Tuan, 2011). Particularly, IL-1β plays a critical role in OA and is responsible for the further induction of various catabolic enzymes, and inflammatory mediators (Jia et al., 2017). Studies showed that elevated IL-1β was observed in both synovial fluid and cartilage of OA patients (Melchiorri et al., 1998). In this study, our results showed that MT significantly inhibited the level of IL-1β. In addition, MMP-13, and TNF-α production were also inhibited by MT treatment. The results suggested that MT may protect OA by inhibiting proinflammatory cytokines and catabolic enzymes. These results are consistent with the findings of Guo et al. (2017), who found that MT treatment could inhibit IL-1β, TNFα and suppress MMP-13 in OA (Guo et al., 2017). However, data related to TNF-α must be interpreted cautiously because there was substantial heterogeneity ($I^2 = 99\%$) and very wide (95%) CI. One possible explanation for these high degrees of heterogeneity and wider 95% CI is that MT may exert consistently better protective effects in OA pathogenesis where pathogenesis is strongly associated with

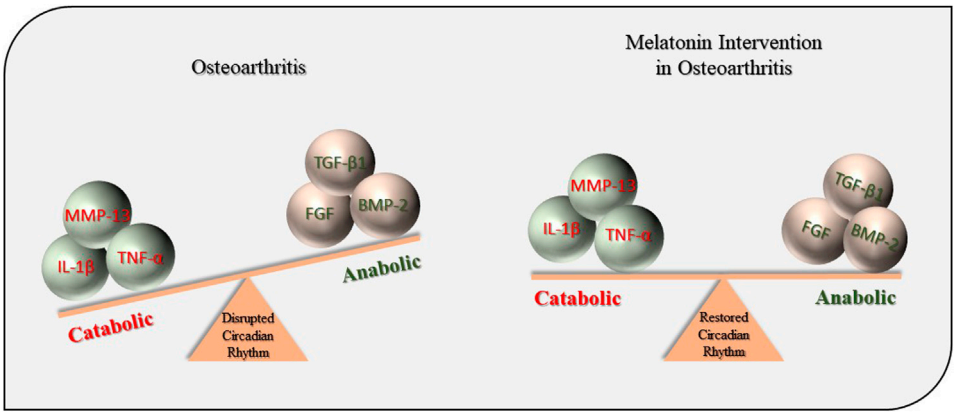


FIGURE 8 | Protective effects of MT during OA pathogenesis. MT, melatonin; OA, osteoarthritis; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; MMP-13, matrix metalloproteinase-13; TGF-β1, transforming growth factor-β1; FGF, fibroblast growth factors; BMP-2, bone morphogenetic protein-2.

circadian disruption. For example, Guo *et al.* (Guo et al., 2015) has shown that exposure to IL-1β severely altered circadian clock gene expression in cartilage, but TNF-α did not have this type of association. Furthermore, others catabolic factors such as IL-6, VEGF, and ADAMTS-4 have been identified as critical players in the pathogenesis and progression of OA (Mueller and Tuan, 2011; Hamilton et al., 2016; Verma et al., 2016), which are also attenuated by MT treatment (Hong et al., 2014, 2017). Our systematic review has reported that MT treatment suppressed COX-2 enzymatic activity in an OA animal model, although the COX-1 level remains unchanged (Savtekin et al., 2018). Previous work demonstrates that COX-2, but not COX-1 induced by catabolic mediators and COX-2 inhibitor showed the most beneficial effects against articular tissue inflammation, whereas COX-1 inhibitor produced unwanted gastrointestinal side effects (Hawkey, 1999; Martel-Pelletier et al., 2003). Hence, MT can be the drug of choice to manage OA pain due to most nonsteroidal anti-inflammatory drugs (NSAIDs) have well-known adverse effects (affecting the gastric mucosa, renal system, cardiovascular system, hepatic system, and hematologic system (Ghlichloo and Gerriets, 2021)).

In the present study, we found that MT might have a protective effect against OA pathogenesis through the upregulation of TGF-β1. Likewise, BMP-2, and bFGF are also unregulated by MT treatment and shown beneficial effects in OA. These findings may be associated with significant reduction of several catabolic factors and regulation of circadian rhythm pathway. Recent studies also support our idea that elevated expression of catabolic cytokines and disruption of circadian core clock genes are responsible for altered anabolic signaling that eventually promotes OA pathogenesis (Akagi et al., 2017; Hong et al., 2017; Tang et al., 2017; Fu et al., 2019; Hu et al., 2020). In addition, our meta-analysis demonstrated that the serum MT level was notably decreased in OA animals both mid-night (2AM) and middle of the day (2PM). The results further strengthen our hypothesis that targeting a/c homeostasis and regulating circadian rhythms by MT would be a promising approach for the treatment of OA. Even though Hong *et al.* (Hong et al., 2017) reported that *Per2* and *Cry1* were upregulated by MT treatment in OA cartilage, the *Bmal1* expression pattern remains unchanged. Interestingly, Fu et al. (2019) (Fu et al., 2019) demonstrated that exogenous MT upregulated *Bmal1*, but downregulated *Per1*, in chondrocytes. Further studies are required to investigate the differential role of MT in different stages of OA pathogenesis. In our systematic review, we reported MT treatment reduced SOX-9 and COL2A1 mRNA expression in one study (Hong et al., 2017), but another study disclosed that COL2A1 protein level increased by MT (Hong et al., 2014). A further study with more focus on mRNA and protein levels of several anabolic factors, including SOX-9 and COL2A1 is therefore suggested.

Another important finding was that MT significantly reduced the histological score when compared to the control group ($p < 0.0001$), but there was statistically significant heterogeneity ($I^2 = 84\%$). Thus, we performed subgroup analysis according to wider-CI versus narrow-CI. Our subgroup analysis indicates that the wider-CI subgroup produced relatively a larger effect size than the narrow-CI subgroup. Importantly, there is no heterogeneity between these subgroups ($I^2 = 0\%$). This finding suggests that MT can be an attractive candidate for the treatment of OA pathogenesis.

In this systematic review, we made great efforts to obtain relatively objective results. First, we searched two legitimate major electronic databases along with Chinese and grey literature databases, and two authors independently examined the retrieved trials to ensure that all relevant studies were incorporated in the meta-analysis. Secondly, two authors assessed the methodological quality of the studies to decrease bias of the included trials and eventually extracted the relevant data. Furthermore, the data extraction was executed by two different authors to ascertain that all of the data outputs were accurately extracted and synthesized for the meta-analysis.

However, this review had several limitations. Although the potential source of heterogeneity has been investigated through subgroup analysis, and leave-one-out sensitivity analyses, there was substantial unexplained heterogeneity between trials for TNF-α and MMP-13 outcomes. Further work is required to know whether gender and age variables modify the MT effects for TNF-α and MMP-13. Our risk of bias analysis using the SYRCLE RoB tool indicated that all included studies had a high risk of detection bias. Information regarding key measures essential for bias reduction (e.g., allocation concealment, random outcome assessment, and blinding) was often missing or insufficiently reported. We strongly recommend improvements to the reporting system for animal models to reduce the risk of bias. Also, deficiency of hormones such as estrogen are directly associated the OA (Roman-Blas et al., 2009). These hormones are also associated with the level of melatonin, catabolic, and anabolic factors. Thus, a research is warranted to explore the role of estrogen and associated hormones, with and without melatonin.

In summary, this current study demonstrates that exogenous MT prevents OA pathogenesis through the regulation of circadian rhythms and a/c balance (**Figure 8**). However, these results should be interpreted in light of the limitations in methodological quality of the studies included in the meta-analysis. Therefore, further studies are warranted to improve study quality through better experimental design and reducing the bias in animal trials.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author, upon reasonable request.

AUTHOR CONTRIBUTIONS

Conceptualization, YH, and DS; investigation, DS, JC and ZK; data extraction, DS and ZK; quality appraisal, DS and JC; writing, YH and DS; visualization, DS, JC, ZK, and GK; administration, JC and YH; editing and supervision, YH; funding acquisition, YH. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was supported by the grants from the National Research Foundation (NRF-2017R1D1A1B0302956514 to YH, NRF-2020R1A2C201215512 to YH), Korea. JC is supported by a post-doctoral fellowship from National Research Foundation (NRF-2019R1A6A3A01091422 to JC), Korea. This work was also supported by the 2016–2017 Creative Research Program of Inje University, Korea.

ACKNOWLEDGMENTS

The authors would like to acknowledge the invaluable support and critical comments of members in "Biological Clock and Aging Control" laboratory. This study was completed as part of the doctoral dissertation by DS.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.714974/full#supplementary-material

REFERENCES

Akagi, R., Akatsu, Y., Fisch, K. M., Alvarez-Garcia, O., Teramura, T., Muramatsu, Y., et al. (2017). Dysregulated Circadian Rhythm Pathway in Human Osteoarthritis: NR1D1 and BMAL1 Suppression Alters TGF-β Signaling in Chondrocytes. *Osteoarthritis Cartilage* 25, 943–951. doi:10.1016/j.joca.2016.11.007

Chang, S. F., Hsieh, R. Z., Huang, K. C., Chang, C. A., Chiu, F. Y., Kuo, H. C., et al. (2015). Upregulation of Bone Morphogenetic Protein-2 Synthesis and Consequent Collagen II Expression in Leptin-Stimulated Human Chondrocytes. *PLoS One* 10, e0144252. doi:10.1371/journal.pone.0144252

Chen, D., Shen, J., Zhao, W., Wang, T., Han, L., Hamilton, J. L., et al. (2017). Osteoarthritis: toward a Comprehensive Understanding of Pathological Mechanism. *Bone Res.* 5, 16044. doi:10.1038/boneres.2016.44

Cui, A., Li, H., Wang, D., Zhong, J., Chen, Y., and Lu, H. (2020). Global, Regional Prevalence, Incidence and Risk Factors of Knee Osteoarthritis in Population-Based Studies. *EClinicalMedicine* 29-30, 100587. doi:10.1016/j.eclinm.2020.100587

de Vries, R. B., Hooijmans, C. R., Tillema, A., Leenaars, M., and Ritskes-Hoitinga, M. (2011). A Search Filter for Increasing the Retrieval of Animal Studies in Embase. *Lab. Anim.* 45, 268–270. doi:10.1258/la.2011.011056

DerSimonian, R., and Laird, N. (1986). Meta-analysis in Clinical Trials. *Control. Clin. Trials* 7, 177–188. doi:10.1016/0197-2456(86)90046-2

Dudek, M., Gossan, N., Yang, N., Im, H. J., Ruckshanthi, J. P., Yoshitane, H., et al. (2016). The Chondrocyte Clock Gene Bmal1 Controls Cartilage Homeostasis and Integrity. *J. Clin. Invest.* 126, 365–376. doi:10.1172/JCI82755

Fu, S., Kuwahara, M., Uchida, Y., Koudo, S., Hayashi, D., Shimomura, Y., et al. (2019). Circadian Production of Melatonin in Cartilage Modifies Rhythmic Gene Expression. *J. Endocrinol.* 241, 161–173. doi:10.1530/JOE-19-0022

Ghlichloo, I., and Gerriets, V. (2021). "Nonsteroidal Anti-inflammatory Drugs (NSAIDs)," in *StatPearls* (Treasure Island (FL): StatPearls Publishing). Available at: http://www.ncbi.nlm.nih.gov/books/NBK547742/(Accessed May 24, 2021)

Global Burden of Disease Study (2016). Global, Regional, and National Incidence, Prevalence, and Years Lived with Disability for 310 Diseases and Injuries, 1990-2015: a Systematic Analysis for the Global Burden of Disease Study 2015. *Lancet* 388, 1545–1602. doi:10.1016/S0140-6736(16)31678-6

Gossan, N., Boot-Handford, R., and Meng, Q. J. (2015). Ageing and Osteoarthritis: a Circadian Rhythm Connection. *Biogerontology* 16, 209–219. doi:10.1007/s10522-014-9522-3

Guo, B., Yang, N., Borysiewicz, E., Dudek, M., Williams, J. L., Li, J., et al. (2015). Catabolic Cytokines Disrupt the Circadian Clock and the Expression of Clock-Controlled Genes in Cartilage via an NFκБ́Гдодмгдмт Оатжхачћ *Osteoarthritis Cartilage* 23, 1981–1988. doi:10.1016/j.joca.2015.02.020

Guo, J. Y., Li, F., Wen, Y. B., Cui, H. X., Guo, M. L., Zhang, L., et al. (2017). Melatonin Inhibits Sirt1-dependent NAMPT and NFAT5 Signaling in Chondrocytes to Attenuate Osteoarthritis. *Oncotarget* 8, 55967–55983. doi:10.18632/oncotarget.18356

Hamilton, J. L., Nagao, M., Levine, B. R., Chen, D., Olsen, B. R., and Im, H. J. (2016). Targeting VEGF and its Receptors for the Treatment of Osteoarthritis and Associated Pain. *J. Bone Miner. Res.* 31, 911–924. doi:10.1002/jbmr.2828

Hawkey, C. J. (1999). COX-2 Inhibitors. *Lancet* 353, 307–314. doi:10.1016/s0140-6736(98)12154-2

Higgins, J. P., Altman, D. G., Gøtzsche, P. C., Jüni, P., Moher, D., Oxman, A. D., et al. (2011). The Cochrane Collaboration's Tool for Assessing Risk of Bias in Randomised Trials. *BMJ* 343, d5928. doi:10.1136/bmj.d5928

Hong, Y., Kim, H., Lee, Y., Lee, S., Kim, K., Jin, Y., et al. (2014). Salutary Effects of Melatonin Combined with Treadmill Exercise on Cartilage Damage. *J. Pineal Res.* 57, 53–66. doi:10.1111/jpi.12143

Hong, Y., Kim, H., Lee, S., Jin, Y., Choi, J., Lee, S. R., et al. (2017). Role of Melatonin Combined with Exercise as a Switch-like Regulator for Circadian Behavior in Advanced Osteoarthritic Knee. *Oncotarget* 8, 97633–97647. doi:10.18632/oncotarget.19276

Hooijmans, C. R., Tillema, A., Leenaars, M., and Ritskes-Hoitinga, M. (2010). Enhancing Search Efficiency by Means of a Search Filter for Finding All Studies on Animal Experimentation in PubMed. *Lab. Anim.* 44, 170–175. doi:10.1258/la.2010.009117

Hooijmans, C. R., Rovers, M. M., de Vries, R. B., Leenaars, M., Ritskes-Hoitinga, M., and Langendam, M. W. (2014). SYRCLE's Risk of Bias Tool for Animal Studies. *BMC Med. Res. Methodol.* 14, 43. doi:10.1186/1471-2288-14-43

Hossain, F. M., Hong, Y., Jin, Y., Choi, J., and Hong, Y. (2019). Physiological and Pathological Role of Circadian Hormones in Osteoarthritis: Dose-Dependent or Time-Dependent? *J. Clin. Med.* 8, 1415. doi:10.3390/jcm8091415

Hu, P., Du, J., Zhang, S., Wang, T., Li, J., Chen, G., et al. (2020). Oral Administration of Strontium Gluconate Effectively Reduces Articular Cartilage Degeneration through Enhanced Anabolic Activity of Chondrocytes and Chondrogenetic Differentiation of Mesenchymal Stromal Cells. *Biol. Trace Elem. Res.* 193, 422–433. doi:10.1007/s12011-019-01711-9

Huang, C., Liu, J., and Hu, Y. (2010a). The Effect of Melatonin on the Expression of TGF-β 1 and IL-1β in the Articular Cartilage of Osteoarthritis Rats. *Chin. J. Orthopaedics* 18, 1635–1639.

Huang, C., Liu, J., and Xia, C. (2010b). Melatonin Combined with Compound Betamethasone for Articular Cartilage Lesions in Rats with Knee Osteoarthritis. *Chin. Tissue Eng. Res. Clin. Rehabil.* 14, 8737–8740.

Huang, C., Liu, J., Xia, C., and Hu, Y. (2010c). Effects of Melatonin on Expression of Bone Morphogenetic Protein 2 and Interleukin 1beta in Articular Cartilage of Rat with Osteoarthritis. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 24, 1082–1087.

Hwang, H. S., Lee, M. H., and Kim, H. A. (2020). TGF-β1-induced Expression of Collagen Type II and ACAN Is Regulated by 4E-BP1, a Repressor of Translation. *FASEB J.* 34, 9531–9546. doi:10.1096/fj.201903003R

Jahanban-Esfahlan, R., Mehrzadi, S., Reiter, R. J., Seidi, K., Majidinia, M., Baghi, H. B., et al. (2018). Melatonin in Regulation of Inflammatory Pathways in Rheumatoid Arthritis and Osteoarthritis: Involvement of Circadian Clock Genes. *Br. J. Pharmacol.* 175, 3230–3238. doi:10.1111/bph.13898

Jia, T., Qiao, J., Guan, D., and Chen, T. (2017). Anti-Inflammatory Effects of Licochalcone A on IL-1β-Stimulated Human Osteoarthritis Chondrocytes. *Inflammation* 40, 1894–1902. doi:10.1007/s10753-017-0630-5

Jiang, N. (2014). *The Effect of Melatonin on the Expression of bFGF and IL-1β in the Articular Cartilage of Rats with Osteoarthritis*. Qingdao: Qingdao University. Postgraduate dissertation

Kang, D., Shin, J., Cho, Y., Kim, H. S., Gu, Y. R., Kim, H., et al. (2019). Stress-activated miR-204 Governs Senescent Phenotypes of Chondrocytes to Promote Osteoarthritis Development. *Sci. Transl. Med.* 11, eaar6659. doi:10.1126/scitranslmed.aar6659

Kc, R., Li, X., Voigt, R. M., Ellman, M. B., Summa, K. C., Vitaterna, M. H., et al.(2015). Environmental Disruption of Circadian Rhythm Predisposes Mice to Osteoarthritis-like Changes in Knee Joint. *J. Cel. Physiol.* 230, 2174–2183. doi:10.1002/jcp.24946

Kim, J. H., Jeon, J., Shin, M., Won, Y., Lee, M., Kwak, J. S., et al. (2014). Regulation of the Catabolic Cascade in Osteoarthritis by the Zinc-ZIP8-MTF1 Axis. *Cell* 156, 730–743. doi:10.1016/j.cell.2014.01.007

Lemon, R., and Dunnett, S. B. (2005). Surveying the Literature from Animal Experiments. *BMJ* 330, 977–978. doi:10.1136/bmj.330.7498.977

Li, X., Su, G., Wang, J., Zhou, Z., Li, L., Liu, L., et al. (2013). Exogenous bFGF Promotes Articular Cartilage Repair via Up-Regulation of Multiple Growth Factors. *Osteoarthritis Cartilage* 21, 1567–1575. doi:10.1016/j.joca.2013.06.006

Li, S., Niu, G., Wu, Y., Du, G., Huang, C., Yin, X., et al. (2016). Vitamin D Prevents Articular Cartilage Erosion by Regulating Collagen II Turnover through TGF-B1 in Ovariectomized Rats. *Osteoarthritis Cartilage* 24, 345–353. doi:10.1016/j.joca.2015.08.013

Lim, H. D., Kim, Y. S., Ko, S. H., Yoon, I. J., Cho, S. G., Chun, Y. H., et al. (2012). Cytoprotective and Anti-inflammatory Effects of Melatonin in Hydrogen Peroxide-Stimulated CHON-001 Human Chondrocyte Cell Line and Rabbit Model of Osteoarthritis via the SIRT1 Pathway. *J. Pineal Res.* 53, 225–237. doi:10.1111/j.1600-079X.2012.00991.x

Ling, L., Alattar, A., Tan, Z., Shah, F. A., Ali, T., Alshaman, R., et al. (2020). A Potent Antioxidant Endogenous Neurohormone Melatonin, Rescued MCAO by Attenuating Oxidative Stress-Associated Neuroinflammation. *Front. Pharmacol.* 11, 1220. doi:10.3389/fphar.2020.01220

Liu, X., Xu, Y., Chen, S., Tan, Z., Xiong, K., Li, Y., et al. (2014). Rescue of Proinflammatory Cytokine-Inhibited Chondrogenesis by the Antiarthritic Effect of Melatonin in Synovium Mesenchymal Stem Cells via Suppression of Reactive Oxygen Species and Matrix Metalloproteinases. *Free Radic. Biol. Med.* 68, 234–246. doi:10.1016/j.freeradbiomed.2013.12.012

Loeser, R. F., Collins, J. A., and Diekman, B. O. (2016). Ageing and the Pathogenesis of Osteoarthritis. *Nat. Rev. Rheumatol.* 12, 412–420. doi:10.1038/nrrheum.2016.65

Loeser, R. F. (2017). The Role of Aging in the Development of Osteoarthritis. *Trans. Am. Clin. Climatol. Assoc.* 128, 44–54.

Lotz, M., and Loeser, R. F. (2012). Effects of Aging on Articular Cartilage Homeostasis. *Bone* 51, 241–248. doi:10.1016/j.bone.2012.03.023

Martel-Pelletier, J., Pelletier, J. P., and Fahmi, H. (2003). Cyclooxygenase-2 and Prostaglandins in Articular Tissues. *Semin. Arthritis Rheum.* 33, 155–167. doi:10.1016/s0049-0172(03)00134-3

Melchiorri, C., Meliconi, R., Frizziero, L., Silvestri, T., Pulsatelli, L., Mazzetti, I., et al. (1998). Enhanced and Coordinated *In Vivo* Expression of Inflammatory Cytokines and Nitric Oxide Synthase by Chondrocytes from Patients with Osteoarthritis. *Arthritis Rheum.* 41, 2165–2174. doi:10.1002/1529-0131(199812)41:12<2165:AID-ART11>3.0.CO;2-O

Mobasheri, A., Rayman, M. P., Gualillo, O., Sellam, J., van der Kraan, P., and Fearon, U. (2017). The Role of Metabolism in the Pathogenesis of Osteoarthritis. *Nat. Rev. Rheumatol.* 13, 302–311. doi:10.1038/nrrheum.2017.50

Mueller, M. B., and Tuan, R. S. (2011). Anabolic/Catabolic Balance in Pathogenesis of Osteoarthritis: Identifying Molecular Targets. *PM&R.* 3, S3–S11. doi:10.1016/j.pmrj.2011.05.009

Nambi, G. (2021). Does Low Level Laser Therapy Has Effects on Inflammatory Biomarkers IL-1β, IL-6, TNF-α, and MMP-13 in Osteoarthritis of Rat Models-A Systemic Review and Meta-Analysis. *Lasers Med. Sci.* 36, 475–484. doi:10.1007/s10103-020-03124-w

Nummenmaa, E., Hämäläinen, M., Moilanen, T., Vuolteenaho, K., and Moilanen, E. (2015). Effects of FGF-2 and FGF Receptor Antagonists on MMP Enzymes, Aggrecan, and Type II Collagen in Primary Human OA Chondrocytes. *Scand. J. Rheumatol.* 44, 321–330. doi:10.3109/03009742.2014.1000372

Asanjani Oskoii, M., Khatami, N., Majidinia, M., Rezazadeh, M.-A., Mir, S. M., Sadeghpour, A., et al. (2020). Serum Level of Melatonin in Patients with Osteoarthritis and its Relation with 8-Hydroxy-2-Deoxyguanosine and Vitamin D. *J. Res. Clin. Med.* 8, 34. doi:10.34172/jrcm.2020.034

Ouzzani, M., Hammady, H., Fedorowicz, Z., and Elmagarmid, A. (2016). Rayyan-a Web and mobile App for Systematic Reviews. *Syst. Rev.* 5, 210. doi:10.1186/s13643-016-0384-4

Pap, T., and Korb-Pap, A. (2015). Cartilage Damage in Osteoarthritis and Rheumatoid Arthritis-Ttwo Unequal Siblings. *Nat. Rev. Rheumatol.* 11, 606–615. doi:10.1038/nrrheum.2015.95

Patsopoulos, N. A., Evangelou, E., and Ioannidis, J. P. (2008). Sensitivity of Between-Study Heterogeneity in Meta-Analysis: Proposed Metrics and Empirical Evaluation. *Int. J. Epidemiol.* 37, 1148–1157. doi:10.1093/ije/dyn065

Paulino Silva, K. M., de Sousa, F. L., Alves, A. C. B., Rocha, P. A., da Costa, H. N. A. F., Ferreira, W. R., et al. (2021). Chondroprotective Effect of Melatonin and Strontium Ranelate in Animal Model of Osteoarthritis. *Heliyon* 7, e06760. doi:10.1016/j.heliyon.2021.e06760

Pei, M., He, F., Wei, L., and Rawson, A. (2009). Melatonin Enhances Cartilage Matrix Synthesis by Porcine Articular Chondrocytes. *J. Pineal Res.* 46, 181–187. doi:10.1111/j.1600-079X.2008.00646.x

Pelletier, J. P., Martel-Pelletier, J., Rannou, F., and Cooper, C. (2016). Efficacy and Safety of Oral NSAIDs and Analgesics in the Management of Osteoarthritis: Evidence from Real-Life Setting Trials and Surveys. *Semin. Arthritis Rheum.* 45, S22–S27. doi:10.1016/j.semarthrit.2015.11.009

Rahmati, M., Nalesso, G., Mobasheri, A., and Mozafari, M. (2017). Aging and Osteoarthritis: Central Role of the Extracellular Matrix. *Ageing Res. Rev.* 40, 20–30. doi:10.1016/j.arr.2017.07.004

Roman-Blas, J. A., Castañeda, S., Largo, R., and Herrero-Beaumont, G. (2009). Osteoarthritis Associated with Estrogen Deficiency. *Arthritis Res. Ther.* 11, 241. doi:10.1186/ar2791

Savtekin, G., Serakinci, N., Erzik, C., Cetinel, S., and Sehirli, A. O. (2018). Effects of Circadian Rhythm Hormones Melatonin and 5-Methoxytryptophol on COXs, Raf-1 and STAT3. *Int. J. Pharmacol.* 14, 787–795. doi:10.3923/ijp.2018.787.795

Sumsuzzman, D. M., Choi, J., Khan, Z. A., and Hong, Y. (2020). Protective Effects of Melatonin against Severe Burn-Induced Distant Organ Injury: A Systematic Review and Meta-Analysis of Experimental Studies. *Antioxidants (Basel)* 9, 1196. doi:10.3390/antiox9121196

Sumsuzzman, D. M., Khan, Z. A., Choi, J., and Hong, Y. (2021a). Differential Role of Melatonin in Healthy Brain Aging: A Systematic Review and Meta-Analysis of the SAMP8 Model. *Aging (Albany NY)* 13, 9373–9397. doi:10.18632/aging.202894

Sumsuzzman, D. M., Choi, J., Jin, Y., and Hong, Y. (2021b). Neurocognitive Effects of Melatonin Treatment in Healthy Adults and Individuals with Alzheimer's Disease and Insomnia: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Neurosci. Biobehav. Rev.* 127, 459–473. doi:10.1016/j.neubiorev.2021.04.034

Tang, Q., Zheng, G., Feng, Z., Tong, M., Xu, J., Hu, Z., et al. (2017). Wogonoside Inhibits IL-1β Induced Catabolism and Hypertrophy in Mouse Chondrocyte and Ameliorates Murine Osteoarthritis. *Oncotarget* 8, 61440–61456. doi:10.18632/oncotarget.18374

van der Kraan, P. M. (2017). The Changing Role of TGFβ in Healthy, Ageing and Osteoarthritic Joints. *Nat. Rev. Rheumatol.* 13, 155–163. doi:10.1038/nrrheum.2016.219

Verma, P., Dalal, K., and Chopra, M. (2016). Pharmacophore Development and Screening for Discovery of Potential Inhibitors of ADAMTS-4 for Osteoarthritis Therapy. *J. Mol. Model.* 22, 178. doi:10.1007/s00894-016-3035-8

Wang, X., Fan, J., Ding, X., Sun, Y., Cui, Z., and Liu, W. (2019). Tanshinone I Inhibits IL-1β-Induced Apoptosis, Inflammation and Extracellular Matrix Degradation in Chondrocytes CHON-001 Cells and Attenuates Murine Osteoarthritis. *Drug Des. Devel. Ther.* 13, 3559–3568. doi:10.2147/DDDT.S216596

Yang, W., Kang, X., Liu, J., Li, H., Ma, Z., Jin, X., et al. (2016). Clock Gene Bmal1 Modulates Human Cartilage Gene Expression by Crosstalk with Sirt1. *Endocrinology* 157, 3096–3107. doi:10.1210/en.2015-2042

Zhang, Y., Lin, J., Zhou, X., Chen, X., Chen, A. C., Pi, B., et al. (2019). Melatonin Prevents Osteoarthritis-Induced Cartilage Degradation via Targeting MicroRNA-140. *Oxid. Med. Cell. Longev.* 2019, 9705929. doi:10.1155/2019/9705929

Zhong, L., Huang, X., Karperien, M., and Post, J. N. (2016). Correlation between Gene Expression and Osteoarthritis Progression in Human. *Int. J. Mol. Sci.* 17, 1126. doi:10.3390/ijms17071126

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 10 January 2022
doi: 10.3389/fphar.2021.781389

Composition Analysis and Pharmacological Activity of Avocado/Soybean Unsaponifiable Products Used in the Treatment of Osteoarthritis

Cécile Lambert[1]*, *Gaëlle Bellemère*[2], *Gaëtan Boyer*[2], *Frank Ponelle*[2], *Thierry Bauer*[2], *Marie-Christine Legeny*[2], *Caroline Baudouin*[2] and *Yves Henrotin*[1,3,4]

[1]MusculoSKeletal Innovative Research Lab, Center for Interdisciplinary Research on Medicines, University of Liège, Liège, Belgium, [2]Innovation, Research and Development, Laboratoires Expanscience, Epernon, France, [3]Department of Physical Therapy and Rehabilitation, Princess Paola Hospital, Vivalia, Marche-en-Famenne, Belgium, [4]Artialis S.A., Liège, Belgium

OPEN ACCESS

Edited by:
Raquel Largo,
Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain

Reviewed by:
Sadiq Umar,
University of Illinois at Chicago, United States
Waqas Ahmad,
University of Science Malaysia, Malaysia

***Correspondence:**
Cécile Lambert
cecile.lambert@uliege.be

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 22 September 2021
Accepted: 13 December 2021
Published: 10 January 2022

Citation:
Lambert C, Bellemère G, Boyer G, Ponelle F, Bauer T, Legeny M-C, Baudouin C and Henrotin Y (2022) Composition Analysis and Pharmacological Activity of Avocado/Soybean Unsaponifiable Products Used in the Treatment of Osteoarthritis. Front. Pharmacol. 12:781389. doi: 10.3389/fphar.2021.781389

Objective: Avocado/soybean unsaponifiables (ASUs) are commonly used to treat OA symptoms. However, there are many ASU mixtures on the market with differing compositions and pharmacological activities. This study aimed to compare the composition and pharmacological activity of seven commercially available ASU products on human osteoarthritis chondrocytes.

