frontiers | Research Topics

Novel biological synthesis of nutrients for chronic diseases intervention

Edited by
Wenzhen Liao, Yi Cao, Miaomiao Yuan, Xian Wu and Silvia Turroni

Published in
Frontiers in Bioengineering and Biotechnology

ISSN 1664-8714
ISBN 978-2-83250-991-3
DOI 10.3389/978-2-83250-991-3

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

Novel biological synthesis of nutrients for chronic diseases intervention

Topic editors

Wenzhen Liao — Southern Medical University, China
Yi Cao — Xiangtan University, China
Miaomiao Yuan — Sun Yat-sen University, China
Xian Wu — Miami University, United States
Silvia Turroni — University of Bologna, Italy

Citation

Liao, W., Cao, Y., Yuan, M., Wu, X., Turroni, S., eds. (2022). *Novel biological synthesis of nutrients for chronic diseases intervention*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-991-3

Table of contents

frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 06 August 2020
doi: 10.3389/fbioe.2020.00904

Extract Derived From Black Rice Functions as a Photothermal Agent for Suppressing Tumor Growth and Metastasis

Muzhou Teng[1†], Shuyi Zhou[1†], Rongjun Zhang[1†], Yu Zhang[2], Yang Xu[1,2] and Xuemei Fu[2*]*

[1] School of Basic Medical Sciences, Southern Medical University, Guangzhou, China, [2] The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, China

It remains a challenge to develop an effective therapeutic agent with low cost and good biocompatibility for cancer therapy. Based on its dark color, we hypothesized that, the extraction from black rice grains, denoted BRE, could serve as a photothermal conversion agent. The results showed that BRE confers a high photothermal conversion efficiency up to 54.13%. The combination of BRE and near infrared (NIR) treatment enables effective photothermal tumor ablation, and suppress tumor metastasis via inhibiting the epithelial-mesenchymal transition (EMT) pathway. In addition, BRE exhibits no obvious toxicity *in vivo*. Therefore, BRE could serve as a promising photothermal therapy agent with a low toxicity to treat cancer.

Keywords: black rice, photothermal therapy, tumor, lung metastasis, epithelial-mesenchymal transition

OPEN ACCESS

Edited by:
Yi Cao,
Xiangtan University, China

Reviewed by:
Chaohua Wu,
Texas A&M University, United States
Yingbin Shen,
Guangzhou University, China

****Correspondence:***
Yang Xu
smuccd@163.com
Xuemei Fu
fxmzj2004@163.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *19 June 2020*
Accepted: *14 July 2020*
Published: *06 August 2020*

Citation:
Teng M, Zhou S, Zhang R, Zhang Y, Xu Y and Fu X (2020) Extract Derived From Black Rice Functions as a Photothermal Agent for Suppressing Tumor Growth and Metastasis. Front. Bioeng. Biotechnol. 8:904. doi: 10.3389/fbioe.2020.00904

INTRODUCTION

Despite great efforts that have been devoted to fight against cancer, it still poses a major threat to human health (Siegel et al., 2019). The current available strategies of cancer treatment include surgical therapy, chemotherapy (Yang et al., 2019), radiation therapy (RT) (Peng et al., 2020), photothermal therapy (PTT) (Jia et al., 2019), photodynamic therapy (PDT) (Wu et al., 2020), and immunotherapy (Feng et al., 2020). Among these cancer therapeutics, PTT has been developed vigorously in recent years which could convert the absorbed light energy into heat (Chen Y. et al., 2019). The advantage of PTT is that near infrared (NIR) could irradiate the subcutaneous and local tumor directly, so the constant high temperature could kill tumor cells precisely and ablate solid tumors(Chen D. et al., 2019). The black phosphorus (Chen et al., 2017), CuS–MnS_2 nanomaterials (Chen W. et al., 2019), Au nanoparticle (Depciuch et al., 2020), and other metallic materials (Yuan et al., 2020) have been used as photothermal agent in cancer treatment. However, the metallic materials have certain drawbacks such as serious toxicity, expensive reagents, and complicated preparation procedure (Li et al., 2018), which have limited their further applications (Deng et al., 2019). Therefore, it is crucial to develop novel photothermal biomaterials with lower cost and better biocompatibility.

Compared with chemically synthetic biomaterials, natural biomaterials with excellent biocompatibility, and biodegradability are preferred in recent clinical trials. Currently, many biomaterials have been studied in photothermal ablation of tumors. For instance, natural sodium humate, biodegraded from the humic acid, had been applied as an excellent photothermal agents to induce tumor cell death (Miao et al., 2018). In addition, nanoparticles extracted from cuttlefish

ink have been used to inhibit tumor growth by synergizing immunotherapy and PTT, showing excellent ability in the repolarization of tumor-associated macrophages and enhanced recruitment of cytotoxic T lymphocytes as well as photothermal killing effect (Deng et al., 2019). Inspired by studies to develop natural biomaterials, we are interested in developing cheaper and more accessible food-sourced PTT agents.