Methods: The contents of the lipidic part of ASUs were characterized by gas chromatography analysis using a VARIAN 3400 chromatograph. The pharmacological activity of the ASU products was tested on human osteoarthritis chondrocytes cultured in alginate beads. Their effects were evaluated on aggrecan, interleukin (IL)-6 and -8, and matrix metalloproteases (MMP)-3 using immunoassays and on nitric oxide through measurement of nitrite *via* spectrometry.

Results: PIASCLEDINE-ExpASU® showed a specific profile with the presence of chromatographic peaks corresponding to an alkyl furan fraction and alkyl triols. PIASCLEDINE-ExpASU®, Persemax, Insaponifiable 300, Arthrocen, and Arthocare contained quantifiable amounts of tocopherol, while tocopherol was undetectable in Avovida and Saponic. Squalene was found only in PIASCLEDINE-ExpASU®. The abundance of sterols varied depending on the product. PIASCLEDINE-ExpASU® was the most active of the tested ASU products in inhibiting nitric oxide, IL-6, and IL-8 production by chondrocytes. With the exception of Saponic and Persemax, all the ASU mixtures either slightly or significantly increased aggrecan production. MMP-3

Abbreviations: AGG, aggrecan; ASU, avocado and soybean unsaponifiables; CM, cells with their associated matrix; DMEM, Dulbecco's modified Eagle's medium; DNA, deoxyribonucleic acid; FID, flame ionization detector; FRM, further-removed matrix; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); IL-, interleukin; LDH, lactate dehydrogenase; MMP, matrix metalloproteases; $NaNO_2$, sodium nitrite; NO, nitric oxide; OA, osteoarthritis; PG, prostaglandin; RCT, randomized controlled trials; SPI, septum-equipped programmable injector; SVOC, semi-volatile organic compounds; SYSADOA, symptomatic slow-acting drugs in osteoarthritis.

levels were significantly decreased by Insaponifiable 300 and PIASCLEDINE-ExpASU® and significantly increased by Saponic.

Conclusion: The composition of PIASCLEDINE-ExpASU® is different to that of the other evaluated ASU mixtures. This specific composition explains its better pharmacological activity, including the higher inhibitory effect on pro-inflammatory and pro-catabolic factors. Our findings are helpful in providing a basis for understanding the symptomatic effect of PIASCLEDINE-ExpASU® in patients with osteoarthritis.

Keywords: unsaponifiable, avocado, soybean, osteoarthritis, chondrocytes

INTRODUCTION

Osteoarthritis (OA) affects around 500 million people worldwide (Vos et al., 2017; Hunter et al., 2020) and is one of the most common causes of physical disability among older adults. Unfortunately, the chance of developing OA increases with age and is also associated with other common diseases such as diabetes (type 2) and cardiovascular diseases (Swain et al., 2020). The main hallmark of the disease is a progressive degradation of cartilage which is driven by a combination of mechanical and biochemical factors (Loeser et al., 2012). Chondrocytes play a key role by secreting locally abnormal quantities of catabolic and pro-inflammatory mediators. Therefore, regulation of chondrocyte metabolism remains a target for OA treatments, with a potential structure-modifying effect (Rahmati et al., 2016).

To date, there are few pharmaceutical options that can help to safely relieve symptoms and none that can cure OA (Glyn-Jones et al., 2015). Therefore, there is an important need for efficient treatments that can delay both the structural and clinical progression of the disease. One of the most widely used treatments for OA is a product composed of avocado and soybean unsaponifiables (ASUs), a member of the symptomatic slow-acting drugs in osteoarthritis (SYSADOA) family (Edgard Henrotin, 2018; Honvo et al., 2019). This product, called PIASCLEDINE-ExpASU® is commercialized as a drug in many countries (PIASCLEDINE®300, Laboratoires Expanscience, Courbevoie, France). This is the only ASU product that has been rigorously investigated in robust randomized controlled trials (RCTs) (Maheu et al., 1998; Appelboom et al., 2001; Karel et al., 2010). It is a pharmaceutical-grade product composed of a specific ratio of avocado and soybean oil unsaponifiables (1:2 w/w) obtained by a patented process that influences the composition of the product (Maheu et al., 2014). Indeed, while the extraction of unsaponifiable fractions from soy oil does not represent any major hurdle, the extraction of unsaponifiables from the avocado pericarp is technically more complex because the high water content in the fruit tends to chemically modify a number of bioactive lipids *via* uncontrolled peroxidation and oxidization during the extraction process. To circumvent this, the process for extracting PIASCLEDINE-ExpASU® has been adapted to include a drying step, whereby heat is used to remove the water from the avocado fruit before initiating ASU extraction (patent number: 10688142). It has been shown that a fraction of native furanic unsaponifiables specific to the avocado pericarp, identified as comprising persin compounds, undergoes cyclization when the fruit is submitted to heating (Kashman et al., 1969). This chemical transformation occurs during the PIASCLEDINE-ExpASU® extraction process. The resulting persin-derived compounds represent a major unsaponifiable fraction in PIASCLEDINE-ExpASU® that cannot be found in any other type of avocado extraction process, such as cold pressure and centrifugation, or in soy unsaponifiables (Farines et al., 1995). Similarly, the specific process used for preparation of PIASCLEDINE-ExpASU® leads to enrichment of the avocado unsaponifiable fraction by alkyl triols, which has been previously described (Kashman et al., 1969; Néeman et al., 1970; Brown, 1973) (Laboratoires Expanscience, Internal data). The composition of PIASCLEDINE-ExpASU® is complex and typically contains 35% sterols, 25% tocopherols, and 25% molecules from avocados that are obtained specifically for this process. The pharmacological activities of this compound have been well investigated, and the findings have been summarized in a narrative review study (Edgard Henrotin, 2018). In summary, PIASCLEDINE-ExpASU® was found to stimulate aggrecan (AGG) and inhibit the production of interleukin (IL)-6 and -8, prostaglandin (PG) E2, and some matrix metalloproteases (MMPs) in chondrocytes. It also positively modulates the altered phenotype of OA subchondral bone osteoblasts and reduces the production of collagenases by synovial cells. At this time, four robust RCTs have all demonstrated a beneficial symptomatic effect of PIASCLEDINE-ExpASU® in the treatment of hip or knee OA (Maheu et al., 1998; Appelboom et al., 2001; Karel et al., 2010; Anon, 2021a).

However, in the past years, many food supplements based on ASUs that have emerged on the market in recent years are claiming to have similar pharmacological activities and alleging more or less clearly the same clinical and safety profile as PIASCLEDINE-ExpASU® (Ghasemian et al., 2016).

This study aimed to compare the composition and pharmacological activity of products composed of ASUs available on the market, including PIASCLEDINE-ExpASU®. These products were chosen because they were the most representative products in different regions. More precisely, we studied the chromatographic pattern of these compounds and their effects on human OA chondrocytes in alginate bead cultures.

MATERIALS AND METHODS

Products Investigated

The following products were included in the study: Arthocare (Bonapharm S.A.C., Lima, Peru), Arthrocen (Pharmin United States, LLC, San Jose, CA, United States), Avovida (Pharma Nature, Saint Hippolyte du Fort, France), Insaponifiable 300 (GIPHAR Group, Paris, France), Persemax (Laboratorios Synthesis SAS, Bogota, Colombia), PIASCLEDINE-ExpASU® (Laboratoires Expanscience, Courbevoie, France), and Saponic (Laboratorio Gador La Paz, Montevideo, Uruguay). All products were presented as 300 mg capsules.

Analytical Assays

For each product, the mass of 20 individual capsules was weighed and recorded. The content of the capsules was collected and subjected to Soxhlet extraction using chloroform. Soxhlet extraction is a very standard and well-recognized method that leads to the extraction of water-insoluble and slightly water-soluble organics identified as semi-volatile organic compounds (SVOCs) (Abubakar and Haque, 2020). The mass of the SVOC fraction was determined by gravimetry and recorded as the lipidic part. The contents of the lipidic part were characterized by gas chromatography analysis using a VARIAN 3400 chromatograph equipped with a septum-equipped programmable injector (SPI), a flame ionization detector (FID), and a capillary column with 5%-phenyl-95%-methylpolysiloxane as the stationary phase and helium as the mobile phase. A solution of squalene at 0.2% (m/v) in hexane and a solution of avocado oil unsaponifiables at 0.4% (m/v) and soybean oil unsaponifiables at 0.8% (m/V) in chloroform were used as standards for the identification of unsaponifiables. Quantification was performed by gas chromatography using purified alkyl furans and alkyl triols in addition to aliphatic alkane fractions from avocado, purified sterol fractions from soy, and tocopherol standards.

Patients

OA human articular cartilage was obtained from 12 different patients (five women and seven men; mean age of 62 ± 6.2 years) with knee OA at the time of total knee joint replacement surgery. All the subjects provided their informed consent, and ethical approval (ethics committee agreement of Liège University, no. B70720108313) was granted for this study.

Cartilage Processing and Chondrocyte Culture in Alginate Beads

To allow testing the product on primary human OA chondrocytes, which are one of the therapeutic targets of ASU, we used freshly isolated chondrocytes expressing inflammatory phenotypes defined by a high rate of IL-6 production (up to 40 ng/μg DNA). To obtain OA chondrocytes, the following process was used: 1. On dissection, the femoral, patellar, and tibial articular surfaces were evaluated for gross morphological modifications of cartilage according to the Collins diagram (Collins D and McElligott T, 1960). The severity of lesions was, thus, recorded for each sample. Different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow–gray area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; III, subchondral bone denudation on one or more articular surfaces, in less than 50% of the most damage compartment; and IV, subchondral bone denudation on more than 50% of the articular surface at least in one tibial plateau. All donors showed OA cartilage lesions of grade III to IV. 2. We evaluated the IL-6 levels produced by cultured chondrocytes using an immunoassay.

Full-depth articular cartilage was excised and immersed in Dulbecco's modified Eagle's medium (DMEM) (with phenol red and 4.5 g/L of glucose) supplemented with N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) 10 mM, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (all from Biowest, Nuaillé, France). After three washes, the chondrocytes were released from the cartilage by sequential enzymatic digestions with 0.5 mg/ml of hyaluronidase type IV-S (Sigma-Aldrich, Bornem, Belgium) for 30 min at 37°C, 1 mg/ml of pronase E (Merck, Leuven, Belgium) for 1 h at 37°C, and 0.5 mg/ml clostridial collagenase IA (Sigma-Aldrich) for 16–20 h at 37°C. The enzymatically isolated cells were then suspended in alginate beads, as previously described (Sanchez et al., 2002), and maintained in culture for 12 days in DMEM supplemented with 1% ITS+ (Lonza, Verviers, Belgium), 10 mM HEPES, penicillin (100 U/ml) and streptomycin (0.1 mg/ml), 200 μg/ml of glutamine (Lonza), 50 μg/ml of ascorbic acid (Sigma-Aldrich), and 2 mM proline (Sigma-Aldrich). The cells remained in this culture medium (washout medium) for 48 h. After this washout period, the culture medium was changed every 3 days, and the collected supernatants were stored at −20°C until analysis. The chondrocytes were cultured for 12 days either without any ASU (negative control, Ctl) or with 10 μg/ml PIASCLEDINE-ExpASU® or one of the six other nutraceutical products being evaluated in comparison, namely, Arthocare, Avovida, Arthrocen, Insaponifiable 300, Persemax, or Saponic (3 wells/condition). At the end of the culture period, the culture medium was carefully discarded, and the beads were dissolved in 1 ml of 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1,200 rpm for 10 min. With this method, two fractions were collected: the supernatant containing macromolecules originating from the further-removed matrix (FRM) and a pellet containing cells with their associated matrix (CM).

Twelve cultures have been performed. Each culture was performed with chondrocytes coming from one single cartilage specimen. Given the limited number of cells per specimen, it was not possible to test all products on the same specimen. Therefore, the products were separated into two groups. Each group was composed of 3 products and the reference product PIASCLEDINE-ExpASU®. Each product group has been tested on six independent cultures. Each culture condition was performed in triplicates.

TABLE 1 | Product composition by comparison of gas chromatography profiles. The results are expressed in milligrams per capsule (mg/capsule) for each indicated fraction.

	Piascledine E-ASU	Persemax	Saponic	Arthrocen	Arthocare	Avovida	Insaponifiable 300
Mass (mg/capsule)	307.0	356.6	352.0	372.5	773.3	296.8	381.0
Lipid content (mg/capsule)	305.5	185.1	133.0	212.0	666.8	220.8	201.4
Alkyl furans (mg/capsule)	65.2 ± 2.0	Absence	Absence	Absence	Absence	Absence	Absence
Alkyl triols & aliphatic alkanes (mg/capsule)	3.0–15.0	Absence	Absence	Absence	Absence	Absence	Absence
Tocopherols (mg/capsule)	73.7 ± 4.1	1.8	Absence	1.5	0.1	Absence	1.8
Sterols (mg/capsule)	106.0 ± 5.1	124.0	102.0	126.8	195.4	138.4	154.2
Squalene (mg/capsule)	2.0–30.0	Absence	Absence	Traces	Traces (0.2)	Absence	Traces (0.4)

DNA Assay

The DNA content of the culture was measured according to the fluorometric method using Hoechst (Labarca and Paigen, 1980).

Immunoassay for Aggrecan, Interleukin-6 and -8, and MMP-3

The total AGG production corresponded to the AGG accumulated in the alginate beads. AGG at D12 and IL-6, IL-8, and MMP-3 only in the supernatant (cumulative production D3_D6_D9 and D12) were measured by specific enzyme-amplified sensitivity immunoassays (Invitrogen, Merelbeke, Belgium).

LDH Assay

Cell viability was estimated by quantifying the release of LDH in the culture supernatant, as previously described (Mathy-Hartert et al., 2009). A sample of the supernatant or dilutions of the standard solution (LDH from rabbit muscle) was mixed with Tris buffer [10 mM Tris-HCl (pH 8.5) and, 0.1% bovine serum albumin] containing 800 mM lactate. Then, a colorimetric reagent containing 1.6 mg/ml iodonitrotetrazolium chloride (Sigma-Aldrich), 4 mg/ml nicotinamide adenine dinucleotide (Roche Diagnostics, Brussels, Belgium), and 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich) was added, and the absorbance at 492 nm was read after 10 min of incubation at room temperature.

Nitric Oxide Assay

Nitrite and nitrate are stable end products of nitric oxide. Nitrate was reduced to nitrite by its addition to the supernatant of nitrate reductase (0.25 U/ml) for 20 min at 37°C. The cumulative nitrite concentrations in conditioned culture supernatants after 3, 6, 9, and 12 days of culture were determined *via* a spectrophotometric method based upon the Griess reaction. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

Statistical Analysis

Data from *in vitro* experiments were analyzed and compared using a paired Student's *t*-test and bilateral hypothesis followed by Bonferroni adjustment. Differences were considered significant when $p < 0.05$. Data are presented as histograms. The exact *p* values are provided, and asterisk representations were also performed. All data were analyzed by R software.

RESULTS

ASU Products Composition

For all the products except Piascledine®300, the lipidic part extracted by the Soxhlet device represented less than 300 mg/capsule lipidic part, while the PIASCLEDINE-ExpASU® capsules contained 305.5 mg of the lipidic part (**Table 1**).

The composition of the products was compared by superimposing their chromatographic profiles (**Figure 1**). The purified standard of unsaponifiable fractions from avocados and soybeans was used for characterization and quantification. PIASCLEDINE-ExpASU® showed a specific profile with the presence of chromatographic peaks corresponding to alkyl furans and alkyl triols. Subsequent quantification showed that the PIASCLEDINE-ExpASU® product contained 65.2 ± 2.0 mg/capsule of alkyl furans and 3.0–15.0 mg/capsule of alkyl triols and aliphatic alkane fractions. All other products investigated in our study were devoid of these unsaponifiable compounds (**Table 1**).

Arthocare (0.1 mg/capsule), Arthrocen (1.5 mg/capsule), Insaponifiable 300 (1.8 mg/capsule), Persemax (1.8 mg/capsule), and PIASCLEDINE-ExpASU® (73.7 mg/capsule) contained quantifiable amounts of tocopherol, while tocopherol was undetectable in Avovida and Saponic. Only PIASCLEDINE-ExpASU® contained squalene (2 mg/capsule). The sterol content varied depending on the product: 102 mg/capsule in Saponic, 106 mg/capsule in PIASCLEDINE-ExpASU®, 124 mg/capsule in Persemax, 126.8 mg/capsule in Arthrocen, 138.4 mg/capsule in Avovida, 154.2 mg/capsule in Insaponifiable 300, and 195.4 mg/capsule in Arthocare.

NO Production

Primary OA human chondrocytes were cultured for 12 days in alginate beads with or without ASU products. Cell viability, evaluated by LDH release and DNA content, were not affected by the tested compounds (data not shown).

Under basal conditions, the NO production was 69.11 ± 20.08 nmoL/μg of DNA. As illustrated in **Figure 2**, Arthrocen, PIASCLEDINE-ExpASU®, and Persemax significantly decreased NO production (Ctl vs. PIASCLEDINE-ExpASU®: $p < 0.0001$; Ctl vs. Arthrocen: $p = 0.01563$; Ctl vs. Persemax: $p = 0.00044$). However, the effects of PIASCLEDINE-ExpASU® on NO production were significantly higher than those of other compounds ($p < 0.0001$). In contrast, Arthrocare, Avovida, Insaponifiable 300, and Saponic had no significant effect on NO production.

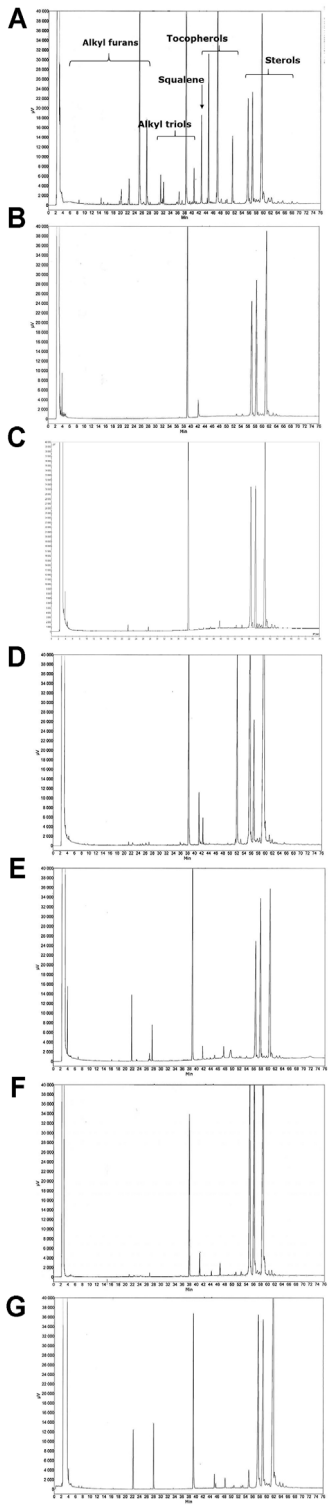


FIGURE 1 | Typical gas chromatography profiles of the products. **(A)** PIASCLEDINE-ExpASU®; **(B)** Saponic; **(C)** Persemax; **(D)** Arthocare; **(E)** Avovida; **(F)** Insaponifiable 300; and **(G)** Arthrocen. For each product, the mass of 20 individual capsules was weighed and recorded. The content of the capsules was collected and subjected to a Soxhlet extraction using chloroform. The mass of the SVOC fraction was determined by gravimetry and

(*Continued*)

FIGURE 1 | recorded as the lipidic part. The contents of the lipidic part were characterized by gas chromatography analysis using a VARIAN 3400 chromatograph. The composition of the products was compared by superimposing their chromatographic profiles. The purified standard of unsaponifiable fractions from avocado and soybean was used for characterization and quantification.

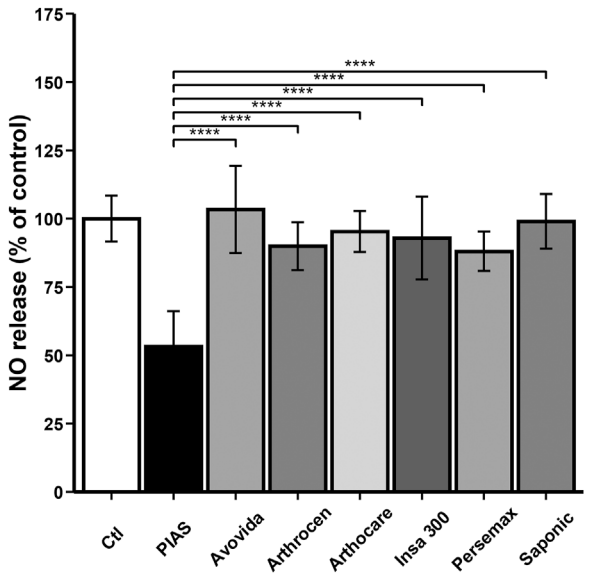


FIGURE 2 | Effect of ASU products tested on total NO production. The chondrocytes were cultured for 12 days either without any ASU (negative control, Ctl) or with 10 µg/ml of either PIASCLEDINE-ExpASU® (PIAS) or one of the six other nutraceutical products being evaluated in comparison, namely, Avovida, Arthrocen, Arthocare, Insaponifiable 300 (Insa 300), Persemax, or Saponic. Each product has been tested on six independent cultures. Each culture condition was performed in triplicates. The results are expressed as the percent control and represented by the mean ± SD. ****$p <$ 0.0001 versus PIASCLEDINE-ExpASU®.

AGG Production

Except for Persemax and Saponic, all other products either slightly or significantly increased the total AGG production by human OA chondrocytes (Ctl vs. PIASCLEDINE-ExpASU®: p = 0.10116; Ctl vs. Arthrocen: p = 0.04979; Ctl vs. Arthocare: p = 0.00010; Ctl vs. Persemax: p = 0.00063) (**Figure 3**).

Interleukin-6

Except for Avovida and Saponic, all other products significantly decreased IL-6 production by human OA chondrocytes (Ctl vs. PIASCLEDINE-ExpASU®: $p < 0.0001$; Ctl vs. Arthrocen: p = 0.00031; Ctl vs. Arthocare: p = 0.00244; Ctl vs. Insaponifiable 300: p = 0.00038; Ctl vs. Persemax: p = 0.00014) (**Figure 4**). However, the inhibitory effect of PIASCLEDINE-ExpASU® was significantly higher than that of the other compounds ($p < 0.0001$).

Interleukin-8

As illustrated in **Figure 5**, except for Avovida and Arthocare, all other ASU products significantly decreased IL-8 production by

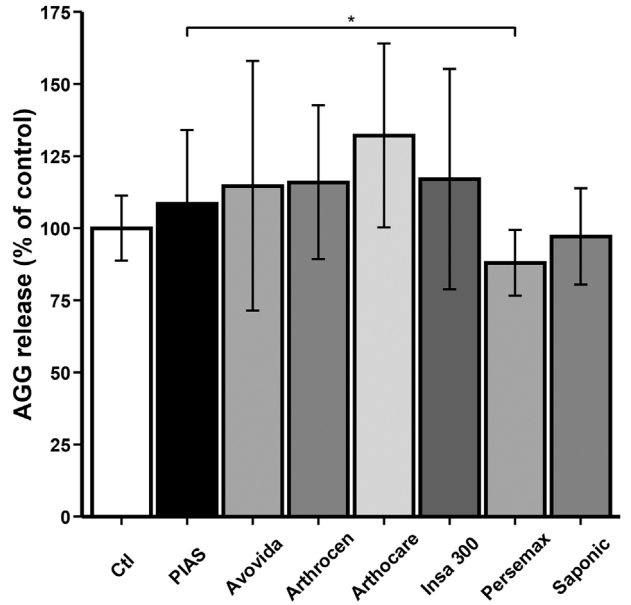


FIGURE 3 | Effect of ASU products tested on total AGG production. The chondrocytes were cultured for 12 days either without any ASU (negative control, Ctl) or with 10 µg/ml of either PIASCLEDINE-ExpASU® (PIAS) or one of the six other nutraceutical products being evaluated in comparison, namely, Avovida, Arthrocen, Arthocare, Insaponifiable 300 (Insa 300), Persemax, or Saponic. Each product has been tested on six independent cultures. Each culture condition was performed in triplicates. The results are expressed as the percent control and represented by the mean ± SD. *p < 0.05 versus PIASCLEDINE-ExpASU®.

chondrocytes (Ctl vs. PIASCLEDINE-ExpASU®: $p < 0.0001$; Ctl vs. Arthrocen: $p = 0.00711$; Ctl vs. Insaponifiable 300: $p = 0.00658$; Ctl vs. Persemax: $p = 0.00192$; Ctl vs. Saponic: $p = 0.00603$). However, the inhibitory effect of PIASCLEDINE-ExpASU® was significantly higher than that of the other compounds ($p < 0.0001$).

Matrix Metalloprotease-3

PIASCLEDINE-ExpASU® and Insaponifiable 300 significantly decreased the basal production of MMP-3 (Ctl vs. PIASCLEDINE-ExpASU®: $p = 0.00019$; Ctl vs. Insaponifiable 300: $p = 0.00204$) while Arthocare, Arthrocen, Avovida, and Persemax had no significant effects, and Saponic significantly increased MMP-3 production (Ctl vs. Saponic: $p = 0.04103$) (**Figure 6**).

DISCUSSION

ASU products are commonly used to treat OA patients. They are members of a class of drugs called SYSADOA, which have been recommended by many scientific and medical societies for relieving OA symptoms (Jordan et al., 2003; Zhang et al., 2005; Anon 2021b). However, ASU products may differ in their composition as a consequence of their manufacturing process. Until now, the composition of the main ASU products available on the market had never been compared. Moreover, it has never been demonstrated how the composition of ASU products might influence their pharmacological activity. For the first time, our study compared the chromatographic profiles of seven ASU products commercially available with the aim of identifying differences in their pharmacological activity on human OA chondrocytes. As chondrocytes are key players in cartilage degradation in OA, our study also contributes to identifying which ASU product components may be significant in explaining differences in the pharmacological activity of ASU products.

Our characterization clearly of ASU products shows that PIASCLEDINE-ExpASU® has a unique composition characterized by the presence of alkyl furans, alkyl triols, and squalene. These compounds were absent in all of the other mixtures, indicating that these molecules could be responsible for the different pharmacological activity profiles of PIASCLEDINE-ExpASU®. Indeed, PIASCLEDINE-ExpASU® was the most effective inhibitor of NO, IL-6, IL-8, and MMP-3. The unique formulation of PIASCLEDINE-ExpASU® is obtained by a particular proprietary-patented process. Our results confirmed those of a previous study conducted by the manufacturer (Msika et al., 2008), which demonstrated that the commercial nutraceutical products called Dasuquin and Avoca ASU (Nutramax, United States) totally differ in composition from the common and natural sterol-based avocado and

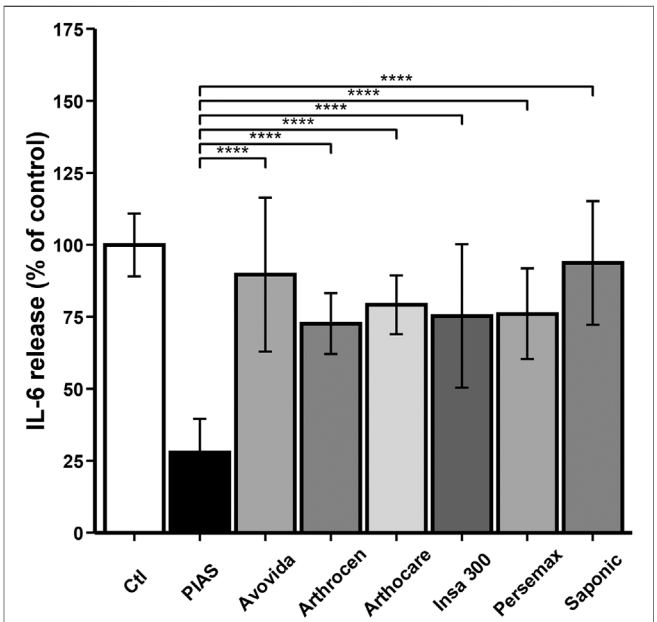


FIGURE 4 | Effect of ASU products tested on total IL-6 production. The chondrocytes were cultured for 12 days either without any ASU (negative control, Ctl) or with 10 µg/ml of either PIASCLEDINE-ExpASU® (PIAS) or one of the six other nutraceutical products being evaluated in comparison, namely, Avovida, Arthrocen, Arthocare, Insaponifiable 300 (Insa 300), Persemax, or Saponic. Each product has been tested on six independent cultures. Each culture condition was performed in triplicates. The results are expressed as the percent control and represented by the mean ± SD. ****p < 0.0001 versus PIASCLEDINE-ExpASU®.

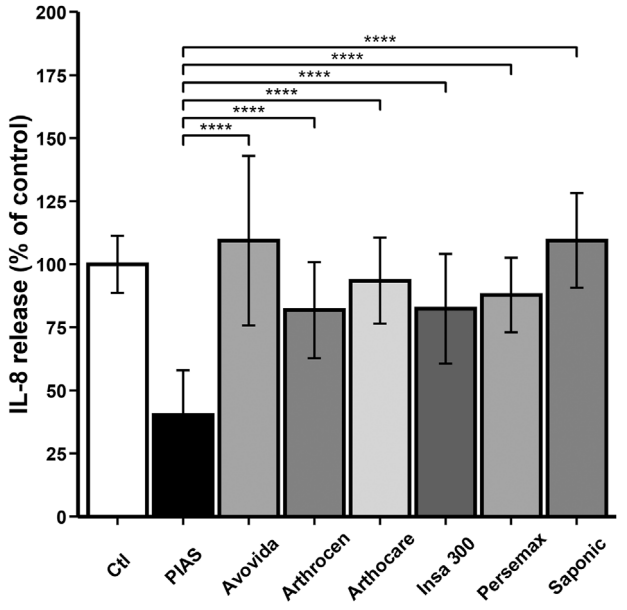


FIGURE 5 | Effect of ASU products tested on total IL-8 production. The chondrocytes were cultured for 12 days either without any ASU (negative control, Ctl) or with 10 µg/ml of either PIASCLEDINE-ExpASU® (PIAS) or one of the six other nutraceutical products being evaluated in comparison, namely, Avovida, Arthrocen, Arthocare, Insaponifiable 300 (Insa 300), Persemax, or Saponic. Each product has been tested on six independent cultures. Each culture condition was performed in triplicates. The results are expressed as the percent control and represented by the mean ± SD. ****$p <$ 0.0001 versus PIASCLEDINE-ExpASU®.

soybean unsaponifiables and also from Piascledine®300, namely, in the absence of or not having detectable levels of specific molecules from common natural avocado unsaponifiables such as the key molecule citrostadienol, the absence of alkyl furans, alkyl triols, and squalene and finally, the presence of brassicasterol, which is highly specific for rapeseed oil and related unsaponifiables (Msika et al., 2008).

Another major constituting element of ASU mixtures are the phytosterols beta-sitosterol, campesterol, and stigmasterol. Lippiello et al. compared the sterol composition of NMX-1000™ and Piascledine®300 and tested the influence of the sterol content on the upregulation of glycosaminoglycan and collagen synthesis by bovine chondrocytes *in vitro* and on the upregulation of PGE_2 in an IL-1–induced *in vitro* model of articular cartilage breakdown (Lippiello et al., 2008). They reported that PIASCLEDINE-ExpASU® has a different gas chromatographic profile to NMX-1000™ with three additional major unidentified peaks, while the total content of sterols was similar. This confirms our data showing that PIASCLEDINE-ExpASU® has a unique composition. However, in contrast to our study, they showed that both ASU products, equalized to contain equivalent sterol contents, had similar metabolic effects on articular chondrocytes. The two products inhibited prostaglandin synthesis, metalloprotease activity, and the release of radiolabeled sulfate from pre-labeled cartilage. This discrepancy can be explained by differences in the methodological approach used to test the pharmacological activity of ASUs. We used human chondrocytes in alginate beads, while Lippiello et al. used bovine monolayer chondrocytes and cartilage explants. Furthermore, the length of time of cell exposure to the tested components also drastically differed. In our study, cells were treated for 12 days while in the study of Lippiello et al., the exposure time was shorter. Another major difference between the two studies was that we used doses of ASUs by weight in contrast to Lippiello et al. who standardized according to the sterol content. Finally, the investigated parameters were different. We tested the effects of ASUs on NO, IL-6, and IL-8, main inflammatory mediators implicated in OA pathogenesis. Through cytokine-induced MAP kinases, NF-κB activation, and oxidative phosphorylation, these mediators contribute to systemic inflammation (Lambert et al., 2021). These parameters were not explored by Lippiello et al. Interestingly, in our study, this product did not have the highest level of sterols of the evaluated ASUs but was the most active. Indeed, compared to PIASCLEDINE-ExpASU®, Arthocare contained almost twice the amount ofsterols but did not demonstrate effective inhibition of MMP-3, NO, and IL-8 and was significantly less effective on IL-6 than PIASCLEDINE-ExpASU®. Furthermore, surprisingly, there was an absence of effects for Avovida. This can be explained by the absence of four key components in its composition. In addition to the absence of alkyl furans, alkyl triols, and squalene, Avovida does not contain

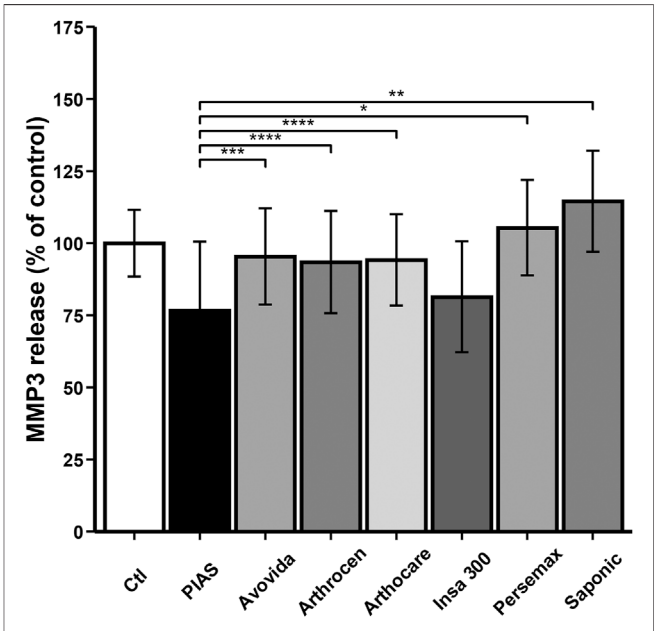


FIGURE 6 | Effect of ASU products tested on total MMP-3 production. The chondrocytes were cultured for 12 days either without any ASU (negative control, Ctl) or with 10 µg/ml of either PIASCLEDINE-ExpASU® (PIAS) or one of the six other nutraceutical products being evaluated in comparison, namely, Avovida, Arthrocen, Arthocare, Insaponifiable 300 (Insa 300), Persemax, or Saponic. Each product has been tested on six independent cultures. Each culture condition was performed in triplicates. The results are expressed as the percent control and represented by the mean ± SD. *$p <$ 0.05 versus PIASCLEDINE-ExpASU®, **$p <$ 0.01 versus PIASCLEDINE-ExpASU®, ***$p <$ 0.001 versus PIASCLEDINE-ExpASU®, and ****$p <$ 0.0001 versus PIASCLEDINE-ExpASU®.

tocopherols. These data suggest that tocopherols could play a role in determining the pharmacological activities of ASU mixtures.

There are some limitations to our study with regard to interpreting the impact of composition on the pharmacological activity. One is the lack of definitive identification of the various minor components of the ASUs. Another is the small number of parameters investigated. It is possible that when assessing other parameters, the activity of ASU products would show different trends. Finally, we investigated a limited number of products. We cannot exclude the possibility that other commercially available products not studied here may show effects comparable to PIASCLEDINE-ExpASU®.

In conclusion, our study demonstrates that PIASCLEDINE-ExpASU® has a unique composition characterized by the presence of alkyl furans, alkyl triols, and squalene. This specific composition can explain its higher efficacy on pro-inflammatory and pro-catabolic mediators compared to other ASU products. Our data also confirm that PIASCLEDINE-ExpASU® shows beneficial effects on the chondrocyte metabolism *via* AGG, which could explain, in part, its clinical efficacy in OA patients. Therefore, to extrapolate the clinical data obtained with one ASU formulation to another one remains fully speculative. Our findings justify investigating each ASU product in clinical trials before claiming any efficacy with respect to OA symptoms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics committee agreement of Liège University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CL, GBe, GBo, FP, TB, M-CL, CB, and YH conceived and planned the experiments. CL and GBe carried out the experiments. CL, GBe, and YH analyzed the results. CL, GBe, GBo, and YH performed the statistical analysis. CL, GBe, and YH wrote the manuscript in consultation with GBo, FP, TB, M-CL, and CB. All authors contributed to the article and approved the submitted version.

FUNDING

The cost of the present work was supported by Laboratoires Expanscience.

ACKNOWLEDGMENTS

The authors would like to thank the team and surgeons of the orthopedic surgery department of Bois de l'Abbaye (Seraing, Belgium) for their help in this study.

REFERENCES

Abubakar, A. R., and Haque, M. (2020). Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. *J. Pharm. Bioallied Sci.* 12, 1–10. Available at: /pmc/articles/PMC7398001/ (Accessed August 31, 2021). doi:10.4103/jpbs.JPBS_175_19

Anon (2021a). Efficacy and Safety of Avocado/soybean Unsaponifiables in the Treatment of Symptomatic Osteoarthritis of the Knee and Hip. A Prospective, Multicenter, Three-Month, Randomized, Double-Blind, Placebo-Controlled Trial - PubMed. Available at: https://pubmed.ncbi.nlm.nih.gov/9476272/ (Accessed May 3, 2021).

Anon (2021b). Recommendations For the Medical Management of Osteoarthritis of the Hip and Knee: 2000 Update. American College of Rheumatology Subcommittee on Osteoarthritis Guidelines - PubMed. Available at: https://pubmed.ncbi.nlm.nih.gov/11014340/ (Accessed September 6, 2021). .

Appelboom, T., Schuermans, J., Verbruggen, G., Henrotin, Y., and Reginster, J. Y. (2001). Symptoms Modifying Effect of Avocado/soybean Unsaponifiables (ASU) in Knee Osteoarthritis. A Double Blind, Prospective, Placebo-Controlled Study. *Scand. J. Rheumatol.* 30, 242–247. Available at: https://pubmed.ncbi.nlm.nih.gov/11578021/ (Accessed May 3, 2021). doi:10.1080/030097401316909602

Brown, B. I. (1973). Qualitative and Quantitative Estimation by Thin-Layer and Gas Chromatography of a Series of C17 Oxygenated Aliphatic Compounds in the Avocado (Persea Americana). *J. Chromatogr. A* 86, 239–245. doi:10.1016/s0021-9673(01)81264-4

Edgard Henrotin, Y. (2018). Avocado/Soybean Unsaponifiables (Piacledine300) Show Beneficial Effect on the Metabolism of Osteoarthritic Cartilage, Synovium and Subchondral Bone: An Overview of the Mechanisms. *AIMS Med. Sci.* 5, 33–52. Available at: http://www.aimspress.com/article/doi/10.3934/medsci.2018.1.33 (Accessed May 3, 2021). doi:10.3934/medsci.2018.1.33

Farines, M., Soulier, J., Rancurel, A., Montaudoin, M. G., and Leborgne, L. (1995). Influence of Avocado Oil Processing on the Nature of Some Unsaponifiable Constituents. *J. Am. Oil Chem. Soc.* 72, 473–476. Available at: https://link.springer.com/article/10.1007/BF02636092 (Accessed August 31, 2021). doi:10.1007/bf02636092

Ghasemian, M., Owlia, S., and Owlia, M. B. (2016). Review of Anti-inflammatory Herbal Medicines. *Adv. Pharmacol. Sci.* 2016, 9130979, 2016 . Available at: https://pubmed.ncbi.nlm.nih.gov/27247570/ (Accessed April 23, 2021). doi:10.1155/2016/9130979

Glyn-Jones, S., Palmer, A. J. R., Agricola, R., Price, A. J., Vincent, T. L., Weinans, H., et al. (2015). "Osteoarthritis,"in *The Lancet*, 386 9991), 376–387. Epub of Print. doi:10.1016/s0140-6736(14)60802-3

Honvo, G., Reginster, J. Y., Rabenda, V., Geerinck, A., Mkinsi, O., Charles, A., et al. (2019). Safety of Symptomatic Slow-Acting Drugs for Osteoarthritis: Outcomes of a Systematic Review and Meta-Analysis. *Drugs Aging* 36, 65–99. Available at: /pmc/articles/PMC6509099/ (Accessed April 23, 2021). doi:10.1007/s40266-019-00662-z

Hunter, D. J., March, L., and Chew, M. (2020). Osteoarthritis in 2020 and beyond: a Lancet Commission. *Lancet* 396, 1711–1712. Available at: https://pubmed.ncbi.nlm.nih.gov/33159851/ (Accessed September 6, 2021). doi:10.1016/S0140-6736(20)32230-3

Jordan, K. M., Arden, N. K., Doherty, M., Bannwarth, B., Bijlsma, J. W., Dieppe, P., et al. (2003). EULAR Recommendations 2003: an Evidence Based Approach to the Management of Knee Osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). *Ann. Rheum. Dis.* 62,

1145–1155. Available at: https://pubmed.ncbi.nlm.nih.gov/14644851/ (Accessed September 6, 2021). doi:10.1136/ard.2003.011742

Karel, P., Coste, P., Géher, P., and Krejci, G. (2010). Efficacy and Safety of Piascledine 300 versus Chondroitin Sulfate in a 6 Months Treatment Plus 2 Months Observation in Patients with Osteoarthritis of the Knee. *Clin. Rheumatol.* 29, 659–670. Available at: https://pubmed.ncbi.nlm.nih.gov/20179981/(Accessed May 3, 2021).

Kashman, Y., Néeman, I., and Lifshitz, A. (1969). New Compounds from Avocado Pear. *Tetrahedron* 25, 4617–4631. doi:10.1016/s0040-4020(01)83005-2

Labarca, C., and Paigen, K. (1980). A Simple, Rapid, and Sensitive DNA Assay Procedure. *Anal. Biochem.* 102, 344–352. Available at: https://pubmed.ncbi.nlm.nih.gov/6158890/ (Accessed April 23, 2021). doi:10.1016/0003-2697(80)90165-7

Lambert, C., Zappia, J., Sanchez, C., Florin, A., Dubuc, J.-E., and Henrotin, Y. (2021). The Damage-Associated Molecular Patterns (DAMPs) as Potential Targets to Treat Osteoarthritis: Perspectives from a Review of the Literature. *Front. Med.* 7, 607186. doi:10.3389/fmed.2020.607186

Lippiello, L., Nardo, J. V., Harlan, R., and Chiou, T. (2008). Metabolic Effects of Avocado/soy Unsaponifiables on Articular Chondrocytes. *Evidence-Based Complement. Altern. Med.*, 5, 191–197. Available at: https://pubmed.ncbi.nlm.nih.gov/18604259/ (Accessed September 6, 2021). doi:10.1093/ecam/nem132

Loeser, R. F., Goldring, S. R., Scanzello, C. R., and Goldring, M. B. (2012). Osteoarthritis: a Disease of the Joint as an Organ. *Arthritis Rheum.* 64, 1697–1707. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22392533. doi:10.1002/art.34453

Maheu, E., Cadet, C., Marty, M., Moyse, D., Kerloch, I., Coste, P., et al. (2014). Randomised, Controlled Trial of Avocado-Soybean Unsaponifiable (Piascledine) Effect on Structure Modification in Hip Osteoarthritis: The ERADIAS Study. *Ann. Rheum. Dis.* 73, 376–384. Available at: https://pubmed.ncbi.nlm.nih.gov/23345601/ (Accessed April 23, 2021). doi:10.1136/annrheumdis-2012-202485

Maheu, E., Mazières, B., Valat, J. P., Loyau, G., Le Loët, X., Bourgeois, P., et al. (1998). Symptomatic Efficacy of Avocado/soybean Unsaponifiables in the Treatment of Osteoarthritis of the Knee and Hip: a Prospective, Randomized, Double-Blind, Placebo-Controlled, Multicenter Clinical Trial with a Six-Month Treatment Period and a Two-Month Followup Demonstrating a Persistent Effect. *Arthritis Rheum.* 41, 81–91. Available at: https://pubmed.ncbi.nlm.nih.gov/9433873/ (Accessed May 3, 2021). doi:10.1002/1529-0131(199801)41:1<81::AID-ART11>3.0.CO;2-9

Mathy-Hartert, M., Jacquemond-Collet, I., Priem, F., Sanchez, C., Lambert, C., and Henrotin, Y. (2009). Curcumin Inhibits Pro-inflammatory Mediators and Metalloproteinase-3 Production by Chondrocytes. *Inflamm. Res.* 58, 899–908. Available at: https://pubmed.ncbi.nlm.nih.gov/19579007/ (Accessed April 23, 2021). doi:10.1007/s00011-009-0063-1

Msika, P., Baudouin, C., Saunois, A., and Bauer, T. (2008). Avocado/soybean Unsaponifiables, ASU EXPANSCIENCE, Are Strictly Different from the Nutraceutical Products Claiming ASU Appellation. *Osteoarthritis Cartilage* 16, 1275–1276. Available at: https://pubmed.ncbi.nlm.nih.gov/18420430/ (Accessed September 6, 2021). doi:10.1016/j.joca.2008.02.017

Néeman, I., Lifshitz, A., and Kashman, Y. (1970). New Antibacterial Agent Isolated from the Avocado Pear. *Appl. Microbiol.* 19, 470–473. Available at: https://pubmed.ncbi.nlm.nih.gov/4985830/ (Accessed August 31, 2021). doi:10.1128/am.19.3.470-473.1970

Rahmati, M., Mobasheri, A., and Mozafari, M. (2016). Inflammatory Mediators in Osteoarthritis: A Critical Review of the State-Of-The-Art, Current Prospects, and Future Challenges. *Bone* 85, 81–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26812612. doi:10.1016/j.bone.2016.01.019

Sanchez, C., Mateus, M. M., Defresne, M. P., Crielaard, J. M., Reginster, J. Y., and Henrotin, Y. E. (2002). Metabolism of Human Articular Chondrocytes Cultured in Alginate Beads. Longterm Effects of Interleukin 1beta and Nonsteroidal Antiinflammatory Drugs. *J. Rheumatol.* 29, 772–782. Available at: https://pubmed.ncbi.nlm.nih.gov/11950021/ (Accessed April 23, 2021).

Swain, S., Sarmanova, A., Coupland, C., Doherty, M., and Zhang, W. (2020). Comorbidities in Osteoarthritis: A Systematic Review and Meta-Analysis of Observational Studies. *Arthritis Care Res. (Hoboken)* 72, 991–1000. Available at: https://pubmed.ncbi.nlm.nih.gov/31207113/ (Accessed September 6, 2021). doi:10.1002/acr.24008

Vos, T., Abajobir, A. A., Abbafati, C., Abbas, K. M., Abate, K. H., Abd-Allah, F., et al. (2017). Global, Regional, and National Incidence, Prevalence, and Years Lived with Disability for 328 Diseases and Injuries for 195 Countries, 1990-2016: A Systematic Analysis for the Global Burden of Disease Study 2016. *Lancet* 390, 1211–1259. Available at: https://pubmed.ncbi.nlm.nih.gov/28919117/ (Accessed May 3, 2021) doi:10.1016/S0140-6736(18)32279-7

Zhang, W., Doherty, M., Arden, N., Bannwarth, B., Bijlsma, J., Gunther, K. P., et al. (2005). EULAR Evidence Based Recommendations for the Management of Hip Osteoarthritis: Report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT). *Ann. Rheum. Dis.* 64, 669–681. Available at: /pmc/articles/PMC1755499/ (Accessed September 6, 2021). doi:10.1136/ard.2004.028886

Conflict of Interest: GBe, GBo, FP, TB, M-CL, and CB are employees of Laboratoires Expanscience. YH received consulting fees from Laboratoires Expanscience, Artialis, Tilman, Biose, Immubio, Naturex, and GeneQuine Biotherapeutics. CL declared no conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 17 January 2022
doi: 10.3389/fphar.2021.753240

A Nitrobenzoyl Sesquiterpenoid Insulicolide A Prevents Osteoclast Formation *via* Suppressing c-Fos-NFATc1 Signaling Pathway

Yanhui Tan[1,2†], Minhong Ke[2†], Zhichao Li[1], Yan Chen[2], Jiehuang Zheng[2], Yiyuan Wang[2], Xuefeng Zhou[3], Gang Huang[4*] and Xiaojuan Li[2*]*

[1]State Key Laboratory for Chemistry and Molecular Engineering of Medicinal Resources, Collaborative Innovation Center for Guangxi Ethnic Medicine, School of Chemistry and Pharmaceutical Sciences, Guangxi Normal University, Guilin, China, [2]Laboratory of Anti-inflammatory and Immunomodulatory Pharmacology, Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China, [3]CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China, [4]Integrated Traditional Chinese and Western Medicine Hospital, Southern Medical University, Guangzhou, China

OPEN ACCESS

Edited by:
Raquel Largo,
Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain

Reviewed by:
Yuanli Chen,
Hefei University of Technology, China
Ting Zheng,
Qilu University of Technology, China
Mervyn Weitzmann,
Emory University, United States

***Correspondence:**
Xuefeng Zhou
xfzhou@scsio.ac.cn
Gang Huang
huanggang@smu.edu.cn
Xiaojuan Li
lixiaoj@smu.edu.cn

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 04 August 2021
Accepted: 19 November 2021
Published: 17 January 2022

Citation:
Tan Y, Ke M, Li Z, Chen Y, Zheng J, Wang Y, Zhou X, Huang G and Li X (2022) A Nitrobenzoyl Sesquiterpenoid Insulicolide A Prevents Osteoclast Formation via Suppressing c-Fos-NFATc1 Signaling Pathway. Front. Pharmacol. 12:753240. doi: 10.3389/fphar.2021.753240

It is a viable strategy to inhibit osteoclast differentiation for the treatment of osteolytic diseases such as osteoporosis, rheumatoid arthritis and tumor bone metastases. Here we assessed the effects of insulicolide A, a natural nitrobenzoyl sesquiterpenoid derived from marine fungus, on receptor activator of nuclear factor-κB ligand (RANKL)-stimulated osteoclastogenesis *in vitro* and its protective effects on LPS-induced osteolysis mice model *in vivo*. The results demonstrated that insulicolide A inhibited osteoclastogenesis from 1 μM *in vitro*. Insulicolide A could prevent c-Fos and nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) nuclear translocation and attenuate the expression levels of osteoclast-related genes and DC-STAMP during RANKL-stimulated osteoclastogenesis but have no effects on NF-κB and MAPKs. Insulicolide A can also protect the mice from LPS-induced osteolysis. Our research provides the first evidence that insulicolide A may inhibit osteoclastogenesis both *in vitro* and *in vivo*, and indicates that it may have potential for the treatment of osteoclast-related diseases.

Keywords: insulicolide A, osteoclast, receptor activator of nuclear factor-κB ligand (RANKL), LPS, nuclear factor of activated T-cells cytoplasmic 1 (NFATc1)

INTRODUCTION

Bone resorption and formation are keeping a dynamic balance to maintain skeletal renewal and integrity. However, hyperactivity of osteoclast can lead to bone osteoclastic diseases such as osteoporosis, rheumatoid arthritis (RA), and tumor bone metastases (Perpetuo et al., 2017; Tsukasaki and Takayanagi, 2019; Győri and Mócsai, 2020; Kim et al., 2020; Liu et al., 2021). Therefore, targeting osteoclast formation has been regarded as a practicable treatment strategy to improve the prognosis of patients with bone destructive diseases (Broadhead et al., 2011; Stickeler and Fehm, 2014).

Osteoclasts, originate from bone marrow mononuclear macrophage lineage, are multinuclear and functioning as bone-resorbing cells (Boyle et al., 2003). Both receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are essential for proliferation

and differentiation of osteoclast (Arai et al., 1999; Cappellen et al., 2002; Lorenzo, 2017). Once RANKL is bound to its homologous receptor RANK, it first engages the adaptor protein tumor necrosis factor receptor-associated factor 6 (TRAF6) (Tanaka et al., 2005), then quickly triggers the signal cascade including nuclear factor NF-κB, mitogen-activated protein kinases (MAPKs) (Matsumoto et al., 2000; Li et al., 2003; Wada et al., 2006; Lee et al., 2016), followed by activating c-Fos (Grigoriadis et al., 1994). Activated NF-κB or c-Fos can induce the activation and amplification of the downstream nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), which can initiate the expression of osteoclast-related genes including OSCAR, Blimp1, DC-STAMP, cathepsin K, TRAP, and so on (Takayanagi et al., 2002; Edwards and Mundy, 2011).

Natural products from marine fungus have become a rich source for developing novel drugs for the treatment of various diseases (Kang et al., 2015; Malve, 2016). Nitrobenzoyl sesquiterpenoids (NSs) represent a novel and rare class of compounds isolated from marine fungi, and only seven are identified until now (Wu et al., 2012; Zhao et al., 2016; Tan et al., 2018). We have identified one NS compound, 6β,9α-dihydroxy-14-*p*-nitrobenzoylcinnamolide (NS4), having the potential to suppress osteoclast formation by inhibiting NF-κB/RelB signaling pathway *via* binding to Arg B246 of NF-κB P65 (Tan et al., 2020). Another NS compound, insulicolide A, isolated from the marine-derived fungus *Aspergillus ochraceus*, has demostrated antiinflammation and antitumor activity *in vitro* (Wang et al., 2014; Guo et al., 2018). However, the influence of insulicolide A on osteoclast differentiation *in vitro* and bone lysis *in vivo* is not yet known.

Here, our study evaluated the suppressive effect of insulicolide A on RANKL-stimulated bone marrow monocytes (BMMs)-derived osteoclastogenesis *in vitro*, and tested the potential protective effects of insulicolide A on a LPS-induced osteolysis mice model *in vivo*. Insulicolide A mitigated osteoclastogenesis by preventing activation of c-Fos and NFATc1 but not affecting NF-κB signaling pathway.

METHODS

Reagents and antibodies

Insulicolide A was extracted from the cultured *Aspergillus ochraceus* Jcma1F17 derived from marine fungus according to the methods described previously (Tan et al., 2018). The structure of the compound was identified by both high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR). The purity of the sample was more than 95% as analyzed by high-performance liquid chromatography (HPLC). The compound was kept under −20°C for long-term storage and dissolved in dimethyl sulfoxide to a concentration of 10 mM as a reserve before usage. Our previous study described the extracting craft and structure of insulicolide A (Wu et al., 2012; Zhao et al., 2016; Tan et al., 2018). RAW264.7 cells stably transfected with luciferase reporter genes of NF-κB and NFATc1 is a gift from Professor Xu (University of Western Australia, Nedlands, Australia). Dulbecco's modified Eagle's medium (DMEM) and alpha minimum essential medium (α-MEM) were provided by Gibco (Rockville, MD, USA). Recombinant mouse M-CSF and RANKL are both from R&D (Minneapolis, Minnesota, USA). MTT, TRAP assay Kit, BAY11-7082, and CSA were all provided by Sigma- Aldrich (St. Louis, MO, USA). Osteo Assay Surface Polystyrene Microplates were provided by Corning (St. Lowell, MA, USA). qPCR Master Mix were obtained from Promega (United States). RNeasy kit and PrimeScript RT reagent kit were purchased from TaKaRa (China). Nuclear isolation kit was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Rabbit mAbs of p65 (#49445), NFATc1 (#8032), p-ERK (#4370), ERK (#4695), p-p38 (#9215), p38 (#9255), p-JNK (#9255), JNK (#9215), c-Fos (#2250), β-actin (#3700), and murine mAb of lamin A/C (#4777) were all provided by CST (Beverly, MA, USA).

Mice

Mice of C57BL/6J and ICR were provided by the medical animal center in Guangdong province, China. The mice were housed in an environment with the temperature of 22–24°C, a light/dark cycle of 12 h and 50–55% humidity. Water and food were provided at liberty. Animal studies were approved by the Committee of the animal protection and utilization of Southern Medical University and institutional animal protection and utilization of Guangxi Normal University.

Cell culture

The marrow cavity of C57BL/6J mice with 6–8 weeks of age was exposed and flushed with sterile PBS under sterile conditions. Cells were then collected, and red blood cells were lysed accordingly. The obtained cells were incubated in α-MEM medium including 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 50 ng/ml of M-CSF, and then nonadherent cells were collected for subsequent use. The mouse RAW264.7 cells transfected with luciferase reporter gene of NFATc1 were incubated in complete DMEM with 10% FBS in an incubator with 5% CO_2 at 37°C.

Cell viability assay

BMMs (1×10^3 cells/well) with or without insulicolide A were treated in α-MEM medium supplemented with 50 ng/ml of M-CSF for 4 days. MTT assay was used to test cell viability following the instructions of the manufacturer.