Black rice, mostly planted in the East Asia, is deemed a traditional and natural food (Samyor et al., 2017). It is widely used due to the low cost, easy accessible, and high nutritious value (Park et al., 2017), outstanding physicochemical characteristics and antioxidant potential (Pang et al., 2018). In addition, black rice is rich in water-soluble bioactive compounds such as phenolic acids, tocopherols, polyphenols, B vitamins, and anthocyanins (Wu et al., 2019) with excellent antioxidant, anti-obesity, and anti-diabetic capacity. These characteristics enabled black rice valuable for health and widely used as a food additive in the food processing industry (Aprodu et al., 2019). Anthocyanin, one of the major components of black rice, could significantly inhibit the proliferation, migration, and metastasis of breast cancer cells through targeting the RAS/RAF/MAPK pathway *in vitro* (Chen X.Y. et al., 2015). Moreover, black rice could enhance the immune response through inducing the proliferation and differentiation of immune cells *in vivo* (Fan et al., 2017). However, it remains unclear whether the black rice could be applied as a photothermal agent for tumor treatment.

The epithelial-mesenchymal transition (EMT) is curial for the metastatic behaviors of tumor cells (Hennessy et al., 2009). EMT involves a series of genetic and phenotypic changes, which contribute to the transformation of early epithelial cells into invasive malignant cancer cells (Lamouille et al., 2014). The process of EMT can be activated by genetic changes or other responses to external environment (Georgakopoulos-Soares et al., 2020). EMT also could promote early cancer cell to transdifferentiate into mesenchymal-like cells such as carcinoma cells and cancer stem cells, thus the cancer cell could acquire migration and invasion ability and detach from epithelial cell mass. During EMT process, the epithelial cells could lead to cell depolarization, reduced or even lost of cell–cell contacts and changing into a fibroblast-like morphology (Xu et al., 2018).

Developing therapeutic strategies with high efficiency as well as low toxicity and cost to drastically eliminate tumors is the ultimate goal in the cancer treatment. In this study, we have studied BRE as a photothermal agent for its high photothermal conversion efficiency up to 54.13%. This natural food could efficiently inhibit tumor growth and metastasis via EMT pathway with low toxicity. Prospectively, BRE might be a promising photothermal agent for tumor therapy.

MATERIALS AND METHODS

Materials

BRE (containing 25% anthocyanin) was purchased from TIANXINGJIAN biochemical technology company (Xi An, China). Crystal violet was obtained from Beyotime biotechnology company (Shanghai, China). Fetal bovine serum (FBS), Phosphate buffer solution (PBS), Pyridine and dimethyl sulfoxide (DMSO), and Dulbecco's modified eagle medium (DMEM) were provided by Gibco-BRL (Grand Island, New York, United States). LIVE/DEAD^{TM} Cell Imaging Kit was bought from Thermo Fisher Scientific (Waltham, MA, United States). Snail, vimentin, N-Cadherin, β-Actin antibody, radioimmunoprecipitation assay buffer (RIPA), and protease/phosphatase inhibitor cocktail (100×) were obtained from CST (Boston, MA, United States). 4% paraformaldehyde was obtained from Fude biotechnology company (Hangzhou, China). Matrigel was bought from Corning (Kangning, New York, United States).

BRE Solutions Preparation

The concentration of the BRE solution was set as 20 mg/mL and stored in 4°C for further use.

Cell Culture

Murine breast cancer cell 4T1 cell line (4T1 cells) was obtained from ATCC and cultured with DMEM containing 10% FBS and 1% penicillin-streptomycin solution (100×) at 37°C with 5% CO_2 humidified atmosphere.

The Detection of UV-vis-NIR Absorption Spectra

To explore the absorption spectra of BRE in the near infrared region (700–900 nm), 3 mL BRE solutions with concentrations of 1, 2, 5, 10, 15, and 20 mg/mL were determined using the UV-2600 spectrophotometer (Shimadzu Co., Japan).

Evaluation of Photothermal Effect and Photostability

To measure the photothermal effect of BRE, a series concentration of 1, 2, 5, 10, 15, and 20 mg/mL BRE were irradiated (808 nm, 1 W/cm^2) for 10 min, and the temperature change was recorded by an infrared thermal imaging camera (Shanghai Xilong Optoelectronics Technology Co. Ltd., China). Besides, the 10 mg/mL concentration of BRE was irradiated and recorded at different power density (0.5, 0.75, 1, and 2 W/cm^