Osteoclastogenesis and tartrate-resistant acidic phosphatase assay

For osteoclastogenesis assay, BMMs (1×10^4 cells/well) were first incubated with different levels of insulicolide A in a 96-well plate, then stimulated with 100 ng/ml of RANKL or 100 ng/ml of LPS and 50 ng/ml of M-CSF for 3 days. After that, the cells were fixed in 2.5% glutaraldehyde and then stained to assay tartrate-resistant acidic phosphatase (TRAP) activity. Images were taken, and quantitation of osteoclasts (nuclei > 5 for BMMs) were counted.

Bone resorption pit assay

BMMs (1×10^4 cells/well) were first incubated in Osteo Assay Surface Polystyrene Microplate, then administered with insulicolide A at different concentrations, stimulated by 50 ng/ml of M-CSF and 100 ng/ml of RANKL for 7 days. After that, 10% bleach solution was used to wash the cells. The resorption areas of osteoclast were quantified by Image-Pro Plus 6.0.

Nuclear factor of activated T-cell cytoplasmic 1 luciferase reporter assay

The activity of luciferase reporter gene of NFATc1 induced by RANKL was measured as mentioned earlier. RAW264.7 cells, which were transfected with a NFATc1-responsive luciferase construct, were pretreated with insulicolide A and CsA (NFATc1 inhibitor, 1 μM) for 4 h. After incubated by 100 ng/ml of RANKL for 12 h, the activity of luciferase was assayed.

Quantitative real-time polymerase chain reaction

Briefly, BMMs (1×10^6 cells/ml) were treated by insulicolide A at different levels for 4 h, followed by stimulation by RANKL and M-CSF for 24 h. The RNeasy mini kit was first used to isolate the total RNA, then, PrimeScript RT kit was prepared for synthesis of cDNA. Real-time PCR was performed using qPCR Master Mix. Polymerase chain reaction was executed by a procedure of 95°C (30 s), 95°C (5 s), and 60°C (34 s) in 40 cycles. The mouse primers used are as follows: DC-STAMP (forward: AGACGTGGTTTA GGAATGCAGCTC; reverse: TCCTCCATGAACAAACAGTTC CAA), cathepsin K (forward: GGCCAACTCAAGAAGAAAAC; reverse: GTGCTTGCTTCCCTTCTGG), GAPDH (forward: ACACATTGGGGGTAGGAACA; reverse: AACTTTGGCATT GTGGAAGG), OSCAR (forward: CCTAGCCTCATACCC CCAG; reverse: CGTTGATCCCAGGAGTCACAA). The comparative $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of target genes. The mean Ct value of target genes in the experimental groups were normalized to the Ct values of GAPDH.

Western blot analysis

BMMs (1×10^6 cells/ml) were treated with different concentrations of Insulicolide A for 4 h, then incubated with 100 ng/ml RANKL for additional 30 min or 24 h. Total proteins were extracted by using RIPA buffer and cytoplasmic and nuclear proteins were prepared with a nuclear extraction kit. Proteins were separated on SDS-PAGE, and then transferred to PVDF membranes. The primary antibodies were used to incubate with membranes at 4°C overnight after blocked by 5% non-fat milk for 1 h. On the next day, TBST was used to wash the membranes and then the secondary antibodies were used to incubate for another 1 h at room temperature. Finally, the blotted protein bands were obtained with a chemilunimescence kit (Yeasen Biotech, China) and quantification of the band intensities was analyzed by ImageJ software. The expression of p65, p38, ERK, JNK, p-ERK, p-p38, p-JNK were measured after treatment of RANKL for 30 min and the expression of c-Fos, NFATc1, DC-STAMP were measured after RANKL treatment for 24 h.

LPS-induced murine inflammatory osteolysis model *in vivo*

Female ICR mice aged 8–9 weeks were randomly divided into four groups with six mice in each group: the control group (served with PBS), model group (served with LPS), model with low dose Insulicolide A (5 mg/kg), and model with high dose Insulicolide A (10 mg/kg). LPS administration was by intraperitoneal injection (5 mg/kg body weight) on days 1 and 4. Insulicolide A or PBS was administered once daily *via* gavage for 8 days. Eight days later, the ICR mice were euthanized. The left femur of all the animals were obtained and scanned by a micro-CT (CT80, ScancoMedical, Zurich, Switzerland) with the following instrument parameters: 50 kV, 500 μA, and 0.7° rotation step. The parameters of trabecular bone contains the ratio of bone volume to tissue volume (BV/TV), trabecular number (Tb.N), Mean density of TV and trabecular separation (Tb.Sp). Removal of the right femur from experimental mice to fix in 4% PFA at 4°C for 24 h, and then the femurs embedded in paraffin after decalcification in 12% EDTA for 1 month were sectioned for H&E and TRAP. As for the H&E and TRAP staining images, there were $n = 12$ images taken in total per group (two images from each mouse). A 600-μm × 600-μm region of interest located 150 μm below the growth plate of the femur metaphysis was employed for the assessment of the number of TRAP-positive multinucleated cells and osteoclastic surface/bone surface (Oc.S/BS). Histomorphometry analysis was performed using Image Pro Plus 6.0 (IPP) software.

Data analysis

All data were expressed as the mean ± standard deviation of three or more experiments. For multiple comparisons, differences were tested using a regular one-way ANOVA, followed by a Tukey multiple comparison for groups with a Gaussian distribution or with a Friedman test, followed by a Dunn's posttest for multiplicity if a Gaussian distribution could not be assumed. The *p*-values of less than 0.05 were deemed to be statistically significant.

RESULTS

Insulicolide A inhibited receptor activator of nuclear factor-κB ligand-induced osteoclastogenesis in bone marrow monocytes *in vitro*

To detect the effects of insulicolide A (**Figure 1A**) on RANKL-induced osteoclastogenesis, BMMs were first incubated with different concentrations of insulicolide A from 0.5 to 2 μM, then followed by incubation of RANKL and M-CSF. BMMs can differentiate into TRAP-positive osteoclasts in the presence of RANKL (**Figures 1C, E**). However, insulicolide A remarkably reduced osteoclastogenesis induced by RANKL

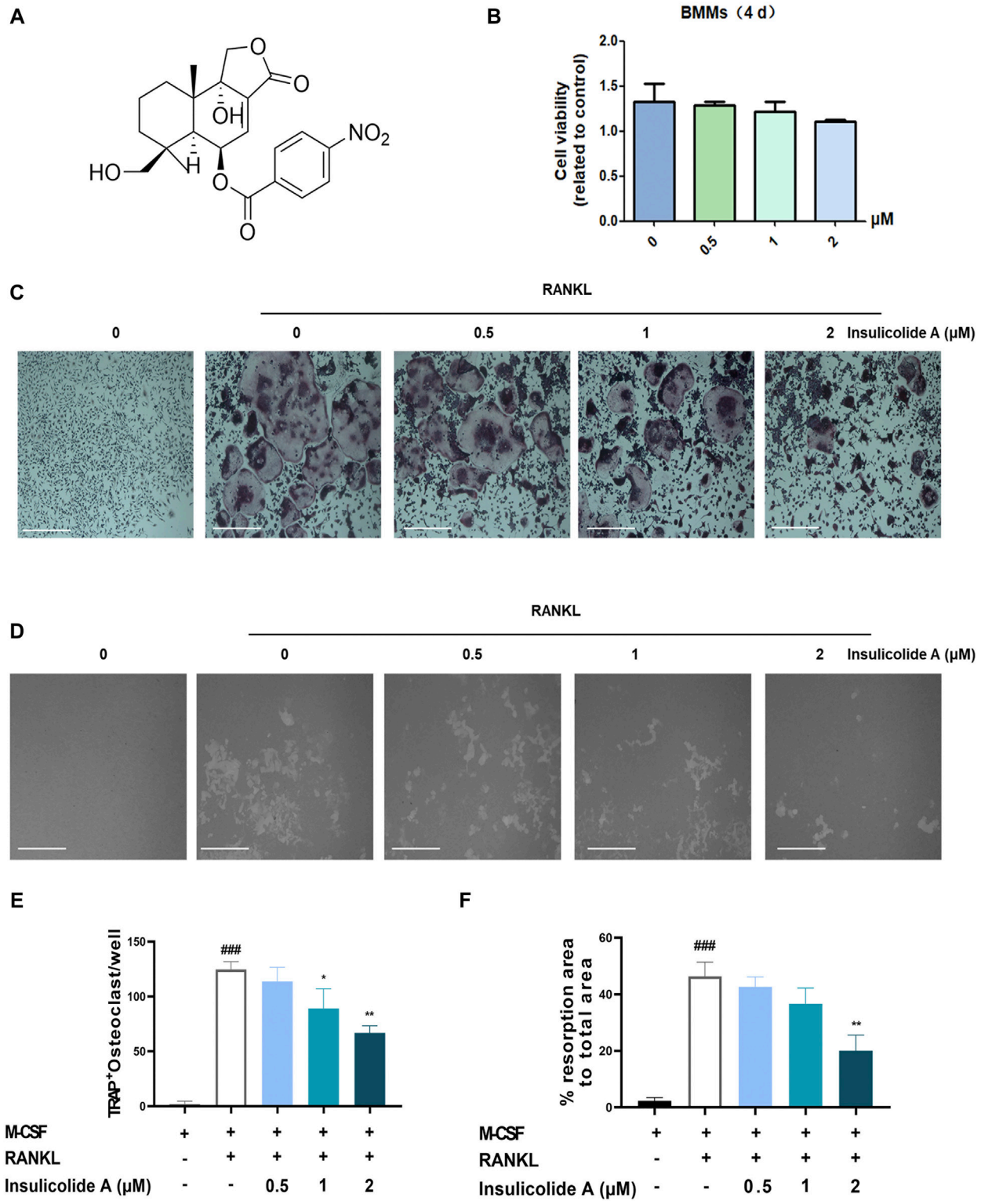


FIGURE 1 | Insulicolide A inhibited receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis *in vitro*. **(A)** The chemical structure of insulicolide A. **(B)** The cell viability of 0.5–2 μM insulicolide A in bone marrow monocytes (BMMs) for 4 days were measured. **(C, E)** Images and number of TRAP-positive multinucleated cells (nuclei > 5) were taken and calculated. **(D, F)** Images and areas of bone resorption by osteoclasts on the hydroxyapatite-coated surfaces were taken and quantified. The data are shown as means ± SD (n = 3 independent experiments, containing three replicate samples each). $^{###}p < 0.001$ vs. nontreatment groups, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. RANKL-induced groups.

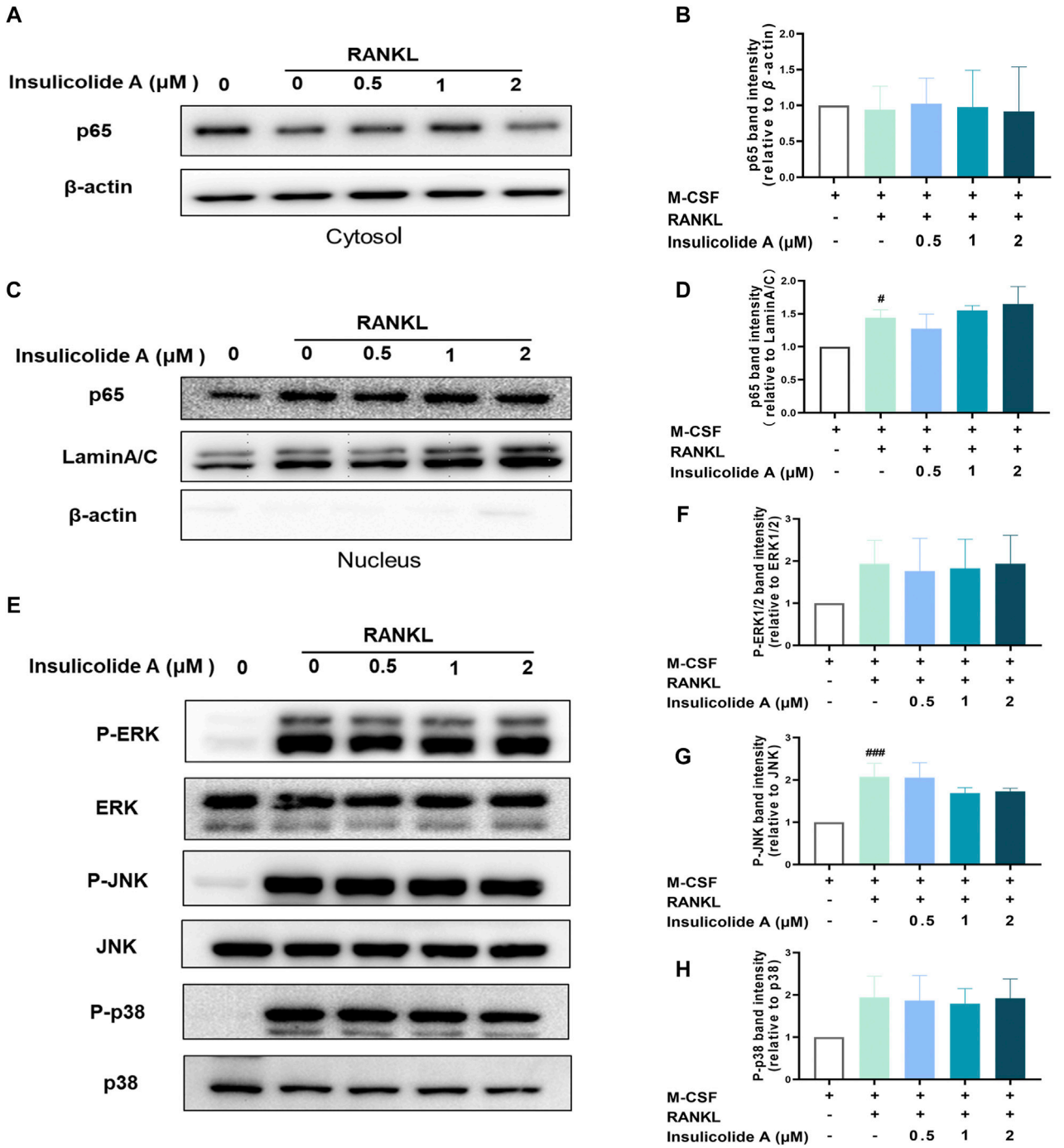


FIGURE 2 | Insulicolide A had little influence on activation of RANKL-induced NF-κB and mitogen-activated protein kinases (MAPKs). Insulicolide A (0.5–2 μM) in BMMs were first cultured for 4 h, then followed by 100 ng/ml of RANKL stimulation for 30 min. After that, the proteins were harvested and detected by p65, LaminA/C, β-actin, ERK, P-ERK, JNK, P-JNK, p38, and P-p38 antibodies. The expression of nuclear or total protein levels of p65 **(A, B)** to β-actin, or p65 **(C, D)** to LaminA/C, and P-ERK to ERK, P-JNK to JNK or P-p38 to p38 **(E–H)** were determined by ImageJ software. The data are shown as means ± SD of three independent experiments. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. nontreatment groups.

in a dose-dependent manner without cytotoxicity (**Figures 1B– C, E**). Additionally, we found that insulicolide A also decreased LPS-induced osteoclastogenesis (**Supplementary Figures S1A, B**). Bone erosion is caused by the increased number of bone-resorbing osteoclasts, so we next evaluated the influence of insulicolide A on bone erosion of BMMs on hydroxyapatite-coated plates. Similar results were obtained, that reduced erosion areas was consistent with the reduced

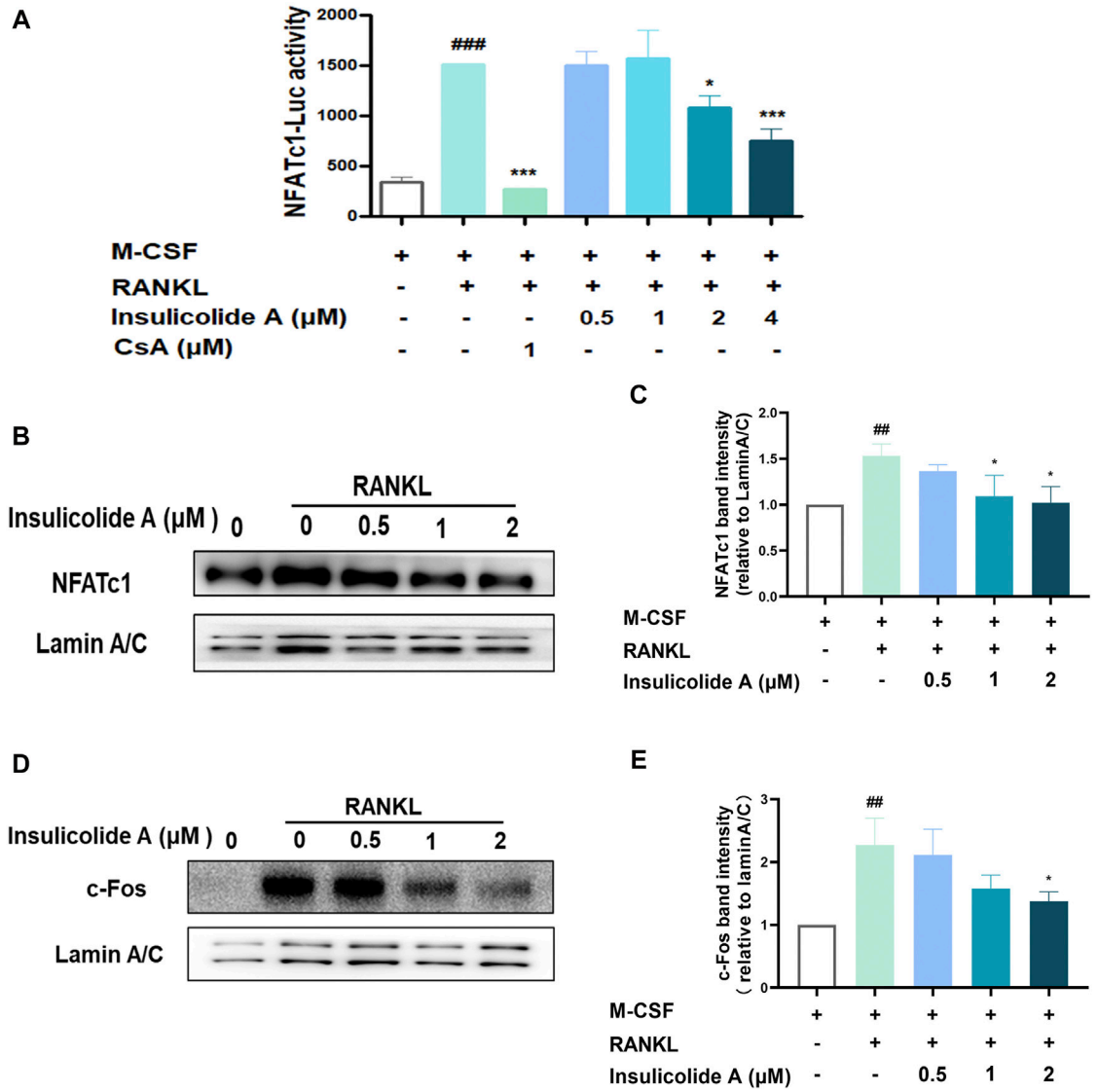


FIGURE 3 | Insulicolide A inhibited RANKL-induced c-Fos/nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) signaling pathway. RAW264.7 cells, stably transfected by NFATc1 luciferase reporter vector, were first pretreated by insulicolide A (0.5–4 μM) for 4 h, then followed by 100 ng/ml of RANKL stimulation for 6 h, and the luciferase activity **(A)** was assayed later. Insulicolide A (0.5–2 μM) in BMMs were first cultured for 4 h, then followed by 100 ng/ml of RANKL stimulation for 30 min. After that, nuclear proteins were harvested and detected by NFATc1, c-Fos, and LaminA/C antibodies. The relative expression of **(B, C)** NFATc1 to LaminA/C or **(D, E)** c-Fos to LaminA/C were analyzed. The data are shown as means ± SD of three independent experiments. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. nontreatment groups, $^{*}p < 0.05$, $^{***}p < 0.001$ vs. RANKL-induced groups.

osteoclast number by insulicolide A, and the erosion areas were almost completely decreased by insulicolide A at 2 μM (**Figures 1D, F**).

Insulicolide A had no statistically significant effects on activation of RANKL-induced NF-κB and MAPKs.

Since we found insulicolide A attenuated osteoclast formation, we next elucidated the mechanisms of insulicolide A during RANKL-induced osteoclastogenesis. As NF-κB and MAPKs signaling pathways are important in RANKL-induced osteoclast formation, we first investigated the effects of insulicolide A on RANKL-induced NF-κB activation including the protein expressions of NF-κB p65 in cytosol and nucleus. Here, Western blotting assays indicated that the nuclear protein expression of NF-κB p65 increased remarkably after RANKL stimulation; however, insulicolide A from 0.5 to 2 μM showed no statistically significant effects on p65 nuclear translocation (**Figures 2A–D**).

Then, we determined whether insulicolide A could attenuate the activation of MAPKs containing ERK/P-ERK, p38/P-p38, and JNK/P-JNK during RANKL-induced osteoclastogenesis. The protein expressions of P-ERK, P-p38, and P-JNK were enhanced rapidly after stimulation with RANKL; however, insulicolide A had no statistically significant effect on the phosphorylation of ERK, p38, and JNK, which was consistent with the influence on NF-κB (**Figures 2E–H**). The above results

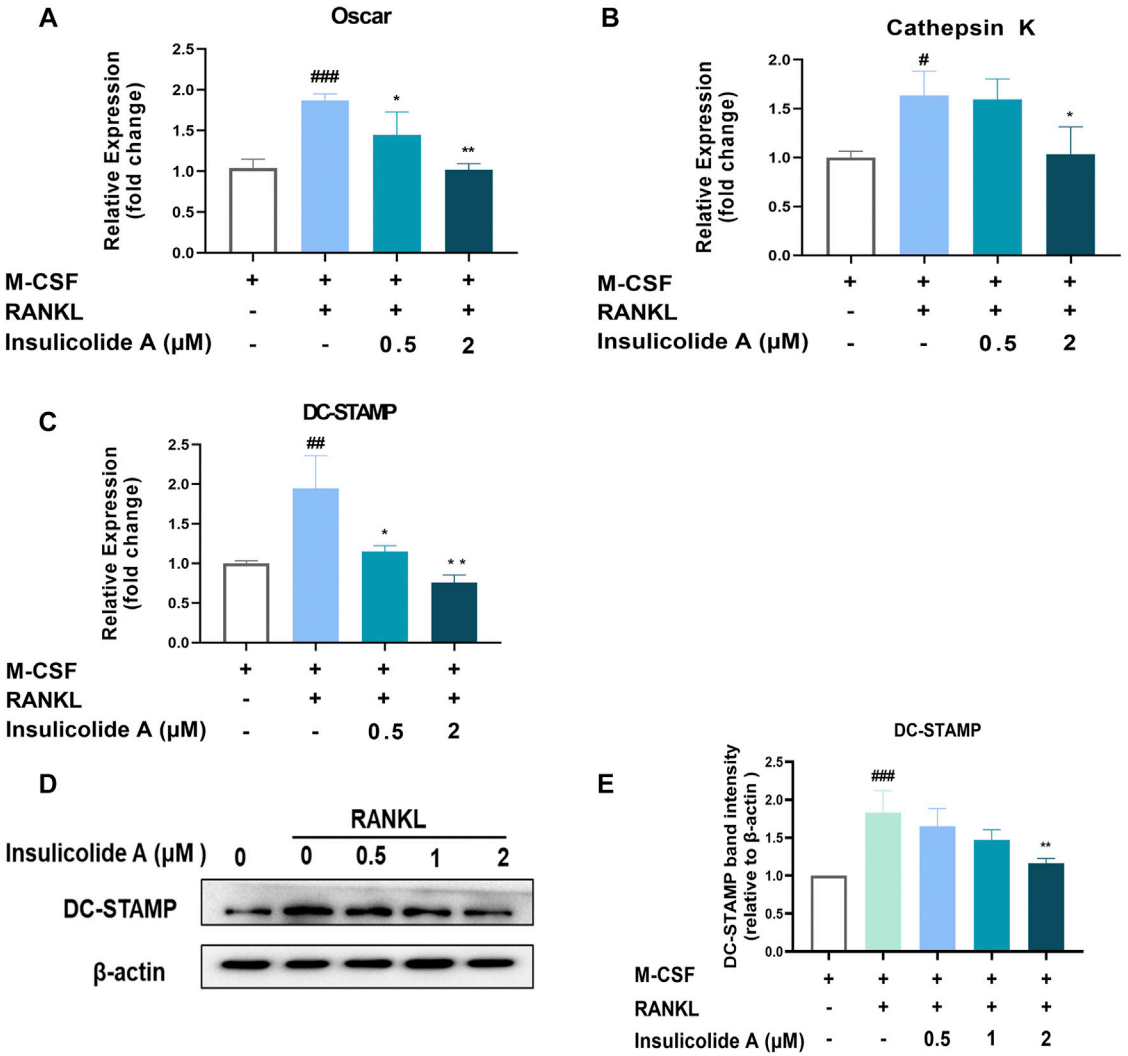


FIGURE 4 | Insulicolide A attenuated the expression of osteoclast-related genes and DC-STAMP induced by RANKL. Insulicolide A (0.5–2 μM) in BMMs were first cultured for 4 h, then followed by 100 ng/ml of RANKL stimulation for 24 h. After that, real-time PCR was used to analyz the gene expression of OSCAR **(A)**, cathepsin K **(B)**, and DC-STAMP **(C)**. Antibodies were used to detect the total proteins expression of DC-STAMP and *β*-actin **(D)**. **(E)** The relative expression of DC-STAMP to *β*-actin was analyzed by ImageJ software. The data are shown as means ± SD of three independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. nontreatment groups, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. RANKL-induced groups.

suggested that insulicolide A inhibits RANKL-induced osteoclast formation through other signaling pathways rather than NF-κB or MAPKs.

Insulicolide A suppressed receptor activator of nuclear factor-κB ligand-induced c-Fos/nuclear factor of activated T-cell cytoplasmic 1 signaling pathway

NFATc1, the master nuclear transcription factor, can activate osteoclastogenesis, and the activation of NFATc1 depends on NF-κB, MAPKs signaling pathways, or its upstream transcriptional regulator c-Fos. Since insulicolide A has little effect on both RANKL-induced NF-κB and MAPKs, we next examined the influence of insulicolide A on the activation of nuclear transcription factor c-Fos and NFATc1. First, we used NFATc1 luciferase reporter assay to determine the influence of insulicolide A on NFATc1 activation, and found that RANKL-induced NFATc1 luciferase activity was markedly suppressed by insulicolide A at 2 and 4 μM concentrations (**Figure 3A**). Then Western blot assay revealed that the nucleus protein expression of RANKL-induced NFATc1 was increased. However, insulicolide A abrogated nucleus protein expression of NFATc1 in a dose-dependent manner (**Figures 3B, C**). NFATc1 self-amplification and activation are produced by the binding of up-stream nuclear transcription factor c-Fos, which combines with the NFATc1 promoter. C-Fos knockout mice showed severe osteosclerosis due to the reduced osteoclasts. In line with NFATc1, c-Fos protein levels were also restrained by insulicolide A (**Figures 3D, E**).

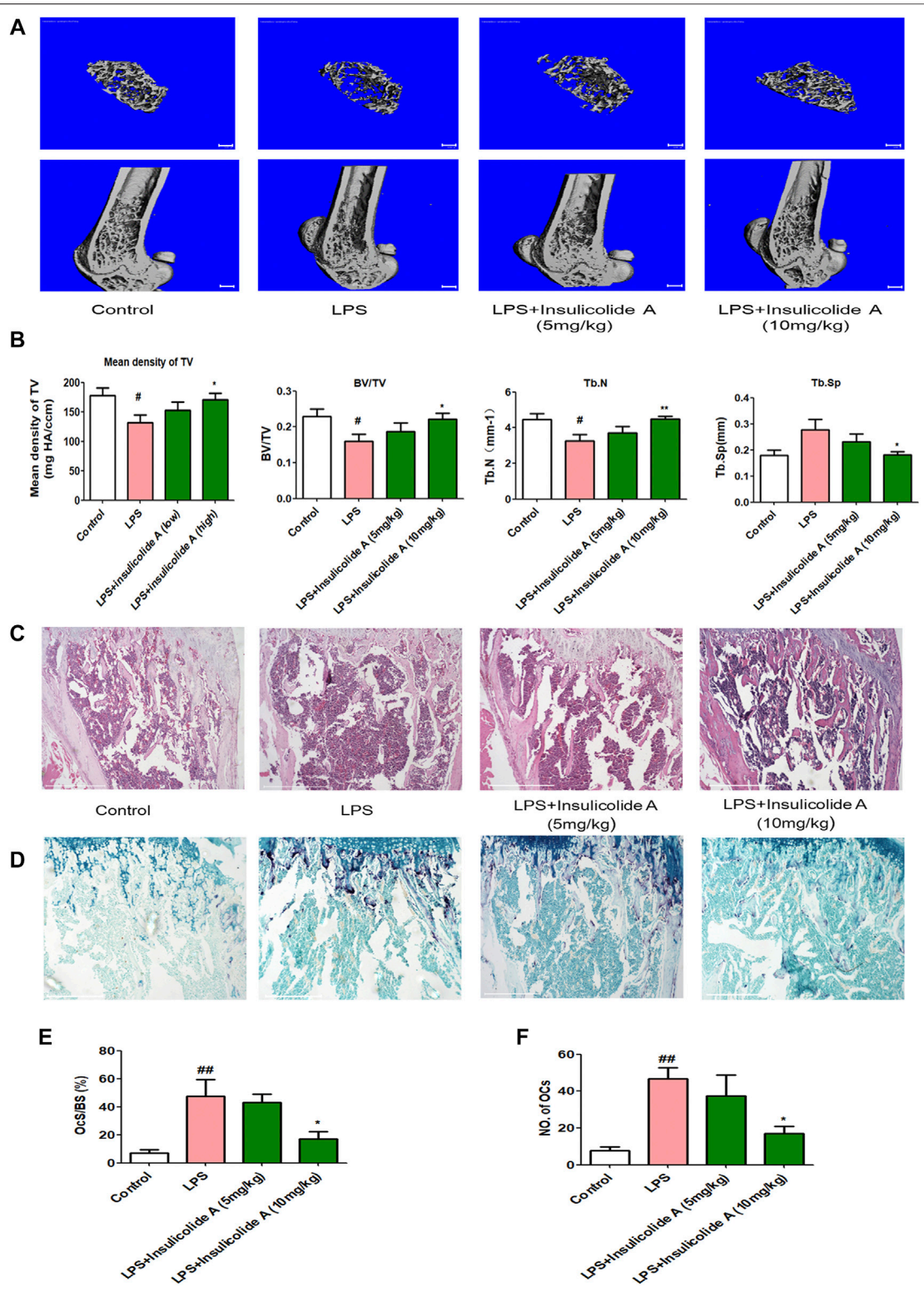


FIGURE 5 | Insulicolide A decreased LPS-induced murine bone loss by suppressing osteoclast activity. All mice were randomly divided into four groups with six mice in each group: control group (injected with PBS), LPS group (injected with LPS), LPS + insulicolide A (5 mg/kg) group, and LPS + insulicolide A (10 mg/kg) group. **(A)** Representative 3D reconstruction micro-CT images of transverse (above) and longitudinal (below) sections of the femur in different groups. **(B)** Quantitative measurements of trabecular bone parameters containing BMD, BV/TV, Tb.N, and Tb.Sp. Representative H&E **(C)** and TRAP staining **(D)** images of the femurs in each group. Osteoclast surface/bone surface **(E)** and the number of osteoclasts in each group **(F)** were analyzed. $n = 6$ mice per group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. PBS-treatment groups, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS-treatment groups.

Together, our results demonstrated that insulicolide A might attenuate RANKL-induced osteoclast formation by targeting the c-Fos/NFATc1 signaling pathway.

Insulicolide A attenuated the expression of osteoclast relative genes and DC-STAMP induced by RANKL.

Once NFATc1 is activated, osteoclast formation related genes, such as TRAP, OSCAR, cathepsin K, and osteoclast function-related genes including DC-STAMP, were strongly enhanced. However, when treated with insulicolide A, the mRNA expression of OSCAR, cathepsin K, and DC-STAMP were inhibited remarkably (**Figures 4A–C**). DC-STAMP, a multi-pass transmembrane molecule, is essential for the fusion and resorptive capacity of preosteoclasts. In DC-STAMP-deficient mice, the number of multinucleated osteoclasts reduced and bone mineral density increased. We then further examined the protein levels of DC-STAMP, consistent with the mRNA levels, insulicolide A also inhibited the protein levels of DC-STAMP induced by RANKL (**Figures 4D, E**). Collectively, these results suggested that insulicolide A inhibited RANKL-induced osteoclastogenesis and bone resorptive function by decreasing osteoclast formation-related genes and fusion-related genes.

Insulicolide A decreased LPS-induced bone loss by inhibiting osteoclast activity

LPS, an effective endotoxin from the cell wall in Gram-negative bacteria, can directly induce osteoblasts to secrete RANKL and then activate osteoclast formation. The activation of osteoclasts can result in osteoclastic diseases. In order to investigate the role of insulicolide A in the suppression of osteoclast function *in vivo*, LPS-induced inflammation bone loss mice model was used. Micro-CT assays showed that LPS-injected mice model suffered a serious bone loss; however, when LPS-injected mice were treated with insulicolide A by gavage, both low-dose group (5 mg/kg) and high-dose group (10 mg/kg) could attenuate LPS-induced bone destruction (**Figure 5A**). Bone parameter analysis indicated that compared with LPS-injected mice, mean density of TV, BV/TV, Tb.N of insulicolide A (10 mg/kg)-treated mice were markedly increased, but Tb.Sp was decreased (**Figure 5B**). H&E-stained bone sections further confirmed the protection of insulicolide A on LPS-induced bone loss. Furthermore, TRAP staining assays and histomorphometric analysis of the number of osteoclasts and the percentage osteoclast surface per bone surface (OcS/BS) in the bone trabecula demonstrated that oral treatment of insulicolide A could remarkably decrease LPS-caused bone destruction and inhibited osteoclast numbers (**Figures 5C–F**). Taken together, our results indicated that oral administration of insulicolide A could prevent inflammatory bone destruction *in vivo*.

DISCUSSION

Sesquiterpenoids from the plants and microorganisms display various biological activities. Marine fungus-derived nitrobenzoyl sesquiterpenoids, rare from natural source, exhibit remarkable pharmacological activities, including antitumor and antiinflammation (Wang et al., 2014; Guo et al., 2018). Here our results indicated that marine-derived nitrobenzoyl sesquiterpenoid, insulicolide A, could attenuate osteoclastogenesis c-Fos-NFATc1 signaling pathway induced by RANKL *in vitro* at doses from 1 to 2 μM. Consistently, we also found that insulicolide A could protect inflammatory osteolysis *in vivo*.

During the process of RANKL-induced osteoclast formation, the combination of RANKL to RANK can recruit TRAF6 followed by activation of NF-κB and MAPKs signaling pathways. NF-κB and MAPKs are crucial pathways of RANKL response in osteoclastogenesis (Ghosh and Karin, 2002; Jimi and Ghosh, 2005). However, our data showed that insulicolide A had no significant reduction on p65 of NF-κB and the phosphorylation of ERK, p38, and JNK of MAPKs, which were incompletely consistent with the effects of NS4 (structural isomer of insulicolide A) on NF-κB. NS4 exhibited inhibitory effects on NF-κB by binding with NF-κB p65 Arg B246 (Tan et al., 2020), the different mechanisms between NS4 and insulicolide A on NF-κB may be due to their different conformations and their affinity with NF-κB. These results suggested that NF-κB and MAPKs might not be a downstream signal by insulicolide A to treat RANKL-induced osteoclast differentiation.

C-Fos, the subunit of activator protein-1 (AP-1), is induced during the process of RANKL-induced osteoclastogenesis (Grigoriadis et al., 1994). As the upstream nuclear transcription factor of NFATc1, activated c-Fos can bind to the promoter region of NFATc1 resulting in the autoamplification and activation of NFATc1 (Takatsuna et al., 2005). C-Fos knockout mice showed decreased NFATc1 nuclear translocation and severe bone sclerosis (Matsuo et al., 2004). Here, our findings determined that the nuclear protein level of c-Fos was drastically inhibited by insulicolide A in the BMMs of RANKL stimulation, which was also different with NS4, while NS4 had little effect on c-Fos nuclear expression during RANKL-induced osteoclast formation (Tan et al., 2020). Thus, the decrease in RANKL-induced c-Fos activation and the following attenuated NFATc1 nuclear protein levels, contributes to impaired osteoclastogenesis after insulicolide A treatment.

NFATc1 is the core transcriptional switch of osteoclast terminal differentiation (Nepal et al., 2013). Activated NFATc1 not only increases the expression levels of osteoclast-related genes including TRAP, OSCAR, and cathepsin K, but also refers to the multinucleation of osteoclast precursors through cell fusion proteins such as DC-STAMP (Yagi et al., 2006; Chiu and Ritchlin, 2016). In our study, the levels of OSCAR and cathepsin K induced by RANKL were remarkably reduced after treatment of insulicolide A. Additionally, RANKL-induced mRNA and protein expressions of DC-STAMP in osteoclast formation were also down-regulated after treatment with insulicolide A. The reduction of nuclear translocation of NFATc1 and the suppression of DC-STAMP caused by insulicolide A might attenuate the expression of osteoclast-specific genes and the fusion of preosteoclast cells to inhibit osteoclasts *in vitro*.

LPS is an effective endotoxin from the cell wall in Gram-negative bacteria, which has an important impact on bone loss (Place et al., 2021). In our experiment, we constructed murine bone loss model induced by LPS to evaluate the influence of insulicolide A on osteoclastogenesis *in vivo*. Micro-CT analysis indicated that oral insulicolide A (10 mg/kg) could recover bone

loss induced by LPS through promoting BMD, BV/TV, and Tb.N, while reducing Tb.Sp. H&E and TRAP-stained bone section analysis further showed that insulicolide A remarkably inhibited bone destruction and osteoclast numbers in LPS-induced mouse model. Therefore, these results further manifested that oral insulicolide A protected bone by decreasing the numbers of osteoclast and improving bone parameters *in vivo*, which were consistent with its inhibitory effects on osteoclasts *in vitro*.

The up-stream signals such as TRAF6, DC-STAMP, and NF-κB/MAPKs regulate the expression of c-Fos during osteoclastogenesis (Yin et al., 2017; Liu et al., 2021; Zou et al., 2021). Here, insulicolide A showed little effect on NF-κB/MAPKs. DC-STAMP knockdown decreases c-Fos and NFATc1 expression in osteoclast precursor cells (Yin et al., 2017), thus, DC-STAMP can also serve as the surface molecule for insulicolide A to suppress osteoclast formation. Furthermore, the analysis of proteins binding with insulicolide A in osteoclastogenesis can be performed to disclose the target of insulicolide A in the future. In addition, we found that insulicolide A protected bone only by resorption *in vivo*. Further studies aimed at bone formation in osteoclast-related mouse models may make a better understanding of this compound for its treatment of osteolytic diseases.

In conclusion, this study discovered that insulicolide A, a natural nitrobenzoyl sesquiterpenoid isolated from the marine-derived *Aspergillus ochraceus* fungus, can suppress RANKL-induced osteoclastogenesis by preventing c-Fos rather than NF-κB and MAPKs, and then decreasing the level of NFATc1 and DC-STAMP *in vitro*. Our data, including the murine femur model experiment, suggest insulicolide A as a substantial osteoclasts inhibitor and indicate its promising application for osteoclast overactivated diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The study was reviewed and approved by the Committee of the animal protection and utilization of Southern Medical University and the institutional animal protection and utilization of Guangxi Normal University.

AUTHOR CONTRIBUTIONS

Study conception and design: XJL, YHT, and MHK. Acquisition, analysis, and interpretation of data: YHT, MHK, ZCL, YC, JHZ, and YYW. Drafting/revision of the work for intellectual content and context: YHT, MHK, XFZ, GH, and XJL. Final approval and overall responsibility for the published work: XFZ, GH and XJL.

FUNDING

This research was funded by the National Natural Science Foundation of China (81773740, U20A20101), the Natural Science Foundation of Guangxi (2021JJB140057), Project to improve the basic research ability of young and middle-aged university teachers of Guangxi (2021KY0062), Finance Science and Technology Project of Hainan Province (ZDKJ202018), Guangdong Local Innovation Team Program (2019BT02Y262), State Key Laboratory for Chemistry and Molecular Engineering of Medicinal Resources (CMEMR2019-A05), and Guangzhou Science and Technology Plan Project (201804010027).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.753240/full#supplementary-material

REFERENCES

Arai, F., Miyamoto, T., Ohneda, O., Inada, T., Sudo, T., Brasel, K., et al. (1999). Commitment and Differentiation of Osteoclast Precursor Cells by the Sequential Expression of C-Fms and Receptor Activator of Nuclear Factor kappaB (RANK) Receptors. *J. Exp. Med.* 190 (12), 1741–1754. doi:10.1084/jem.190.12.1741

Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003). Osteoclast Differentiation and Activation. *Nature* 423 (6937), 337–342. doi:10.1038/nature01658

Broadhead, M. L., Clark, J. C., Dass, C. R., Choong, P. F., and Myers, D. E. (2011). Therapeutic Targeting of Osteoclast Function and Pathways. *Expert Opin. Ther. Targets* 15 (2), 169–181. doi:10.1517/14728222.2011.546351

Cappellen, D., Luong-Nguyen, N. H., Bongiovanni, S., Grenet, O., Wanke, C., and Susa, M. (2002). Transcriptional Program of Mouse Osteoclast Differentiation Governed by the Macrophage colony-stimulating Factor and the Ligand for the Receptor Activator of NFkappa B. *J. Biol. Chem.* 277 (24), 21971–21982. doi:10.1074/jbc.M200434200

Chiu, Y. H., and Ritchlin, C. T. (2016). DC-STAMP: A Key Regulator in Osteoclast Differentiation. *J. Cel Physiol* 231 (11), 2402–2407. doi:10.1002/jcp.25389

Edwards, J. R., and Mundy, G. R. (2011). Advances in Osteoclast Biology: Old Findings and New Insights from Mouse Models. *Nat. Rev. Rheumatol.* 7 (4), 235–243. doi:10.1038/nrrheum.2011.23

Ghosh, S., and Karin, M. (2002). Missing Pieces in the NF-kappaB Puzzle. *Cell* 109 Suppl (Suppl. l), S81–S96. doi:10.1016/s0092-8674(02)00703-1

Grigoriadis, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., et al. (1994). c-Fos: a Key Regulator of Osteoclast-Macrophage Lineage Determination and Bone Remodeling. *Science* 266 (5184), 443–448. doi:10.1126/science.7939685

Guo, L. M., Lyu, J. L., and Zhang, L. B. (2018). [Research Progress on Anti-inflammatory Mechanism of Natural Sesquiterpenoids]. *Zhongguo Zhong Yao Za Zhi* 43 (20), 3989–3999. doi:10.19540/j.cnki.cjcmm.20180726.013

Győri, D. S., and Mócsai, A. (2020). Osteoclast Signal Transduction during Bone Metastasis Formation. *Front Cel Dev Biol* 8, 507. doi:10.3389/fcell.2020.00507

Jimi, E., and Ghosh, S. (2005). Role of Nuclear Factor-kappaB in the Immune System and Bone. *Immunol. Rev.* 208, 80–87. doi:10.1111/j.0105-2896.2005.00329.x

Kang, H. K., Seo, C. H., and Park, Y. (2015). The Effects of marine Carbohydrates and Glycosylated Compounds on Human Health. *Int. J. Mol. Sci.* 16 (3), 6018–6056. doi:10.3390/ijms16036018

Kim, J. M., Lin, C., Stavre, Z., Greenblatt, M. B., and Shim, J. H. (2020). Osteoblast-Osteoclast Communication and Bone Homeostasis. *Cells* 9 (9). doi:10.3390/cells9092073

Lee, K., Chung, Y. H., Ahn, H., Kim, H., Rho, J., and Jeong, D. (2016). Selective Regulation of MAPK Signaling Mediates RANKL-dependent Osteoclast Differentiation. *Int. J. Biol. Sci.* 12 (2), 235–245. doi:10.7150/ijbs.13814

Li, X., Udagawa, N., Takami, M., Sato, N., Kobayashi, Y., and Takahashi, N. (2003). p38 Mitogen-Activated Protein Kinase Is Crucially Involved in Osteoclast Differentiation but Not in Cytokine Production, Phagocytosis, or Dendritic Cell Differentiation of Bone Marrow Macrophages. *Endocrinology* 144 (11), 4999–5005. doi:10.1210/en.2003-0166

Liu, Y., Liu, W., Yu, Z., Zhang, Y., Li, Y., Xie, D., et al. (2021). A Novel BRD4 Inhibitor Suppresses Osteoclastogenesis and Ovariectomized Osteoporosis by Blocking RANKL-Mediated MAPK and NF-Kb Pathways. *Cell Death Dis* 12 (7), 654. doi:10.1038/s41419-021-03939-7

Lorenzo, J. (2017). The many Ways of Osteoclast Activation. *J. Clin. Invest.* 127 (7), 2530–2532. doi:10.1172/JCI94606

Malve, H. (2016). Exploring the Ocean for New Drug Developments: Marine Pharmacology. *J. Pharm. Bioallied Sci.* 8 (2), 83–91. doi:10.4103/0975-7406.171700

Matsumoto, M., Sudo, T., Saito, T., Osada, H., and Tsujimoto, M. (2000). Involvement of P38 Mitogen-Activated Protein Kinase Signaling Pathway in Osteoclastogenesis Mediated by Receptor Activator of NF-Kappa B Ligand (RANKL). *J. Biol. Chem.* 275 (40), 31155–31161. doi:10.1074/jbc.M001229200

Matsuo, K., Galson, D. L., Zhao, C., Peng, L., Laplace, C., Wang, K. Z., et al. (2004). Nuclear Factor of Activated T-Cells (NFAT) Rescues Osteoclastogenesis in Precursors Lacking C-Fos. *J. Biol. Chem.* 279 (25), 26475–26480. doi:10.1074/jbc.M313973200

Nepal, M., Choi, H. J., Choi, B. Y., Yang, M. S., Chae, J. I., Li, L., et al. (2013). Hispidulin Attenuates Bone Resorption and Osteoclastogenesis via the RANKL-Induced NF-Kb and NFATc1 Pathways. *Eur. J. Pharmacol.* 715 (1-3), 96–104. doi:10.1016/j.ejphar.2013.06.002

Perpetuo, I. P., Caetano-Lopes, J., Rodrigues, A. M., Campanilho-Marques, R., Ponte, C., Canhao, H., et al. (2017). Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Rheumatoid Arthritis. *Biomed. Res. Int.* 2017: 2690402. doi:10.1155/2017/2690402

Place, D. E., MalireddiSubbarao, R. K. S., Kim, J., Vogel, P., Yamamoto, M., and Kanneganti, T. D. (2021). Osteoclast Fusion and Bone Loss Are Restricted by Interferon Inducible Guanylate Binding Proteins. *Nat. Commun.* 12 (1), 496. doi:10.1038/s41467-020-20807-8

Stickeler, E., and Fehm, T. (2014). Targeted and Osteo-Oncologic Treatment in Early Breast Cancer: What Is State-Of-The-Art and what Might Become So within the Next 5 years? *Breast Care (Basel)* 9 (3), 161–167. doi:10.1159/000365129

Takatsuna, H., Asagiri, M., Kubota, T., Oka, K., Osada, T., Sugiyama, C., et al. (2005). Inhibition of RANKL-Induced Osteoclastogenesis by (-)-DHMEQ, a Novel NF-kappaB Inhibitor, through Downregulation of NFATc1. *J. Bone Miner Res.* 20 (4), 653–662. doi:10.1359/JBMR.041213

Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., et al. (2002). Induction and Activation of the Transcription Factor NFATc1 (NFAT2) Integrate RANKL Signaling in Terminal Differentiation of Osteoclasts. *Dev. Cel* 3 (6), 889–901. doi:10.1016/s1534-5807(02)00369-6

Tan, Y., Deng, W., Zhang, Y., Ke, M., Zou, B., Luo, X., et al. (2020). A marine Fungus-Derived Nitrobenzoyl Sesquiterpenoid Suppresses Receptor Activator of NF-Kb Ligand-Induced Osteoclastogenesis and Inflammatory Bone Destruction. *Br. J. Pharmacol.* 177 (18), 4242–4260. doi:10.1111/bph.15179

Tan, Y., Yang, B., Lin, X., Luo, X., Pang, X., Tang, L., et al. (2018). Nitrobenzoyl Sesquiterpenoids with Cytotoxic Activities from a Marine-Derived Aspergillus ochraceus Fungus. *J. Nat. Prod.* 81 (1), 92–97. doi:10.1021/acs.jnatprod.7b00698

Tanaka, S., Nakamura, K., Takahasi, N., and Suda, T. (2005). Role of RANKL in Physiological and Pathological Bone Resorption and Therapeutics Targeting the RANKL-RANK Signaling System. *Immunol. Rev.* 208, 30–49. doi:10.1111/j.0105-2896.2005.00327.x

Tsukasaki, M., and Takayanagi, H. (2019). Osteoimmunology: Evolving Concepts in Bone-Immune Interactions in Health and Disease. *Nat. Rev. Immunol.* 19 (10), 626–642. doi:10.1038/s41577-019-0178-8

Wada, T., Nakashima, T., Hiroshi, N., and Penninger, J. M. (2006). RANKL-RANK Signaling in Osteoclastogenesis and Bone Disease. *Trends Mol. Med.* 12 (1), 17–25. doi:10.1016/j.molmed.2005.11.007

Wang, G. W., Qin, J. J., Cheng, X. R., Shen, Y. H., Shan, L., Jin, H. Z., et al. (2014). Inula Sesquiterpenoids: Structural Diversity, Cytotoxicity and Anti-tumor Activity. *Expert Opin. Investig. Drugs* 23 (3), 317–345. doi:10.1517/13543784.2014.868882

Wu, Q. X., Jin, X. J., Draskovic, M., Crews, M. S., Tenney, K., Valeriote, F. A., et al. (2012). Unraveling the Numerous Biosynthetic Products of the Marine Sediment-Derived Fungus, Aspergillus insulicola. *Phytochem. Lett.* 5 (1), 114–117. doi:10.1016/j.phytol.2011.11.005

Yagi, M., Miyamoto, T., Toyama, Y., and Suda, T. (2006). Role of DC-STAMP in Cellular Fusion of Osteoclasts and Macrophage Giant Cells. *J. Bone Miner Metab.* 24 (5), 355–358. doi:10.1007/s00774-006-0697-9

Yin, Y., Tang, L., Chen, J., and Lu, X. (2017). MiR-30a Attenuates Osteoclastogenesis via Targeting DC-STAMP-c-Fos-NFATc1 Signaling. *Am. J. Transl Res.* 9 (12), 5743–5753.

Zhao, H. Y., Anbuchezhian, R., Sun, W., Shao, C. L., Zhang, F. L., Yin, Y., et al. (2016). Cytotoxic Nitrobenzoyloxy-Substituted Sesquiterpenes from Spongederived Endozoic Fungus Aspergillus insulicola MD10-2. *Curr. Pharm. Biotechnol.* 17 (3), 271–274. doi:10.2174/1389201017666151223123424

Zou, B. H., Tan, Y. H., Deng, W. D., Zheng, J. H., Yang, Q., Ke, M. H., et al. (2021). Oridonin Ameliorates Inflammation-Induced Bone Loss in Mice via Suppressing DC-STAMP Expression. *Acta Pharmacol. Sin* 42 (5), 744–754. doi:10.1038/s41401-020-0477-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

MINI REVIEW
published: 17 March 2022
doi: 10.3389/fphar.2022.820971

Glucosamine as a Treatment for Osteoarthritis: What If It's True?

Thierry Conrozier and Thomas Lohse*

Department of Rheumatology, Hôpital Nord Franche-Comté, Belfort, France

No disease-modifying treatments are currently available for osteoarthritis (OA). While many therapeutic approaches are now being investigated it is ethical to resort to alternative solutions as that we already possess. There are many reasons for thinking that, at sufficiently high doses, glucosamine (GlcN) sulphate possesses a clinically relevant effect on OA pain. Wide inter-individual variations in the symptomatic effects of GlcN are explained by the extreme variability of its bioavailability. In studies evaluating its structure-modifying effect, GlcN was more effective than placebo in reducing the rate of joint space narrowing in patients with knee OA. More recent data suggest that GlcN may be effective in the primary prevention of OA in sportsmen. There is no controversy concerning the safety of GlcN which does not differ to that of placebo. Several studies have recently revealed an unexpected effect of GlcN on cardiovascular mortality. After adjusting for confounding factors, the regular consumption of GlcN correlated with a 27% reduction in mortality and a 58% reduction in deaths from cardiovascular causes. These data confirm animal studies demonstrating a protective effect of GlcN against cancer and cardiovascular diseases due to modulation of the O-GlcNAcylation pathway. Disorders in O-GlcNAcylation are involved in diabetes, obesity and cancers, which all feature chronic low-grade inflammation (CLGI). By regulating CLGI, GlcN may be beneficial to the symptoms of OA, its outcome and to that of the concomitant chronic pathologies, making GlcN as a valuable candidate for the treatment of OA in patients with metabolic syndrome, diabetes or cardiovascular diseases.

Keywords: glucosamine, OA, O-GlcNAcylation, treatment, low-grade inflammation, chondroprotective, cardiovascular mortality, metabolic syndrom

OPEN ACCESS

Edited by:
Raquel Largo,
Health Research Institute Foundation Jimenez Diaz (IIS-FJD), Spain

Reviewed by:
Gabriel Herrero-Beaumont,
University Hospital Fundación Jiménez Díaz, Spain
Jordi Monfort,
Parc de Salut Mar, Spain

****Correspondence:***
Thierry Conrozier
thierry.conrozier@hnfc.fr

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *23 November 2021*
Accepted: *15 February 2022*
Published: *17 March 2022*

Citation:
Conrozier T and Lohse T (2022) Glucosamine as a Treatment for Osteoarthritis: What If It's True? Front. Pharmacol. 13:820971. doi: 10.3389/fphar.2022.820971

INTRODUCTION

Osteoarthritis (OA) is a major cause of pain and disability in subjects older than 50 years with a significant impact on physical performance and quality of life. Its prevalence is increasing worldwide with the aging of the population and the increase in risk factors such as obesity, resulting in a significant public health problem and socioeconomic burden. However, OA is not the exclusive preserve of elderly and also affects younger people, especially those having risk factors such as overweight, metabolic syndrome or joint injury (Kloppenburg and Berenbaum, 2020). The pathophysiology of OA is complex and involves, at various levels, interactions between mechanical, genetic, metabolic and inflammatory mechanisms. Anatomically OA is characterized by articular cartilage breakdown, subchondral bone remodelling and synovial low-grade inflammation. To date no disease-modifying therapeutics are currently available for OA due to an insufficient understanding of the pathogenesis, and a delay in the therapeutic management, due to the lack of a sufficiently sensitive biomarker allowing to make the diagnosis during the early

asymptomatic phase of the disease. At an advanced stage the treatment of OA is mainly based on symptomatic measures or joint replacement surgery. On the other hand, if the diagnosis is made early, developing treatments to slow the progression of joint degradation is a reasonable goal. Thanks to recent achievements in understanding the causes of the cartilage degradation many therapeutic approaches are now being investigated (Hochberg et al., 2019; Ghouri and Conaghan, 2019; Stevens et al., 2019; Thorup et al., 2020; Yazici et al., 2020; Fernández-Martín et al., 2021). However, we cannot hope for a structure-modifying treatment to be marketed for several years. In clinical daily practice all the care-givers must legitimately ask them the following question: Is it ethical not to bring forward patients anything at all (except symptomatic treatments, most of which having frequent and sometimes serious side effects) until a real chondroprotective agent has been launched on the market?

This mini review is not a systematic review and does not aim to provide all the data published on this topic, but, as the title indicates, is intended to ask a question. Is it possible that glucosamine actually has a beneficial effect on osteoarthritis?

The committed stance of the authors is to emphasize that, despite controversies, a number of data suggest that the long-term prescription of glucosamine could be beneficial for OA patients, both symptomatically and in terms of cardiovascular status.

Glucosamine (2-amino-2-deoxy-β-d-glucopyranose) is an endogenous amino-monosaccharide synthesized from glucose and utilized for biosynthesis of glycoproteins and glycosaminoglycans of joint cartilage which, when administered orally as the sulphate or hydrochloride, is considered to be a medicinal product of the class of symptomatic slow acting drugs for OA (SYSADOA). It has been used as an OA disease-modifying medicine for over 50 years (Vetter, 1965). Glucosamine is recommended for the treatment of knee by most European scholarly societies (Jordan et al., 2003; Bernetti et al., 2019; Bruyère et al., 2019; Sellam et al., 2020), but not by US ones (Bannuru et al., 2019; Kolasinski et al., 2020). However, with the exception of ESCEO, which recommend glucosamine as first-line treatment along with other SYSADOAs (Jordan et al., 2003), it is commonly considered as an adjunctive treatment, of modest symptomatic efficacy, mainly used for its excellent safety and because of its sparing effect on analgesic medications, notably NSAIDs, potentially much more dangerous (Dougados, 2006). However recent data, in particular studies demonstrating decreased cardiovascular mortality in patients receiving long-term treatment with glucosamine (Li et al., 2020) and the discovery of its mechanism of action on chronic inflammation (Herrero-Beaumont and Largo, 2020), have changed the perspective on this molecule, the indications of which could be considerably expanded.

SYMPTOMATIC EFFECTS IN OA

Despite contradictory results in studies, there are numerous reasons for thinking that, at sufficiently high doses, some medicines containing glucosamine sulphate possess a significant and clinically relevant effect in OA (Gregori et al., 2018). Several randomised controlled studies have shown a symptomatic effect superior to placebo (Noack et al., 1994) and not inferior to that of an NSAID (Müller-Fassbender et al., 1994). The most recent meta-analyses confirm the symptomatic efficacy of pharmaceutical grade glucosamine sulphate, with results showing a standardised mean difference ranging from −0.29 [95% CI −0.49 to −0.09] (Sellam et al., 2020) and −0.35 [−0.54 to −0.16] (Knapik et al., 2018; Beaudart et al., 2020; Ton et al., 2020), which evidences a moderate effect not significantly different from results obtained with other classes of oral symptomatic OA drugs, notably the NSAIDs. OARSI classifies the efficacy of glucosamine as "uncertain," due to a very variable effect size ranging from 0.17 [0.05–0.28] (low) to 0.47 [0.23–0.72] (moderate) depending on study quality and especially depending on the type of glucosamine studied (hydrochloride *versus* sulphate, pharmaceutical grade or not) (Bannuru et al., 2019). The key point, which seems to confirm the objective nature of the improvement, is that the analgesic effect occurs after several weeks' treatment (Conrozier et al., 2019) and that it persists for several weeks after discontinuation of treatment, which does not resemble a placebo response but is rather the definition of an SYSADOA. One can nevertheless question the clinically relevant nature of the clinical improvement, which is often found to be lower than the MCII (Minimum Clinically Important Improvement). However, beyond the data from the studies, the fact that the commercial market for glucosamine is growing despite the wide publicity given to negative clinical trials, is consistent with the possibility that some patients obtain significant clinical benefits (McCarty et al., 2019), as in the PREDOA study where a clinically relevant improvement was noted in 63% of 2,030 patients treated for 6 months (Conrozier et al., 2019). One explanation of the wide interindividual variation in the symptomatic effects of glucosamine is the extreme variability of its bioavailability. (Asthana et al., 2020) Following oral administration, mean bioavailability is low, in the order of 25%, but with considerable variation between subjects according to a ratio of 1:100 (Li et al., 2020). The reasons for these variations remain unknown but an animal study has shown a 1.7-fold increase in plasma concentrations when glucosamine is taken on an empty stomach and in the evening, suggesting an influence of circadian rhythm (Seto et al., 2020). It is also important to know that the concomitant administration of glucosamine and chondroitin reduces the absorption of the former substance by 58% (Jackson et al., 2010). This effect of competition in cells of the intestinal is not without consequence because while glucosamine and chondroitin are both effective on the symptoms of OA, this is not the case when the two are combined, as highlighted by the meta-analysis of Zhu et al. (2018). Following absorption, synovial fluid concentrations of glucosamine are approximately double those in the serum. A possible hypothesis is that sufficient doses of oral glucosamine may have a positive effect on the synthesis of hyaluronic acid by the

human synovial cells, as suggested by *in vitro* studies (Igarashi et al., 2011).

STRUCTURAL EFFECTS IN OA

The structural effect of oral glucosamine in knee OA was assessed in double-blind, randomized, controlled trials, lasting at least 1 year and reporting as outcome measures both the symptom severity and the radiological joint space width progression over time (Reginster et al., 2001; Pavelká et al., 2002; Poolsup et al., 2005). Glucosamine sulphate was found to be more effective than placebo in reducing the rate of joint space narrowing at the tibiofemoral compartment in patients with knee OA, slowing its progression by 54% (RR = 0.46; 95%CI 0.28–0.73; $p = 0.001$) with NNT (number of subjects needed to treat to reach the objective) of 9 (95% CI 6 – 20) (Pavelká et al., 2002). Although the figures appear very encouraging (in comparison the NNT of alendronate for prevention of vertebral fracture is 16 for secondary prevention and 50 for primary prevention) (Holder and Kerley, 2008), they may logically be considered as non-clinically relevant when limiting results to the rate of joint space narrowing compared with placebo, which is only 1/10th of a millimetre per year (−0.31 mm [95% CI −0.48 to −0.13]) for placebo and −0.06 mm [−0.22 to 0.09)] for glucosamine sulphate over a 3-year period) (Reginster et al., 2001). Nevertheless these values are not lower than those obtained by Sprifermin (recombinant FGF-18) (Hochberg et al., 2019) which is nonetheless often presented as the next generation "chrondroprotector". It is particularly interesting to note that in patients with a high cartilage turnover, demonstrated by a very high level of urinary CTX-II before treatment, Christgau et al. noted a significant decrease in CTX-II levels after 12 months of glucosamine treatment and that the change in this level was predictive of radiologically-visible joint narrowing at 3 years (R = 0.43; $p < 0.05$) (Christgau et al., 2004). These data tend to support an effect, admittedly modest, but statistically significant, of glucosamine in secondary prevention of exacerbation of OA of the knee.

More recent data even suggest that glucosamine may be effective in the primary prevention of OA. This was the finding of a Japanese team who studied the effect of glucosamine on markers of the synthesis (CP II) and breakdown (u-CTX-II) of type II collagen in young athletes (rugby and soccer players) and in non-athletes paired for age (Nagaoka et al., 2019). Before treatment, levels of biomarkers were significantly higher in the athletes. After 3 months of treatment with glucosamine 1.5 or 3 g/day, the levels of biomarkers in the athletes had returned to the levels of the control subjects, and 3 months after discontinuation of treatment these had again increased. In a second study (Tsuruta et al., 2018) they randomised the athletes to receive either a dietary supplement containing 2 g of glucosamine or a placebo. After 3 months of treatment the u-CTX-II levels had significantly decreased in the glucosamine group but not in the placebo group. These 2 studies suggest that the administration of high doses of glucosamine (2–3 g per day) has a positive effect on cartilage metabolism *in vivo*, thus confirming the effect observed *in vitro*, where glucosamine inhibits matrix metalloproteinase production and chondrocyte apoptosis (Henrotin et al., 2012) and stimulates haeme oxygenase-1 (HO-1), a key enzyme regulating oxidative stress (Rousset et al., 2013).

SAFETY

In contrast to its efficacy, there is no controversy concerning the safety in use of glucosamine; all meta-analyses and systematic reviews conclude that its safety is excellent, not different from placebo (Zhu et al., 2018). Conventionally, glucosamine is not recommend in patients with shellfish allergy due to its origin (the chitin of crustaceans) but reports of allergic phenomena, skin or respiratory, are not found to any significant extent in the literature. As glucosamine is a molecule similar to glucose its use in diabetics has been discussed, but studies showed no effect on blood glucose levels and hyperinsulinism at the doses used, whether in healthy subjects or diabetics (Simon et al., 2011).

EFFECT ON MORTALITY

Several studies (Simon et al., 2011; King and Xiang, 2020; Li et al., 2020) have recently revealed an unexpected effect of glucosamine. Cardiovascular mortality and mortality all causes, was studied from the clinical data of 16,686 subjects, participants in the American national Survey on health and nutrition from 1999 to 2010. Of these participants, 4% had been taking glucosamine or chondroitin for 1 year or more. During the study period, 20% of participants died. After adjusting for age, sex, race, education level, smoking and physical activity, the intake of glucosamine/chondroitin correlated to a 27% reduction in mortality all causes (HR = 0.73; [0.57 to 0.93]) and a 58% reduction in deaths from cardiovascular causes (HR = 0.42; [0.23 to 0.75]). A Chinese study in a cohort of 500,000 patients followed up for 9 years (Li et al., 2020) found similar results, although slightly less convincing. A notable finding was that the protective effect of glucosamine was particularly marked in smokers ($p = 0.0008$). These results in humans only confirm the very numerous animal studies which demonstrate a protective effect of glucosamine against cancer (lung and colorectal) and cardiovascular diseases (Largo et al., 2009). However, the doses used in animal studies are generally much higher than those authorised in humans.

It is thought that the beneficial effect of glucosamine is due to modulation of the O-GlcNAcylation pathway (Herrero-Beaumont and Largo, 2020) which is a reversible post-translational modification, analogous to phosphorylation, controlling the activity, location or stability of proteins, depending on glucose availability, by adding N-Acetyl-glucosamine to the serine or threonine residues of cytosolic or nuclear proteins. Tardio et al. showed that in human OA cartilage there is an accumulation of O-GlcNAcylated proteins associated to an alteration in the expression of the enzymes that regulate this glycosylation and an overexpression of hypertrophic differentiation markers in chondrocytes (Tardio et al., 2014). Changes in O-GlcNAcylation are involved in human disease such

as diabetes, obesity and some cancers, pathologies which all feature chronic low-grade inflammation causing numerous complications. Hyperglycaemia combined with metabolic syndrome is known to enhance inflammatory processes via oxidative stress and the abnormally high levels of the O-GlcNAc protein which increase the transcriptional activity of nuclear factor kappa B (NFκB), the cause of diabetic complications (Baudoin and Issad, 2015). However now we know that there is a close relationship which links OA, metabolic syndrome, diabetes, obesity and the resulting cardiovascular diseases. It is therefore tempting to think that by regulating the chronic low-grade inflammation which is common to all these pathologies, glucosamine may be beneficial to the symptoms of OA, its outcome and also to that of the concomitant chronic pathologies. As it also acts on oxidative stress by stimulating the production of HO-1 (Rousset et al., 2013), and that its safety is well known, glucosamine is today a valuable candidate for the treatment of OA in patients with metabolic syndrome, diabetes or a history of cardiovascular disease all of which contra-indicate the use of NSAIDs and intra-articular corticosteroids.

CONCLUSION

Well-known and used as a SYSADOA for many years, glucosamine has been given a new lease of life with the demonstration of its beneficial effect in some chronic degenerative pathologies such as diabetes, obesity and atherosclerosis. While we know that its activity regulating O-GlcNAcylation is probably the reason for its effect on chronic inflammation, there are nevertheless numerous points requiring clarification such as optimal posology, the choice of salt (sulphate or hydrochloride), treatment duration, bioavailability and structural efficacy in primary or secondary prevention, before glucosamine can be considered as an anti-osteoarthritic drug of the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Asthana, C., Peterson, G. M., Shastri, M. D., Jones, G., and Patel, R. P. (2020). Variation in Plasma Levels of Glucosamine with Chronic Dosing: A Possible Reason for Inconsistent Clinical Outcomes in Osteoarthritis. *Clin. Ther.* 42 (8), e140–e149. doi:10.1016/j.clinthera.2020.06.009

Bannuru, R. R., Osani, M. C., Vaysbrot, E. E., Arden, N. K., Bennell, K., Bierma-Zeinstra, S. M. A., et al. (2019). OARSI Guidelines for the Non-surgical Management of Knee, Hip, and Polyarticular Osteoarthritis. *Osteoarthr. Cartil.* 27 (11), 1578–1589. doi:10.1016/j.joca.2019.06.011

Baudoin, L., and Issad, T. (2015). O-GlcNAcylation and Inflammation: A Vast Territory to Explore. *Front. Endocrinol. (Lausanne)* 5, 235. doi:10.3389/fendo.2014.00235

Beaudart, C., Lengelé, L., Leclercq, V., Geerinck, A., Sanchez-Rodriguez, D., Bruyère, O., et al. (2020). Symptomatic Efficacy of Pharmacological Treatments for Knee Osteoarthritis: A Systematic Review and a Network Meta-Analysis with a 6-Month Time Horizon. *Drugs* 80 (18), 1947–1959. doi:10.1007/s40265-020-01423-8

Bernetti, A., Mangone, M., Villani, C., Alviti, F., Valeo, M., Grassi, M. C., et al. (2019). Appropriateness of Clinical Criteria for the Use of SYmptomatic Slow-Acting Drug for OsteoArthritis (SYSADOA). A Delphi Method Consensus Initiative Among Experts in Italy. *Eur. J. Phys. Rehabil. Med.* 55 (5), 658–664. doi:10.23736/S1973-9087.19.05633-8

Bruyère, O., Honvo, G., Veronese, N., Arden, N. K., Branco, J., Curtis, E. M., et al. (2019). An Updated Algorithm Recommendation for the Management of Knee Osteoarthritis from the European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases (ESCEO). *Semin. Arthritis Rheum.* 49 (3), 337–350. doi:10.1016/j.semarthrit.2019.04.008

Christgau, S., Henrotin, Y., Tankó, L. B., Rovati, L. C., Collette, J., Bruyere, O., et al. (2004). Osteoarthritic Patients with High Cartilage Turnover Show Increased Responsiveness to the Cartilage Protecting Effects of Glucosamine Sulphate. *Clin. Exp. Rheumatol.* 22 (1), 36–42.

Conrozier, T., Renevier, J. L., Parisaux, J. M., and Balblanc, J. C. (2019). Predictive Factors of Adherence to an Association of Glucosamine Sulfate, Copper, and Ginger Extracts in Patients with Symptomatic Osteoarthritis: a Prospective Open-Label French Noninterventional Study (The PREDOA Study). *Patient Prefer Adherence* 13 (13), 915–921. doi:10.2147/PPA.S200892

Dougados, M. (2006). Symptomatic Slow-Acting Drugs for Osteoarthritis: what Are the Facts? *Jt. Bone Spine* 73 (6), 606–609. doi:10.1016/j.jbspin.2006.09.008

Fernández-Martín, S., López-Peña, M., Muñoz, F., Permuy, M., and González-Cantalapiedra, A. (2021). Bisphosphonates as Disease-Modifying Drugs in Osteoarthritis Preclinical Studies: a Systematic Review from 2000 to 2020. *Arthritis Res. Ther.* 23 (1), 60. doi:10.1186/s13075-021-02446-6

Ghouri, A., and Conaghan, P. G. (2019). Update on Novel Pharmacological Therapies for Osteoarthritis. *Ther. Adv. Musculoskelet. Dis.* 11, 1759720X19864492. doi:10.1177/1759720X19864492

Gregori, D., Giacovelli, G., Minto, C., Barbetta, B., Gualtieri, F., Azzolina, D., et al. (2018). Association of Pharmacological Treatments with Long-Term Pain Control in Patients with Knee Osteoarthritis: A Systematic Review and Meta-Analysis. *JAMA* 320 (24), 2564–2579. doi:10.1001/jama.2018.19319

Henrotin, Y., Mobasheri, A., and Marty, M. (2012). Is There Any Scientific Evidence for the Use of Glucosamine in the Management of Human Osteoarthritis? *Arthritis Res. Ther.* 14 (1), 201. doi:10.1186/ar3657

Herrero-Beaumont, G., and Largo, R. (2020). Glucosamine and O-GlcNAcylation: a Novel Immunometabolic Therapeutic Target for OA and Chronic, Low-Grade Systemic Inflammation? *Ann. Rheum. Dis.* 79 (10), 1261–1263. doi:10.1136/annrheumdis-2020-217454

Hochberg, M. C., Guermazi, A., Guehring, H., Aydemir, A., Wax, S., Fleuranceau-Morel, P., et al. (2019). Effect of Intra-articular Sprifermin vs Placebo on Femorotibial Joint Cartilage Thickness in Patients with Osteoarthritis: The FORWARD Randomized Clinical Trial. *JAMA* 322 (14), 1360–1370. doi:10.1001/jama.2019.14735

Holder, K. K., and Kerley, S. S. (2008). Alendronate for Fracture Prevention in Postmenopause. *Am. Fam. Phys.* 78 (5), 579–581.

Igarashi, M., Kaga, I., Takamori, Y., Sakamoto, K., Miyazawa, K., and Nagaoka, I. (2011). Effects of Glucosamine Derivatives and Uronic Acids on the Production of Glycosaminoglycans by Human Synovial Cells and Chondrocytes. *Int. J. Mol. Med.* 27 (6), 821–827. doi:10.3892/ijmm.2011.662

Jackson, C. G., Plaas, A. H., Sandy, J. D., Hua, C., Kim-Rolands, S., Barnhill, J. G., et al. (2010). The Human Pharmacokinetics of Oral Ingestion of Glucosamine and Chondroitin Sulfate Taken Separately or in Combination. *Osteoarthr. Cartil.* 18 (3), 297–302. doi:10.1016/j.joca.2009.10.013

Jordan, K. M., Arden, N. K., Doherty, M., Bannwarth, B., Bijlsma, J. W., Dieppe, P., et al. (2003). EULAR Recommendations 2003: an Evidence Based Approach to the Management of Knee OA: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). *Ann. Rheum. Dis.* 62 (12), 1145–1155. doi:10.1136/ard.2003.011742

King, D. E., and Xiang, J. (2020). Glucosamine/Chondroitin and Mortality in a US NHANES Cohort. *J. Am. Board Fam. Med.* 33 (6), 842–847. doi:10.3122/jabfm.2020.06.200110

Kloppenburg, M., and Berenbaum, F. (2020). Osteoarthritis Year in Review 2019: Epidemiology and Therapy. *Osteoarthr. Cartil.* 28 (3), 242–248. doi:10.1016/j.joca.2020.01.002

Knapik, J. J., Pope, R., Hoedebecke, S. S., Schram, B., Orr, R., and Lieberman, H. R. (2018). Effects of Oral Glucosamine Sulfate on Osteoarthritis-Related Pain and Joint-Space Changes: Systematic Review and Meta-Analysis. *J. Spec. Oper. Med.* 18 (4), 139–147.

Kolasinski, S. L., Neogi, T., Hochberg, M. C., Oatis, C., Guyatt, G., Block, J., et al. (2020). 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis Care Res. (Hoboken)* 72 (2), 149–162. doi:10.1002/acr.24131

Largo, R., Martínez-Calatrava, M. J., Sánchez-Pernaute, O., Marcos, M. E., Moreno-Rubio, J., Aparicio, C., et al. (2009). Effect of a High Dose of Glucosamine on Systemic and Tissue Inflammation in an Experimental Model of Atherosclerosis Aggravated by Chronic Arthritis. *Am. J. Physiol. Heart Circ. Physiol.* 297 (1), 268–276. doi:10.1152/ajpheart.00142.2009

Li, Z. H., Gao, X., Chung, V. C., Zhong, W. F., Fu, Q., Lv, Y. B., et al. (2020). Associations of Regular Glucosamine Use with All-Cause and Cause-specific Mortality: a Large Prospective Cohort Study. *Ann. Rheum. Dis.* 79 (6), 829–836. doi:10.1136/annrheumdis-2020-217176

McCarty, M. F., O'Keefe, J. H., and DiNicolantonio, J. J. (2019). Glucosamine for the Treatment of Osteoarthritis: The Time Has Come for Higher-Dose Trials. *J. Diet. Suppl.* 16 (2), 179–192. doi:10.1080/19390211.2018.1448920

Müller-Fassbender, H., Bach, G. L., Haase, W., Rovati, L. C., and Setnikar, I. (1994). Glucosamine Sulfate Compared to Ibuprofen in Osteoarthritis of the Knee. *Osteoarthr. Cartil.* 2 (1), 61–69. doi:10.1016/s1063-4584(05)80007-x

Nagaoka, I., Tsuruta, A., and Yoshimura, M. (2019). Chondroprotective Action of Glucosamine, a Chitosan Monomer, on the Joint Health of Athletes. *Int. J. Biol. Macromol.* 132, 795–800. doi:10.1016/j.ijbiomac.2019.03.234

Noack, W., Fischer, M., Förster, K. K., Rovati, L. C., and Setnikar, I. (1994). Glucosamine Sulfate in Osteoarthritis of the Knee. *Osteoarthr. Cartil.* 2 (1), 51–59. doi:10.1016/s1063-4584(05)80006-8

Pavelká, K., Gatterová, J., Olejarová, M., Machacek, S., Giacovelli, G., and Rovati, L. C. (2002). Glucosamine Sulfate Use and Delay of Progression of Knee Osteoarthritis: a 3-year, Randomized, Placebo-Controlled, Double-Blind Study. *Arch. Intern. Med.* 162 (18), 2113–2123. doi:10.1001/archinte.162.18.2113

Poolsup, N., Suthisisang, C., Channark, P., and Kittikulsuth, W. (2005). Glucosamine Long-Term Treatment and the Progression of Knee Osteoarthritis: Systematic Review of Randomized Controlled Trials. *Ann. Pharmacother.* 39 (6), 1080–1087. doi:10.1345/aph.1E576

Reginster, J. Y., Deroisy, R., Rovati, L. C., Lee, R. L., Lejeune, E., Bruyere, O., et al. (2001). Long-term Effects of Glucosamine Sulphate on Osteoarthritis Progression: a Randomised, Placebo-Controlled Clinical Trial. *Lancet* 357 (9252), 251–256. doi:10.1016/S0140-6736(00)03610-2

Rousset, F., Nguyen, M. V., Grange, L., Morel, F., and Lardy, B. (2013). Heme Oxygenase-1 Regulates Matrix Metalloproteinase MMP-1 Secretion and Chondrocyte Cell Death via Nox4 NADPH Oxidase Activity in Chondrocytes. *PLoS One* 8 (6), e66478. doi:10.1371/journal.pone.0066478

Sellam, J., Courties, A., Eymard, F., Ferrero, S., Latourte, A., Ornetti, P., et al. (2020). Recommendations of the French Society of Rheumatology on Pharmacological Treatment of Knee Osteoarthritis. *Jt. Bone Spine* 87 (6), 548–555. doi:10.1016/j.jbspin.2020.09.004

Seto, Y., Yoshihashi, T., Tomonari, M., and To, H. (2020). Absorption of Glucosamine Is Improved by Considering Circadian Rhythm and Feeding in Rats. *Chronobiol. Int.* 37, 1528. doi:10.1080/07420528.2020.1784189

Simon, R. R., Marks, V., Leeds, A. R., and Anderson, J. W. (2011). A Comprehensive Review of Oral Glucosamine Use and Effects on Glucose Metabolism in normal and Diabetic Individuals. *Diabetes Metab. Res. Rev.* 27 (1), 14–27. doi:10.1002/dmrr.1150

Stevens, R. M., Ervin, J., Nezzer, J., Nieves, Y., Guedes, K., Burges, R., et al. (2019). Randomized, Double-Blind, Placebo-Controlled Trial of Intraarticular Trans-capsaicin for Pain Associated with Osteoarthritis of the Knee. *Arthritis Rheumatol.* 71 (9), 1524–1533. doi:10.1002/art.40894

Tardio, L., Andrés-Bergós, J., Zachara, N. E., Larrañaga-Vera, A., Rodriguez-Villar, C., Herrero-Beaumont, G., et al. (2014). O-linked N-Acetylglucosamine (O-GlcNAc) Protein Modification Is Increased in the Cartilage of Patients with Knee Osteoarthritis. *Osteoarthr. Cartil.* 22 (2), 259–263. doi:10.1016/j.joca.2013.12.001

Thorup, A. S., Strachan, D., Caxaria, S., Poulet, B., Thomas, B. L., Eldridge, S. E., et al. (2020). ROR2 Blockade as a Therapy for Osteoarthritis. *Sci. Transl. Med.* 12, eaax3063. doi:10.1126/scitranslmed.aax3063

Ton, J., Perry, D., Thomas, B., Allan, G. M., Lindblad, A. J., McCormack, J., et al. (2020). PEER Umbrella Systematic Review of Systematic Reviews: Management of Osteoarthritis in Primary Care. *Can. Fam. Physi.* 66 (3), e89–e98.

Tsuruta, A., Horiike, T., Yoshimura, M., and Nagaoka, I. (2018). Evaluation of the Effect of the Administration of a Glucosamine-Containing Supplement on Biomarkers for Cartilage Metabolism in Soccer Players: A Randomized Double-Blind Placebo Controlled Study. *Mol. Med. Rep.* 18 (4), 3941–3948. doi:10.3892/mmr.2018.9396

Vetter, G. (1965). Glucosamine in the Therapy of Degenerative Rheumatism. *Dtsch Med. J.* 516 (13), 446–449.

Yazici, Y., McAlindon, T. E., Gibofsky, A., Lane, N. E., Clauw, D., Jones, M., et al. (2020). Lorecivivint, a Novel Intraarticular CDC-like Kinase 2 and Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A Inhibitor and Wnt Pathway Modulator for the Treatment of Knee Osteoarthritis: A Phase II Randomized Trial. *Arthritis Rheumatol.* 72 (10), 1694–1706. doi:10.1002/art.41315

Zhu, X., Sang, L., Wu, D., Rong, J., and Jiang, L. (2018). Effectiveness and Safety of Glucosamine and Chondroitin for the Treatment of Osteoarthritis: a Meta-Analysis of Randomized Controlled Trials. *J. Orthop. Surg. Res.* 13 (1), 170. doi:10.1186/s13018-018-0871-5

Conflict of Interest: TC received fees for scientific and/or board member activities from LABRHA, SANOFI, FIDIA, MEDAC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

REVIEW
published: 05 April 2022
doi: 10.3389/fphar.2022.811808

Icariin: A Potential Molecule for Treatment of Knee Osteoarthritis

Juntao Zhang[1,2†], Fangyang Fan[2†], Aifeng Liu[2], Chao Zhang[2], Qi Li[2], Chenglong Zhang[2], Feng He[1] and Man Shang[3*]*

[1]Academy of Medical Engineering and Traditional Medicine, Tianjin University, Tianjin China, [2]Orthopedics Department, National Clinical Research Center for Chinese Medicine Acupuncture and Moxibustion, The First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China, [3]Department of Pharmacology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

Background: Knee osteoarthritis (KOA) is a degenerative disease that develops over time. Icariin (ICA) has a positive effect on KOA, although the mechanism is unknown. To investigate drug-disease connections and processes, network pharmacology is commonly used. The molecular mechanisms of ICA for the treatment of KOA were investigated using network pharmacology, molecular docking and literature research approaches in this study.

Methods: We gathered KOA-related genes using the DisGeNET database, the OMIM database, and GEO microarray data. TCMSP database, Pubchem database, TTD database, SwissTargetPrediction database, and Pharmmapper database were used to gather ICA-related data. Following that, a protein-protein interaction (PPI) network was created. Using the Metascape database, we performed GO and KEGG enrichment analyses. After that, we built a targets-pathways network. Furthermore, molecular docking confirms the prediction. Finally, we looked back over the last 5 years of literature on icariin for knee osteoarthritis to see if the findings of this study were accurate.

Results: core targets relevant to KOA treatment include TNF, IGF1, MMP9, PTGS2, ESR1, MMP2 and so on. The main biological process involved regulation of inflammatory response, collagen catabolic process, extracellular matrix disassembly and so on. The most likely pathways involved were the IL-17 signaling pathway, TNF signaling pathway, Estrogen signaling pathway.

Conclusion: ICA may alleviate KOA by inhibiting inflammation, cartilage breakdown and extracellular matrix degradation. Our study reveals the molecular mechanism of ICA for the treatment of KOA, demonstrating its potential value for further research and as a new drug.

Keywords: icariin, knee osteoarthritis, network pharmacology, molecular docking, molecular mechanism

OPEN ACCESS

Edited by:
Sara Tenti,
University of Siena, Italy

Reviewed by:
Yanqiong Zhang,
China Academy of Chinese Medical Sciences, China
Sudheer Kumar Ravuri,
Steadman Philippon Research Institute, United States

***Correspondence:**
Feng He
heaven@tju.edu.cn
Man Shang
shangman@tmu.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *09 November 2021*
Accepted: *21 February 2022*
Published: *05 April 2022*

Citation:
Zhang J, Fan F, Liu A, Zhang C, Li Q, Zhang C, He F and Shang M (2022) Icariin: A Potential Molecule for Treatment of Knee Osteoarthritis. Front. Pharmacol. 13:811808. doi: 10.3389/fphar.2022.811808

INTRODUCTION

Knee osteoarthritis (KOA) is a chronic progressive disease with pain, swelling, and even deformity of the knee joint as the main clinical manifestation (Martel-Pelletier et al., 2016; Primorac et al., 2020). OA is the 11th leading cause of disability worldwide, which not only affects patients physically and mentally, but also represents a serious social burden (Palazzo et al., 2016). An epidemiological survey showed that the number of KOA patients in the United States doubled from the 1900's to the 2000's

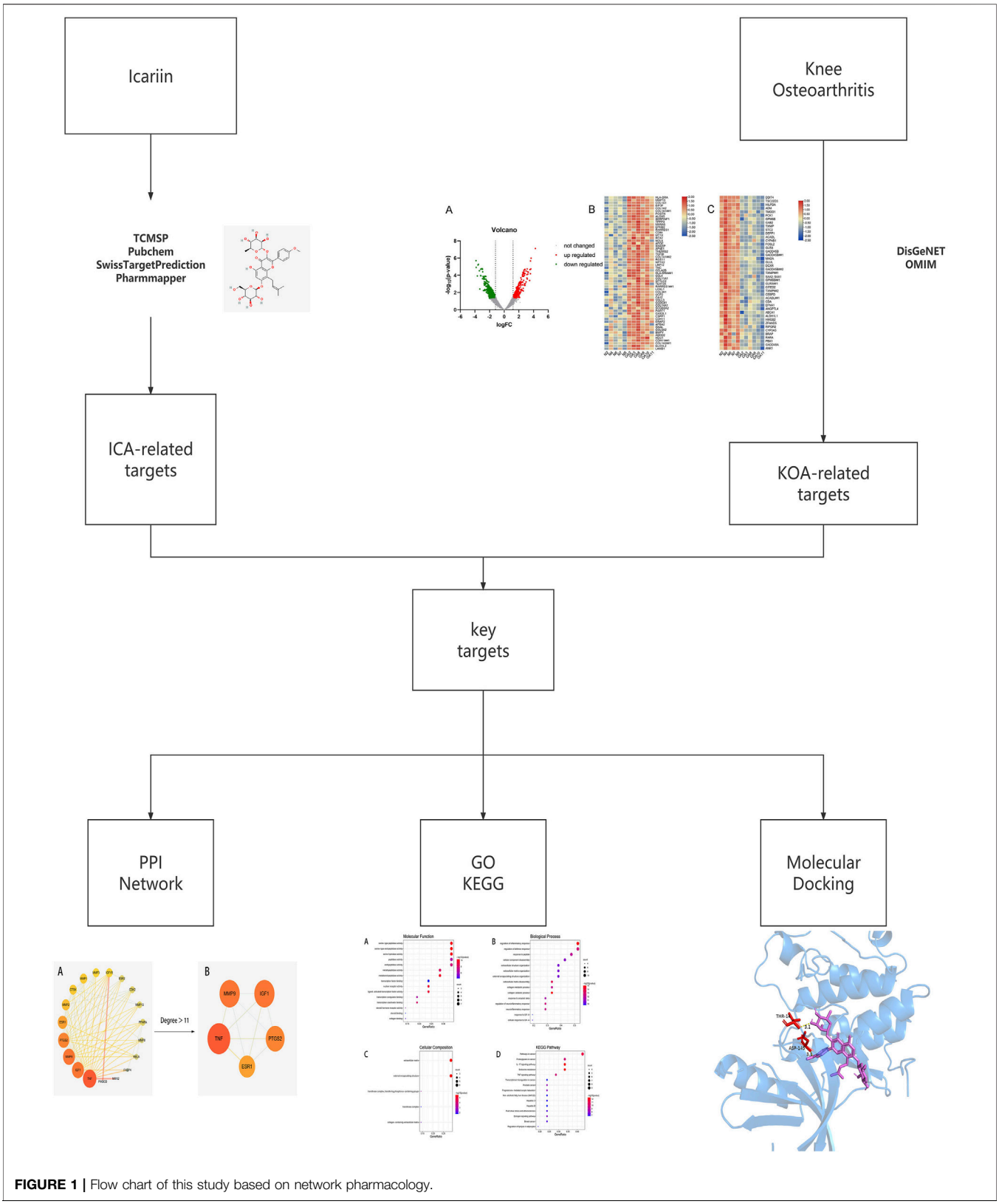


FIGURE 1 | Flow chart of this study based on network pharmacology.

(Wallace et al., 2017). According to research in 2010 (Cross et al., 2014), the global standard age prevalence of KOA was 3.8%, and the prevalence increased sharply with age. KOA pathology mainly involves articular cartilage and synovium (Michael et al., 2010). In the physiological state, the cartilage matrix is in a dynamic balance of cytokine-controlled production and breakdown. When mechanically altered or stimulated by inflammation, cartilage degrades and progresses to KOA if the compensatory capacity of the joint is exceeded (Michael et al., 2010). The pathogenesis of KOA, however, is not yet clear. To better guide the clinical treatment of KOA, its pathogenesis needs to be studied urgently.

Icariin (ICA) is one of the main components of the Chinese medicine Epimedium. Epimedium was first recorded in the Yellow Emperor's Inner Canon in 100 B.C. Traditional Chinese medicine believed that Epimedium could strengthen muscles and bones, and it was widely used in clinical practice. Due to the development of modern medicine, its active ingredients have been further studied. Modern research has discovered that ICA, one of its active ingredients, has strong biological activity on various systems, such as the nervous system (Jin et al., 2019), reproductive system (Zhang et al., 2021), skeletal system (Pan et al., 2017; Wang et al., 2018) and so on. ICA was found to improve bone loss caused by estrogen deficiency through the IGF-1 pathway, the ERK pathway and the JNK pathway (Song et al., 2013; Zhou et al., 2021). It also has the effect of preventing cartilage degradation and promoting cartilage repair, as proved by some *in vivo* and *in vitro* experiments (Wei et al., 2016; Zhang et al., 2019). The most widely used drugs in non-surgical treatment are NSAIDs such as cyclooxygenase-2 (COX-2) inhibitors (Bannuru et al., 2019). However, the long-term use of NSAIDs leads to gastrointestinal complications such as indigestion, gastritis and heartburn (Curtis et al., 2019). Since there is no specific treatment for KOA, there is an urgent need to find a safe and reliable treatment method.

Network pharmacology analysis is one of the most commonly used methods to study the pharmacology of traditional Chinese medicine (TCM). By searching the databases, constructing the networks, enrichment analysis and other steps, we can get the components, potential targets, correlation with diseases and other information of TCM (Yuan et al., 2017). This leads to a more in-depth study of the possible mechanisms of TCM in the treatment of diseases. Molecular docking technology can verify the binding between TCM molecules and predicted targets to validate the accuracy of these targets (Pinzi and Rastelli 2019). Therefore, this study explores the molecular mechanism of ICA for KOA based on network pharmacology, molecular docking, and literature research and the flow chart is shown in **Figure 1**.

MATERIALS AND METHODS

Collection of Osteoarthritis Related Genes

Firstly, microarray information on differential DNA expression between normal subjects and KOA patients was collected from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) (Edgar et al., 2002), Series GSE169077. The microarray data was normalized with $\log_2$ (fold change) > 1 or $\log_2$ (fold change) < -1 as the standard, which was considered to have significant differences in the expression. The KOA related genes were collected by integrating databases through searches with the key words "knee osteoarthritis" and "*Homo sapiens*," followed by taking the intersection. Two databases include: DisGeNET database (https://www.disgenet.org/web/DisGeNET/menu/home) (Piñero et al., 2017) and OMIM database (https://omim.org/) (Amberger et al., 2015). Next, a visual volcano map and a gene expression heat map were created using Graphpad Prism 8.0.2 (GraphPad Software, San Diego, California United States, www.graphpad.com) and TBtools (https://github.com/CJ-Chen/TBtools) (Chen et al., 2020).

Potential Target Genes of Icariin in the Treatment of Knee Osteoarthritis

To evaluate the biological information of ICA, it is chemical name, molecular weight, oral bioavailability (OB) and drug likeness (DL) were obtained from the TCMSP database (https://tcmspw.com/tcmsp.php) (Ru et al., 2014). Studies have shown that OB≥40% is considered to have good bioavailability; DL ≥0.18 is considered to be a drug-like compound (Liu et al., 2013). Then the 2D and 3D molecular structures of ICA were obtained through the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/) (Kim et al., 2021).

Next, the potential targets of ICA were predicted using the SwissTargetPrediction database (http://swisstargetprediction.ch/) (Gfeller et al., 2013), Therapeutic Target Database (http://db.idrblab.net/ttd/) (Wang Y. et al., 2020) and Pharmmapper database (http://www.lilab-ecust.cn/pharmmapper/) (Wang et al., 2017), followed by taking intersection set. After that, all targets were converted into gene symbols standardized through the Uniprot database (https://www.uniprot.org/) (Morgat et al., 2020).

Construction of Protein Interaction Network

The potential targets of ICA were matched with KOA-related genes to obtain the core targets. The venn diagram was drawn through venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) (Oliveros 2007-2015). Next, the targets were analyzed by the String database (https://string-db.org/) (Szklarczyk et al., 2019). After that, the PPI network was edited with Cytoscape 3.7.2 software (http://www.cytoscape.org) and the degree and edge betweenness of the nodes were calculated by the NetworkAnalyzer tool.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis

Based on the above data, GO and KEGG enrichment analysis was performed in the Metascape database (http://metascape.org/gp/index.html) (Zhou et al., 2019). The Gene Ontology (GO) enrichment analysis includes cellular composition, biological process, and molecular function, whereas the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis can profile metabolic pathways of genes in the cell

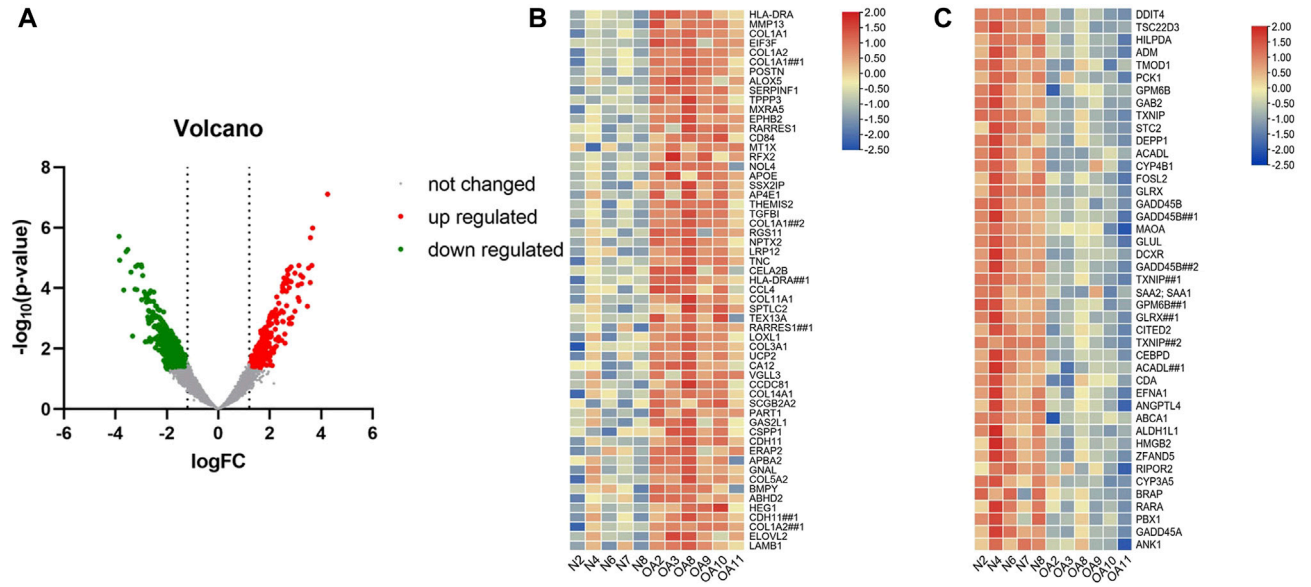
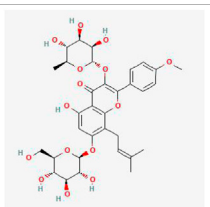
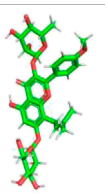


FIGURE 2 | The volcano and heat maps of differential DNA expression in OA patients. **(A)** Red represents up-regulated genes, green represents down-regulated genes, and gray represents unchanged genes. **(B)** The most significantly upregulated genes in cartilage of OA patients versus normal patients. **(C)** The most significantly downregulated genes in cartilage of OA patients versus normal patients.

TABLE 1 | Basic information of ICA.

Molecular Name	Molecular Formula	Molecular Weight	2D Structure	3D Structure
Icariin	$C_{33}H_{40}O_{15}$	676.66 g/mol		

as well as their systemic function. Unlike the last update of the David database in 2016, the Metascape database was updated on 1 February 2021. After this, a visual bubble diagram was drawn using the Bioinformatic online tool (http://bioinformatics.com.cn/).

Construction of Targets-Pathways Network

To investigate the interactions between key genes and pathways of ICA and KOA, the ICA-KOA-targets-pathways network was constructed using the results of metascape database analysis. The degree and edge betweenness were analyzed by topological analysis tools in the cytoscape 3.7.2 software.

Molecular Docking

Proteins corresponding to the top ten key genes were selected to dock with the ICA molecule to verify their affinity. Crystal structures of proteins were downloaded from the PDB database (https://www.rcsb.org/) (Burley et al., 2021) with the search criteria: *Homo sapiens*, refine resolution<3.0 Å and release date 2015–2021. then we imported the protein structures into PyMOL 2.2.0 software (https://pymol.org) (Schrodinger 2015) for modification including removal of water molecules, separation of ligands and addition of hydrogen. Using AutoDockTools 1.5.6 software (http://autodock.scripps.edu/) (Goodsell and Olson 1990) to add charge to protein molecule and set up a docking grid box at the center of the molecule (Seeliger and de Groot 2010). Molecular docking was performed using AutoDock Vina 1.1.2 software (http://vina.scripps.edu/) (Trott and Olson 2010). We chose it as the software for molecular docking because it is efficient, accurate, and has a new way of evaluation. Finally, after analyzing the binding energy of the molecule, choosing the conformation with the lowest binding energy, and observing the formation of hydrogen bonds, we drew a binding diagram with PyMOL 2.2.0.

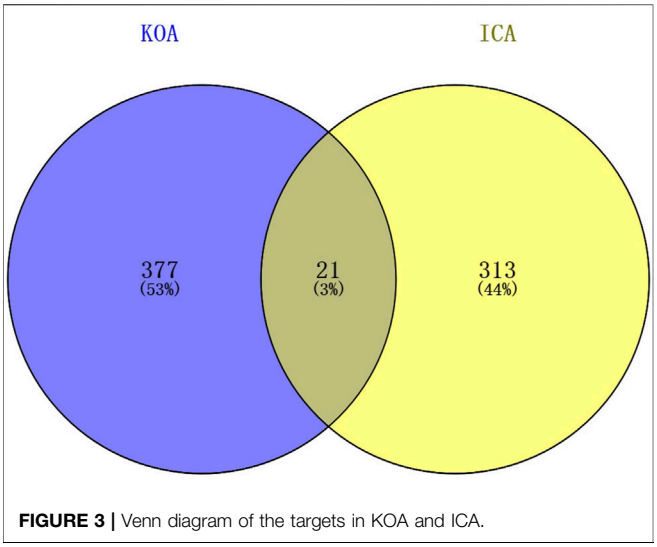


FIGURE 3 | Venn diagram of the targets in KOA and ICA.

Search and Analysis of Literature

To validate the predictions, we also searched for relevant studies over the last 5 years, including *in vivo* studies, *in vitro* studies and reviews. The search terms we used were "icariin", "osteoarthritis", "knee osteoarthritis", "cartilage", "chondrocytes", and "extracellular matrix". To ensure the relevance of the literature to this study, the results were collated by two independent researchers.

RESULT

Acquisition of Knee Osteoarthritis Related Genes

A total of 1105 differentially expressed genes were identified by analysis of gene microarray Series GSE169077, which was used to draw a volcano may and heat map (**Figure 2**). The results showed a large difference in gene expression between OA patients and normal subjects. Then 720 out of 9329 genes from the Genecards database (relevance score >4.26), 368 genes from the Disgenet database, 56 genes from the OMIM database, and finally 398 genes were obtained.

TABLE 2 | Topological information of key targets.

Target	Degree	Betweenness Centrality	Closeness Centrality
TNF	17	0.235	0.947
IGF1	15	0.101	0.857
MMP9	15	0.080	0.857
PTGS2	14	0.070	0.818
ESR1	12	0.035	0.750

Analysis of Icariin and Collection of its Potential Targets

Molecular formula, molecular weight, 2D structure, 3D structures, and ADME information of ICA were collected from the TCMSP database and the Pubchem database (**Table 1**). The OB (41.58%) and DL (0.61) of ICA proved its high bioavailability and drug-like property, which can be used as a drug molecule for the next step of research. A total of 334 potential targets were obtained by combining and removing duplicate ones from the SwissTargetPrediction database, the Pharmmapper database, and the Therapeutic Target Database. Next, the ICA targets were intersected with the KOA genes to obtain the key genes and plotted a Venn diagram (**Figure 3**).

Protein-Protein Interaction Network Analysis

The obtained 21 common genes were uploaded to the String database to produce the PPI network. After that, the results were imported into cytoscape 3.7.2 software and the topological parameters of the network was analyzed. The result showed that the network had a total of 18 nodes and 78 edges (**Figure 4A**). According to the analysis results, TNF was the most important

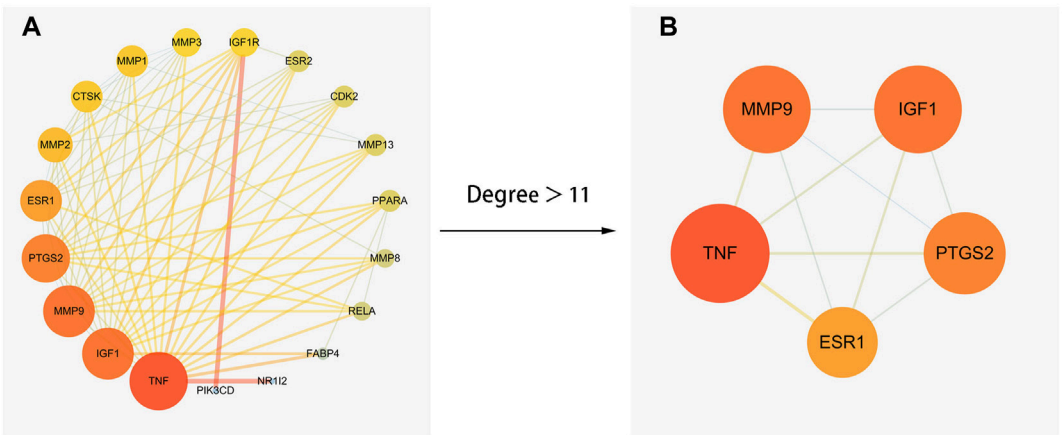


FIGURE 4 | The PPI network of ICA and KOA. **(A)** the network with total 18 nodes and 78 edges. **(B)** the core network with 5 nodes and 7 edges.

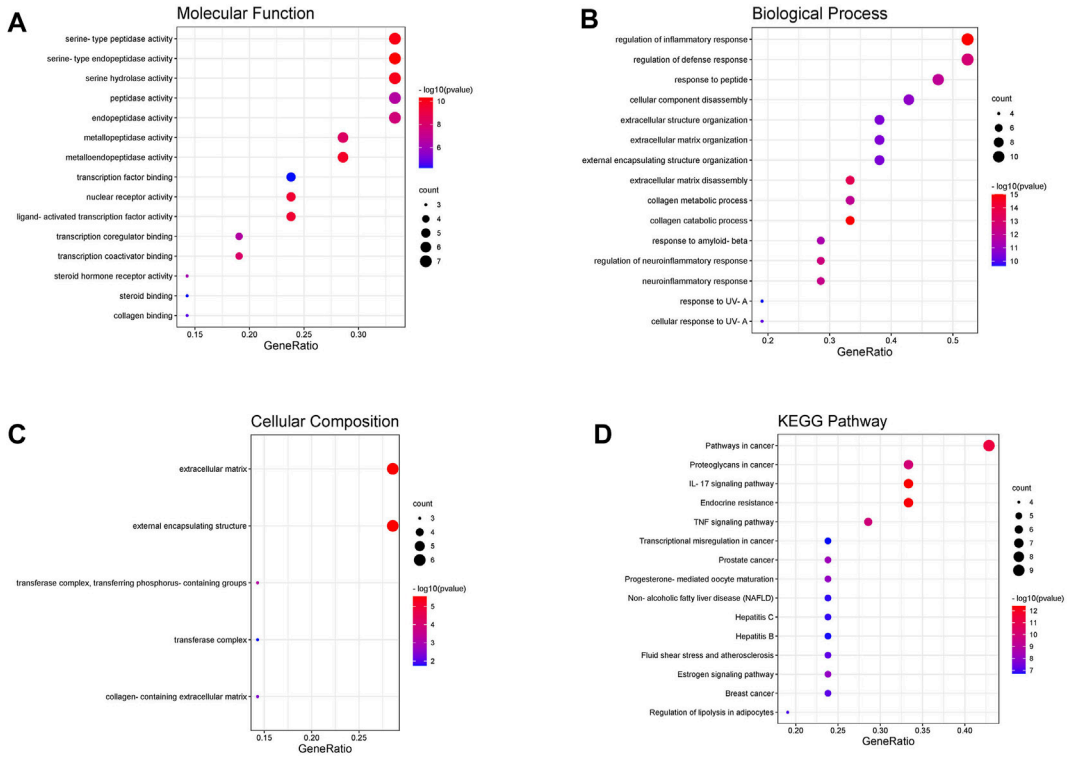


FIGURE 5 | The GO and KEGG enrichment analysis of key targets. Including **(A)** cellular components, **(B)** molecular functions, **(C)** biological processes and **(D)** KEGG pathway analysis.

TABLE 3 | Top 3 KEGG pathways and related targets, excluding other disease.

Term	Pathway	Key Targets in the Pathway	*p* Value
ko04657	IL-17 signaling pathway	MMP1,MMP3,MMP9,MMP13,PTGS2,RELA,TNF,PIK3CD,MMP2,PPARA,CDK2,ESR1,ESR2,CTSK,PDE3B,RORA	3.8E-13
hsa04668	TNF signaling pathway	MMP3,MMP9,PIK3CD,PTGS2,RELA,TNF	1.4E-10
ko04915	Estrogen signaling pathway	ESR1,ESR2,MMP2,MMP9,PIK3CD,MMP1	9.0E-09

target in the network (Degree 17, BC 0.24, CC 0.95). To find the key genes of ICA in treatment of KOA, a core network with 5 nodes and 10 edges was filtered by Degree>11 (**Figure 4B**). The other 4 genes were MMP9, IGF1, PTGS2 and ESR1. Finally, the 5 core genes screened by the NetworkAnalyzer tool and their Degree values and Betweenness Centrality values were shown in **Table 2**.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis

351 GO items were collected through the analysis of the Metascape database (p 0.01, count 3), while the KEGG entries were 112 (p 0.01, count 3). The top 15 most significant entries were filtered in ascending *p*-value order and displayed in a visual bubble diagram, and all results were shown if less than 15 (**Figure 5**). The results show that the most significant molecular function included serine-type endopeptidase activity, serine-type peptidase activity, metallopeptidase activity, nuclear receptor activity and so on. While the biological process was primarily involved in processes such as collagen catabolism, extracellular matrix disassembly, collagen metabolic process, and other extracellular matrix-related processes. Another aspect is the processes associated with inflammation, such as regulation of inflammatory responses, regulation of neuroinflammatory responses and so on. KEGG results showed that genes shared by ICA and KOA were mainly enriched in the IL-17 signaling pathway, TNF signaling pathway and Estrogen signaling pathway, excluding other disease pathways (**Figure 5D**; **Table 3**). Then a targets-pathways network was constructed based on the results (**Figure 6**).

Molecular Docking Analysis

Based on the data above, the 21 core target genes were individually imported into the Autodock Vina 1.1.2 software to dock with the ICA. Affinity was the score used by the software to calculate the

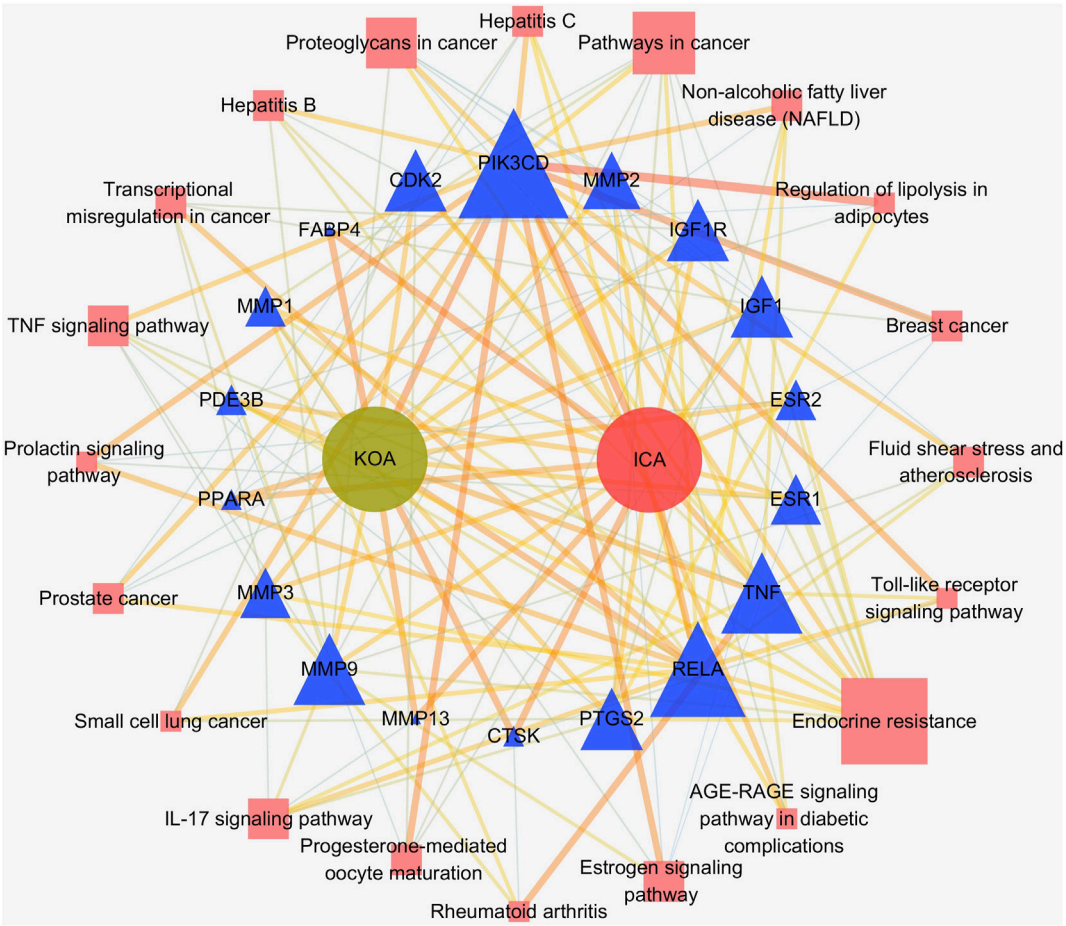


FIGURE 6 | The targets-pathways network of ICA and KOA. Blue triangles represent ICA and KOA core genes, pink squares represent pathways. The size of the shapes represents the value of Degree.

TABLE 4 | Affinity of gene binding to ICA.

The Targets	Affinity (kcal/mol)	The Targets	Affinity (kcal/mol)
CDK2	−10.1	ESR1	−8.2
MMP13	−9.5	PDE3B	−8.2
PTGS2	−9.5	ESR2	−8.0
MMP3	−9.1	CTSK	−8.0
IGF1	−9.1	TNF	−7.7
PIK3CD	−8.8	RELA	−7.5
MMP2	−8.8	MMP1	−7.4
MMP9	−8.4	PPARA	−7.2
IGF1R	−8.3		

binding ability. It is generally believed that affinity < −7 kcal/mol indicated a stronger binding activity (Ferreira et al., 2015; Gromiha et al., 2017; Cui et al., 2020). After calculation, it was found that 17 out of 21 genes met the criteria of affinity < −7 kcal/mol (**Table 4**). The docking diagrams of ICA and genes were drawn using Pymol software (**Figure 7**). In addition, we found the hydrogen bonds formed between molecules and represented by yellow dotted lines in the diagrams.

Results of the Literature Collection

Through our study of the literature, we screened twenty-eight articles from the last 5 years relevant to this study. Of these, 8 papers mentioned that icariin inhibited inflammation, 6 papers indicated that icariin reduced chondrocyte apoptosis and 4 papers indicated that icariin inhibited extracellular matrix degradation. In addition, 9 papers mentioned that icariin promotes chondrocyte proliferation, differentiation, cellular autophagy or cartilage repair (**Figure 8A**). In the literature on the underlying mechanisms, 3 papers suggested that icariin can treat OA via the NF-κB signaling pathway, 3 papers mentioned that icariin inhibited the expression of TNF-α, 3 papers mentioned that icariin downregulated the expression of MMPs, while 2 papers mentioned that icariin reduced the inflammatory response caused by IL-β (**Figure 8B**).

DISCUSSION

Despite the significant developments in the diagnosis and treatment of KOA, single drug development for a single target

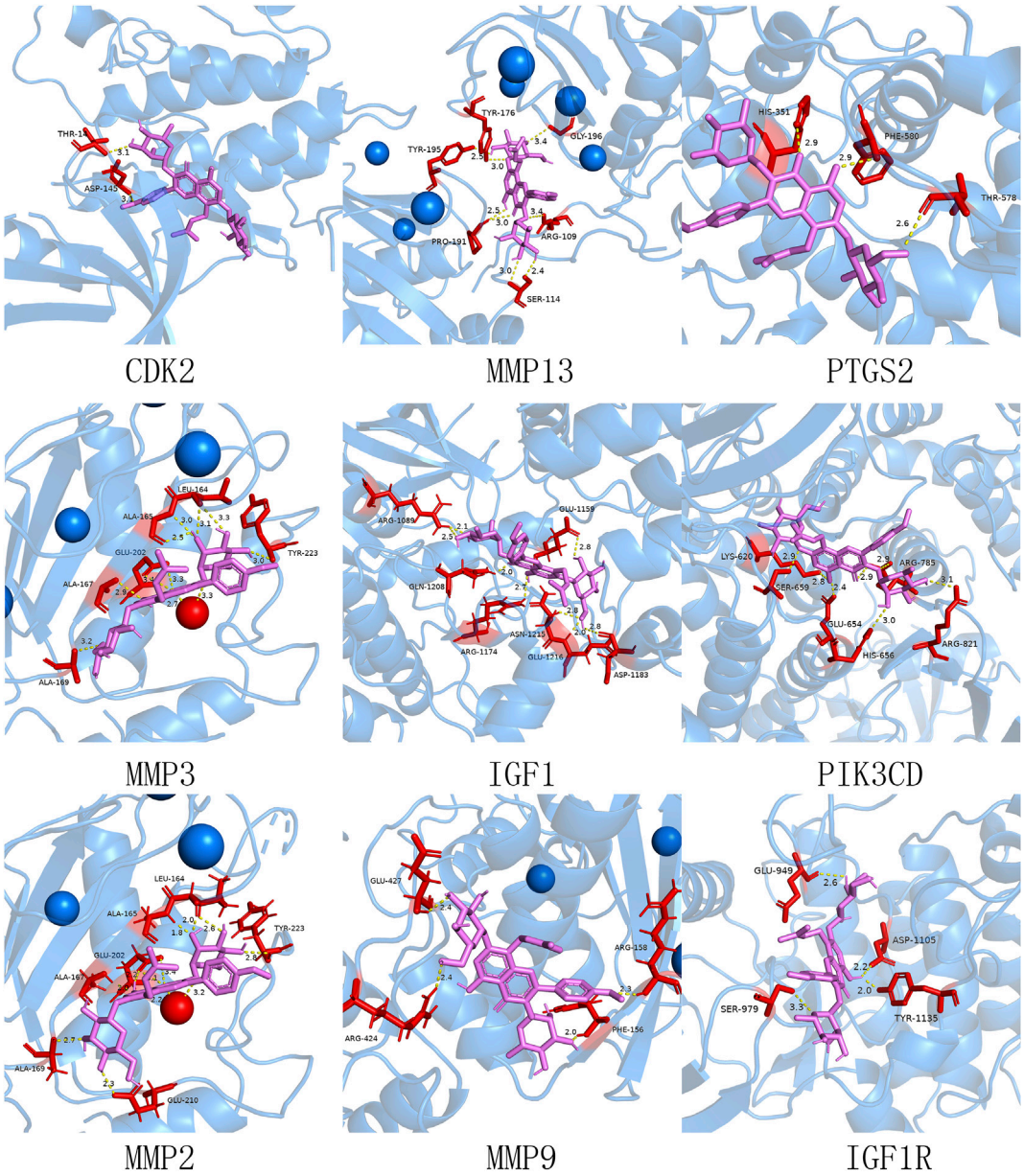


FIGURE 7 | Molecular docking simulation diagram. The ICA is violet stick models and the protein molecules are blue cartoon models. The protein molecules at the docking site are represented as red stick models. The connected hydrogen bonds are indicated by yellow dotted lines. And the blue and red bubbles indicate metal ions.

is still slow (Wang et al., 2015). This is because KOA is a complex pathological process involving multiple substances and regulated by multiple pathways (Abramoff and Caldera 2020). Coincidentally, traditional Chinese medicine has a multi-target and multi-pathway biological effect. As a traditional Chinese medicine, epimedium is widely used in clinical practice. It has also been widely used in the treatment of knee osteoarthritis from ancient times to the present day with good healing properties. As research progressed, ICA, one of the main components of Epimedium, was found to have strong biological activity. Due to its low price and easy availability, it has a high potential value in the treatment of KOA. In the present study, based on network pharmacology, we found that icariin has good bioavailability. Meanwhile, we assembled a number of key targets and pathways, as well as biological functions, and reviewed the literature over the last 5 years to validate them.

Icariin May be Useful for the Treatment of Knee Osteoarthritis by Inhibiting Inflammation

Inflammation is a major factor in the development and progression of KOA. On the one hand, inflammatory mediators promote cartilage apoptosis and extracellular matrix

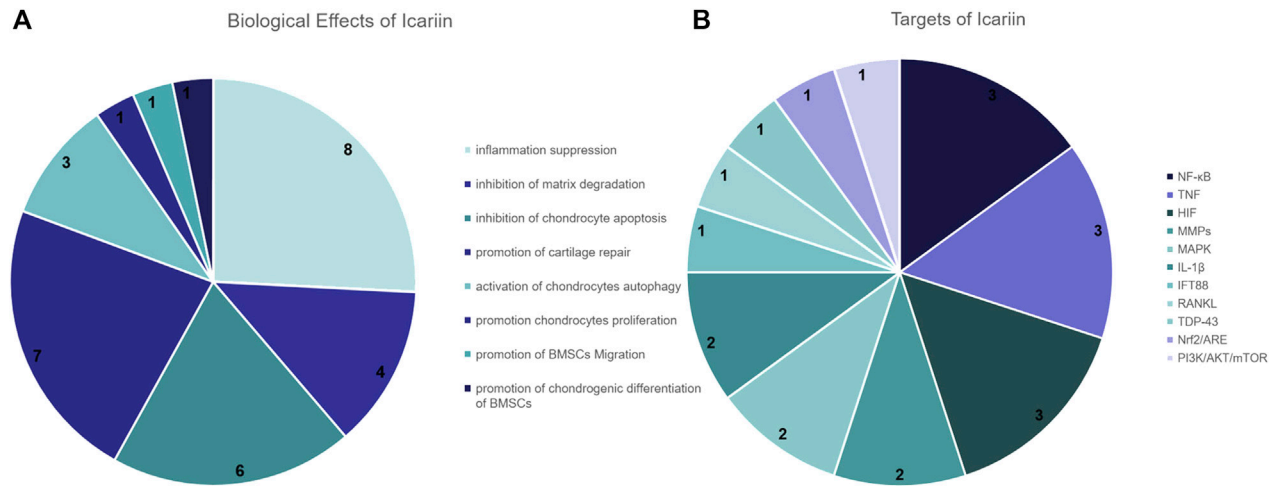


FIGURE 8 | Pie chart of icariin-related literature studies. **(A)** Number of times icariin has been mentioned in the literature for the treatment of KOA effects. **(B)** Number of icariin targets mentioned in the literature.

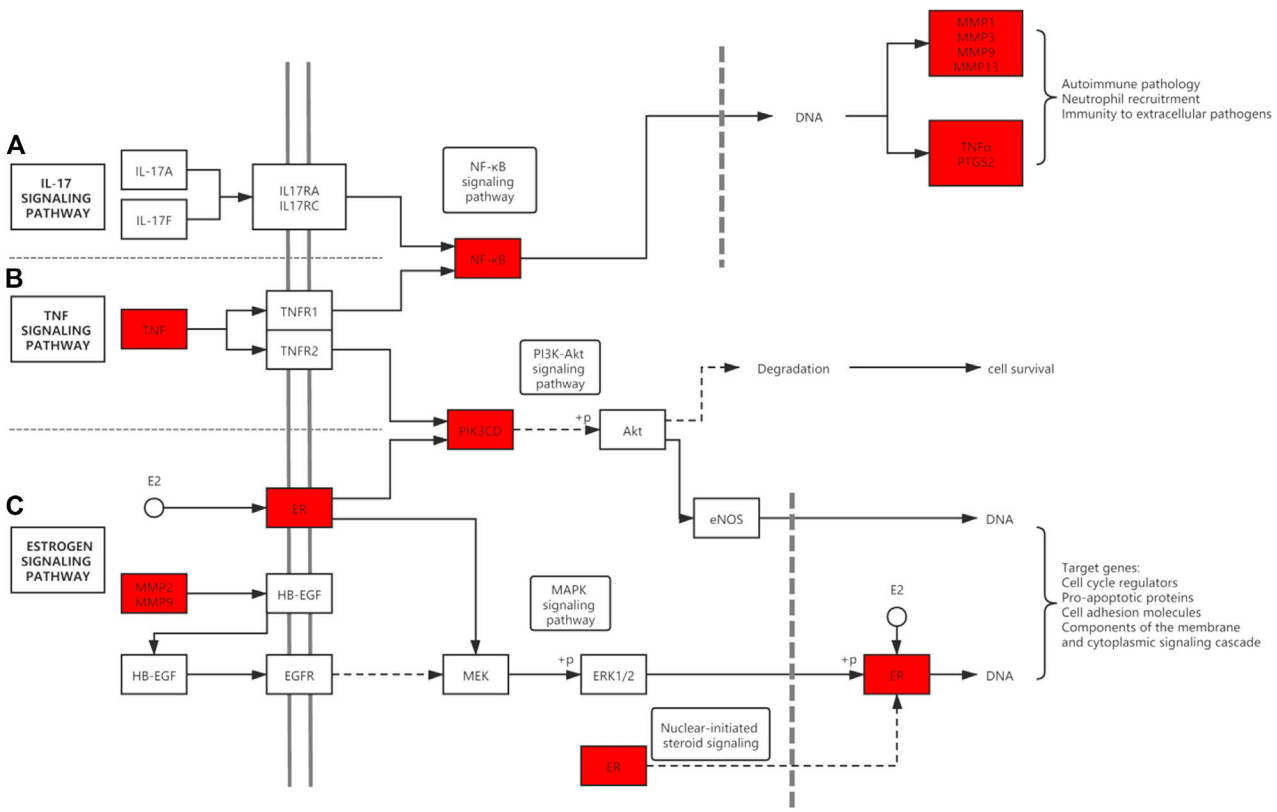


FIGURE 9 | Diagram of ICA potential target genes and pathways. ICA targets are shown in red. **(A)** The IL-17 signaling pathway. **(B)** The TNF signaling pathway. **(C)** The Estrogen signaling pathway. There are also cross talks among pathways.

degradation. On the other hand, the products of chondrocyte apoptosis and matrix breakdown induce the production of inflammatory substances that further aggravate the KOA condition (Martel-Pelletier et al., 2016). These inflammatory mediators include tumour necrosis factor (TNF), interleukin (IL), nitric oxide (NO), prostaglandin E2 (PGE_2) and so on (Fei et al., 2019; Li et al., 2020).

Our study found that the key targets of icariin for the treatment of KOA include TNF, PTGS2 as well as RELA (NF-κB). TNF is a powerful pro-inflammatory factor that binds to two types of receptors, including TNFR1 and TNFR2, which are expressed on the surface of both chondrocytes and synovial cells (Ansari et al., 2020) (**Figure 9**). TNF binding to the receptor activates the downstream NF-κB signaling pathway, thereby up-regulating the synthesis of inflammatory mediators (PTGS2, NO, PGE_2, IL-6, etc.) and matrix degrading enzymes (MMPs, ADAMTS). In addition, it can inhibit the synthesis of type II collagen (Wojdasiewicz et al., 2014). A study reported that icariin can inhibit NF-κB activation and nuclear translocation triggered by TNF-α, as well as reduce chondrocyte apoptosis and inflammation (Mi et al., 2018). In addition, according to the study of Pengzhen Wang et al. (2020), icariin inhibited the activation of NF-κB and HIF-2α in the chondrocytes of OA mice and attenuated the inflammatory response driven by TNF-α. In addition, the expression of MMPs was inhibited. These findings confirm that icariin reduces KOA inflammation by down-regulating the TNF signalling pathway through inhibition of TNF-α, NF-κB. In addition to the above proven targets, we also identified PTGS2 as a potential target for icariin. This could be an area for further research.

Icariin may contribute to the treatment of KOA by promoting cartilage repair and attenuating cartilage extracellular matrix degradation.

In KOA, chondrocyte apoptosis and extracellular matrix degradation are severe pathological changes (Choi et al., 2019). The extracellular matrix is mostly made up of type 2 collagenase and aggrecan, which can be degraded by matrix-degrading enzymes like MMPs and ADAMTS (Guilak et al., 2018). And this is associated with the accumulation of inflammatory substances in KOA joints including TNF-α, IL-6, IL-1β (Krishnan and Grodzinsky 2018). Degradation of the extracellular matrix promotes chondrocyte apoptosis at the same time (Komori 2016).

Our research has found substantial evidence that icariin can promote cartilage repair in KOA over the last 5 years (Wang et al., 2016; Liu et al., 2019; Zhang et al., 2019). Other studies have shown that icariin regulates the TDP-43 signaling pathway and NF-κB signaling pathway to reduce chondrocyte apoptosis (Mi et al., 2018; Huang et al., 2019). In addition, Tang Y, et al. showed that icariin mediated the PI3K/AKT/mTOR pathway to activate chondrocyte autophagy (Tang et al., 2021).

In addition to the above findings, we found that insulin-like growth factor 1 (IGF-1) is also one of the key targets of icariin. It is closely related to the proliferation, differentiation and maintenance of the phenotype of cartilage (Oh and Chun 2003). One study found that icariin enhances IGF-1 signalling, promotes osteogenic differentiation and has estrogen-like effects (Zhou et al., 2021). This suggests that icariin promotes chondrocyte proliferation and differentiation via IGF-1, thereby repairing KOA-damaged cartilage.

We also found that icariin also binds to the estrogen receptors and activates estrogen signaling pathway (**Figure 9**). The estrogen signaling pathway also plays a significant role in the skeletal system. In particular, post-menopausal women have a high incidence of arthritis and cardiovascular disease. Estrogen was found to have good anti-inflammatory effects and reduce cartilage damage (Martín-Millán and Castañeda 2013). This may be one of the reasons why there are more KOA patients among females than males over the age of 50.

Network pharmacology as a new technology has a wide range of applications in exploring the molecular mechanisms of Chinese medicine. In this study, we predicted the molecular mechanism of ICA for the treatment of KOA and obtained 21 relevant targets. Further analysis obtained the core targets as TNF, MMP9, IGF1, PTGS2, ESR1 and so on. After GO and KEGG analysis, we found that the key pathway for ICA treatment of KOA is the IL-17 signaling pathway, TNF signaling pathway and Estrogen signaling pathway with a regulatory effect on the extracellular matrix degradation and inflammation. Molecular docking verified the affinity of key targets. Finally, we reviewed the literature related to icariin for treatment of KOA in the last 5 years, and the results are generally consistent with the prediction.

CONCLUSION

Taken together, ICA is able to inhibit KOA inflammation, chondrocyte apoptosis, matrix degradation, and promote cartilage repair through multiple pathways at multiple targets. Through network pharmacology and molecular docking technology, we obtained the interaction relationship between genes, protein molecules and revealed the molecular mechanism of ICA for the treatment of KOA, making it possible as a new drug.

AUTHOR CONTRIBUTIONS

JZ and FF proposed and designed this study. AL and CZ collected data and drafted this manuscript. QL and CZ performed analysis and interpretation of the data. MS and FH helped in revising and refining the manuscript. All authors discussed and approved the final manuscript.

FUNDING

This study was supported by the National Natural Sciences Foundation of China (grant number 82074470; 51573137).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.811808/full#supplementary-material

REFERENCES

Abramoff, B., and Caldera, F. E. (2020). Osteoarthritis: Pathology, Diagnosis, and Treatment Options. *Med. Clin. North. Am.* 104 (2), 293–311. doi:10.1016/j.mcna.2019.10.007

Amberger, J. S., Bocchini, C. A., Schiettecatte, F., Scott, A. F., and Hamosh, A. (2015). OMIM.org: Online Mendelian Inheritance in Man (OMIM®), an Online Catalog of Human Genes and Genetic Disorders. *Nucleic Acids Res.* 43 (Database issue), D789–D798. doi:10.1093/nar/gku1205

Ansari, M. Y., Ahmad, N., and Haqqi, T. M. (2020). Oxidative Stress and Inflammation in Osteoarthritis Pathogenesis: Role of Polyphenols. *Biomed. Pharmacother.* 129, 110452. doi:10.1016/j.biopha.2020.110452

Bannuru, R. R., Osani, M. C., Vaysbrot, E. E., Arden, N. K., Bennell, K., Bierma-Zeinstra, S. M. A., et al. (2019). OARSI Guidelines for the Non-surgical Management of Knee, Hip, and Polyarticular Osteoarthritis. *Osteoarthritis Cartilage* 27 (11), 1578–1589. doi:10.1016/j.joca.2019.06.011

Burley, S. K., Bhikadiya, C., Bi, C., Bittrich, S., Chen, L., Crichlow, G. V., et al. (2021). RCSB Protein Data Bank: Powerful New Tools for Exploring 3D Structures of Biological Macromolecules for Basic and Applied Research and Education in Fundamental Biology, Biomedicine, Biotechnology, Bioengineering and Energy Sciences. *Nucleic Acids Res.* 49 (D1), D437–D51. doi:10.1093/nar/gkaa1038

Chen, C., Chen, H., Zhang, Y., Thomas, H. R., Frank, M. H., He, Y., et al. (2020). TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* 13 (8), 1194–1202. doi:10.1016/j.molp.2020.06.009

Choi, M. C., Jo, J., Park, J., Kang, H. K., and Park, Y. (2019). NF-κB Signaling Pathways in Osteoarthritic Cartilage Destruction. *Cells* 8 (7). doi:10.3390/cells8070734

Cross, M., Smith, E., Hoy, D., Nolte, S., Ackerman, I., Fransen, M., et al. (2014). The Global burden of Hip and Knee Osteoarthritis: Estimates from the Global burden of Disease 2010 Study. *Ann. Rheum. Dis.* 73 (7), 1323–1330. doi:10.1136/annrheumdis-2013-204763

Cui, Q., Zhang, Y. L., Ma, Y. H., Yu, H. Y., Zhao, X. Z., Zhang, L. H., et al. (2020). A Network Pharmacology Approach to Investigate the Mechanism of Shuxuening Injection in the Treatment of Ischemic Stroke. *J. Ethnopharmacol* 257, 112891. doi:10.1016/j.jep.2020.112891

Curtis, E., Fuggle, N., Shaw, S., Spooner, L., Ntani, G., Parsons, C., et al. (2019). Safety of Cyclooxygenase-2 Inhibitors in Osteoarthritis: Outcomes of a Systematic Review and Meta-Analysis. *Drugs Aging* 36 (Suppl. 1), 25–44. doi:10.1007/s40266-019-00664-x

Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene Expression Omnibus: NCBI Gene Expression and Hybridization Array Data Repository. *Nucleic Acids Res.* 30 (1), 207–210. doi:10.1093/nar/30.1.207

Fei, J., Liang, B., Jiang, C., Ni, H., and Wang, L. (2019). Luteolin Inhibits IL-1β-induced Inflammation in Rat Chondrocytes and Attenuates Osteoarthritis Progression in a Rat Model. *Biomed. Pharmacother.* 109, 1586–1592. doi:10.1016/j.biopha.2018.09.161

Ferreira, L. G., Dos Santos, R. N., Oliva, G., and Andricopulo, A. D. (2015). Molecular Docking and Structure-Based Drug Design Strategies. *Molecules* 20 (7), 13384–13421. doi:10.3390/molecules200713384

Gfeller, D., Michielin, O., and Zoete, V. (2013). Shaping the Interaction Landscape of Bioactive Molecules. *Bioinformatics* 29 (23), 3073–3079. doi:10.1093/bioinformatics/btt540

Goodsell, D. S., and Olson, A. J. (1990). Automated Docking of Substrates to Proteins by Simulated Annealing. *Proteins* 8 (3), 195–202. doi:10.1002/prot.340080302

Gromiha, M. M., Yugandhar, K., and Jemimah, S. (2017). Protein-protein Interactions: Scoring Schemes and Binding Affinity. *Curr. Opin. Struct. Biol.* 44, 31–38. doi:10.1016/j.sbi.2016.10.016

Guilak, F., Nims, R. J., Dicks, A., Wu, C. L., and Meulenbelt, I. (2018). Osteoarthritis as a Disease of the Cartilage Pericellular Matrix. *Matrix Biol.* 71-72, 40–50. doi:10.1016/j.matbio.2018.05.008

Huang, H., Zhang, Z. F., Qin, F. W., Tang, W., Liu, D. H., Wu, P. Y., et al. (2019). Icariin Inhibits Chondrocyte Apoptosis and Angiogenesis by Regulating the TDP-43 Signaling Pathway. *Mol. Genet. Genomic Med.* 7 (4), e00586. doi:10.1002/mgg3.586

Jin, J., Wang, H., Hua, X., Chen, D., Huang, C., and Chen, Z. (2019). An Outline for the Pharmacological Effect of Icariin in the Nervous System. *Eur. J. Pharmacol.* 842, 20–32. doi:10.1016/j.ejphar.2018.10.006

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., et al. (2021). PubChem in 2021: New Data Content and Improved Web Interfaces. *Nucleic Acids Res.* 49 (D1), D1388–D95. doi:10.1093/nar/gkaa971

Komori, T. (2016). Cell Death in Chondrocytes, Osteoblasts, and Osteocytes. *Int. J. Mol. Sci.* 17 (12), 2045. doi:10.3390/ijms17122045

Krishnan, Y., and Grodzinsky, A. J. (2018). Cartilage Diseases. *Matrix Biol.* 71-72, 51–69. doi:10.1016/j.matbio.2018.05.005

Li, L., Li, Z., Li, Y., Hu, X., Zhang, Y., and Fan, P. (2020). Profiling of Inflammatory Mediators in the Synovial Fluid Related to Pain in Knee Osteoarthritis. *BMC Musculoskelet. Disord.* 21 (1), 99. doi:10.1186/s12891-020-3120-0

Liu, H., Wang, J., Zhou, W., Wang, Y., and Yang, L. (2013). Systems Approaches and Polypharmacology for Drug Discovery from Herbal Medicines: an Example Using Licorice. *J. Ethnopharmacol* 146 (3), 773–793. doi:10.1016/j.jep.2013.02.004

Liu, Y., Yang, J., Luo, Z., Li, D., Lu, J., Wang, Q., et al. (2019). Development of an Injectable Thiolated Icariin Functionalized Collagen/hyaluronic Hydrogel to Promote Cartilage Formation *In Vitro* and *In Vivo*. *J. Mater. Chem. B* 7 (17), 2845–2854. doi:10.1039/c9tb00211a

Martel-Pelletier, J., Barr, A. J., Cicuttini, F. M., Conaghan, P. G., Cooper, C., Goldring, M. B., et al. (2016). Osteoarthritis. *Nat. Rev. Dis. Primers* 2, 16072. doi:10.1038/nrdp.2016.72

Martín-Millán, M., and Castañeda, S. (2013). Estrogens, Osteoarthritis and Inflammation. *Jt. bone Spine* 80 (4), 368–373. doi:10.1016/j.jbspin.2012.11.008

Mi, B., Wang, J., Liu, Y., Liu, J., Hu, L., Panayi, A. C., et al. (2018). Icariin Activates Autophagy via Down-Regulation of the NF-Kb Signaling-Mediated Apoptosis in Chondrocytes. *Front. Pharmacol.* 9, 605. doi:10.3389/fphar.2018.00605

Michael, J. W., Schlüter-Brust, K. U., and Eysel, P. (2010). The Epidemiology, Etiology, Diagnosis, and Treatment of Osteoarthritis of the Knee. *Dtsch Arztebl Int.* 107 (9), 152–162. doi:10.3238/arztebl.2010.0152

Morgat, A., Lombardot, T., Coudert, E., Axelsen, K., Neto, T. B., Gehant, S., et al. (2020). Enzyme Annotation in UniProtKB Using Rhea. *Bioinformatics* 36 (6), 1896–1901. doi:10.1093/bioinformatics/btz817

Oh, C. D., and Chun, J. S. (2003). Signaling Mechanisms Leading to the Regulation of Differentiation and Apoptosis of Articular Chondrocytes by Insulin-like Growth Factor-1. *J. Biol. Chem.* 278 (38), 36563–36571. doi:10.1074/jbc.M304857200

Oliveros, J. C. (2007-2015). Venny. An Interactive Tool for Comparing Lists with Venn's Diagrams. Available at: https://bioinfogp.cnb.csic.es/tools/venny/index.html.

Palazzo, C., Nguyen, C., Lefevre-Colau, M. M., Rannou, F., and Poiraudeau, S. (2016). Risk Factors and burden of Osteoarthritis. *Ann. Phys. Rehabil. Med.* 59 (3), 134–138. doi:10.1016/j.rehab.2016.01.006

Pan, L., Zhang, Y., Chen, N., and Yang, L. (2017). Icariin Regulates Cellular Functions and Gene Expression of Osteoarthritis Patient-Derived Human Fibroblast-like Synoviocytes. *Int. J. Mol. Sci.* 18 (12). doi:10.3390/ijms18122656

Piñero, J., Bravo, À., Queralt-Rosinach, N., Gutiérrez-Sacristán, A., Deu-Pons, J., Centeno, E., et al. (2017). DisGeNET: a Comprehensive Platform Integrating Information on Human Disease-Associated Genes and Variants. *Nucleic Acids Res.* 45 (D1), D833–D9. doi:10.1093/nar/gkw943

Pinzi, L., and Rastelli, G. (2019). Molecular Docking: Shifting Paradigms in Drug Discovery. *Int. J. Mol. Sci.* 20 (18). doi:10.3390/ijms20184331

Primorac, D., Molnar, V., Rod, E., Jeleč, Ž., Čukelj, F., Matišić, V., et al. (2020). Knee Osteoarthritis: A Review of Pathogenesis and State-Of-The-Art Non-operative Therapeutic Considerations. *Genes (Basel)* 11 (8), 854. doi:10.3390/genes11080854

Ru, J., Li, P., Wang, J., Zhou, W., Li, B., Huang, C., et al. (2014). TCMSP: a Database of Systems Pharmacology for Drug Discovery from Herbal Medicines. *J. Cheminform* 6, 13. doi:10.1186/1758-2946-6-13

Schrodinger, L. L. C. (2015). The PyMOL Molecular Graphics System. Version 1.8.

Seeliger, D., and de Groot, B. L. (2010). Ligand Docking and Binding Site Analysis with PyMOL and Autodock/Vina. *J. Comput. Aided Mol. Des.* 24 (5), 417–422. doi:10.1007/s10822-010-9352-6

Song, L., Zhao, J., Zhang, X., Li, H., and Zhou, Y. (2013). Icariin Induces Osteoblast Proliferation, Differentiation and Mineralization through Estrogen Receptor-Mediated ERK and JNK Signal Activation. *Eur. J. Pharmacol.* 714 (1-3), 15–22. doi:10.1016/j.ejphar.2013.05.039

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING V11: Protein-Protein Association Networks with Increased

Coverage, Supporting Functional Discovery in Genome-wide Experimental Datasets. *Nucleic Acids Res.* 47 (D1), D607–D13. doi:10.1093/nar/gky1131

Tang, Y., Li, Y., Xin, D., Chen, L., Xiong, Z., and Yu, X. (2021). Icariin Alleviates Osteoarthritis by Regulating Autophagy of Chondrocytes by Mediating PI3K/AKT/mTOR Signaling. *Bioengineered* 12 (1), 2984–2999. doi:10.1080/21655979.2021.1943602

Trott, O., and Olson, A. J. (2010). AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading. *J. Comput. Chem.* 31 (2), 455–461. doi:10.1002/jcc.21334

Wallace, I. J., Worthington, S., Felson, D. T., Jurmain, R. D., Wren, K. T., Maijanen, H., et al. (2017). Knee Osteoarthritis Has Doubled in Prevalence since the Mid-20th century. *Proc. Natl. Acad. Sci. U S A.* 114 (35), 9332–9336. doi:10.1073/pnas.1703856114

Wang, K., Xu, J., Hunter, D. J., and Ding, C. (2015). Investigational Drugs for the Treatment of Osteoarthritis. *Expert Opin. Investig. Drugs* 24 (12), 1539–1556. doi:10.1517/13543784.2015.1091880

Wang, P., Meng, Q., Wang, W., Zhang, S., Xiong, X., Qin, S., et al. (2020). Icariin Inhibits the Inflammation through Down-Regulating NF-Kb/hif-2α Signal Pathways in Chondrocytes. *Biosci. Rep.* 40 (11). doi:10.1042/BSR20203107

Wang, P., Zhang, F., He, Q., Wang, J., Shiu, H. T., Shu, Y., et al. (2016). Flavonoid Compound Icariin Activates Hypoxia Inducible Factor-1α in Chondrocytes and Promotes Articular Cartilage Repair. *PloS one* 11 (2), e0148372. doi:10.1371/journal.pone.0148372

Wang, X., Shen, Y., Wang, S., Li, S., Zhang, W., Liu, X., et al. (2017). PharmMapper 2017 Update: a Web Server for Potential Drug Target Identification with a Comprehensive Target Pharmacophore Database. *Nucleic Acids Res.* 45 (W1), W356–W60. doi:10.1093/nar/gkx374

Wang, Y., Zhang, S., Li, F., Zhou, Y., Zhang, Y., Wang, Z., et al. (2020). Therapeutic Target Database 2020: Enriched Resource for Facilitating Research and Early Development of Targeted Therapeutics. *Nucleic Acids Res.* 48 (D1), D1031–D41. doi:10.1093/nar/gkz981

Wang, Z., Wang, D., Yang, D., Zhen, W., Zhang, J., and Peng, S. (2018). The Effect of Icariin on Bone Metabolism and its Potential Clinical Application. *Osteoporos. Int.* 29 (3), 535–544. doi:10.1007/s00198-017-4255-1

Wei, C. C., Ping, D. Q., You, F. T., Qiang, C. Y., and Tao, C. (2016). Icariin Prevents Cartilage and Bone Degradation in Experimental Models of Arthritis. *Mediators Inflamm.* 2016, 9529630. doi:10.1155/2016/9529630

Wojdasiewicz, P., Poniatowski, Ł. A., and Szukiewicz, D. (2014). The Role of Inflammatory and Anti-inflammatory Cytokines in the Pathogenesis of Osteoarthritis. *Mediators Inflamm.* 2014, 561459. doi:10.1155/2014/561459

Yuan, H., Ma, Q., Cui, H., Liu, G., Zhao, X., Li, W., et al. (2017). How Can Synergism of Traditional Medicines Benefit from Network Pharmacology? *Molecules* 22 (7). doi:10.3390/molecules22071135

Zhang, J., Zhang, C., Liu, A., Ji, Q., Ren, L., Ma, C., et al. (2021). Synthesis of Icariin-Zinc and its Protective Effect on Exercise Fatigue and Reproductive System Related Glands in Male Rats. *Front. Pharmacol.* 12, 611722. doi:10.3389/fphar.2021.611722

Zhang, J., Zhang, D., Wu, C., Liu, A., Zhang, C., Jiao, J., et al. (2019). Icariin-conditioned Serum Engineered with Hyaluronic Acid Promote Repair of Articular Cartilage Defects in Rabbit Knees. *BMC Complement. Altern. Med.* 19 (1), 155. doi:10.1186/s12906-019-2570-0

Zhou, L., Poon, C. C.-W., Wong, K.-Y., Cao, S., Dong, X., Zhang, Y., et al. (2021). Icariin Ameliorates Estrogen-Deficiency Induced Bone Loss by Enhancing IGF-I Signaling via its Crosstalk with Non-genomic ERα Signaling. *Phytomedicine* 82, 153413. doi:10.1016/j.phymed.2020.153413

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., et al. (2019). Metascape Provides a Biologist-Oriented Resource for the Analysis of Systems-Level Datasets. *Nat. Commun.* 10 (1), 1523. doi:10.1038/s41467-019-09234-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership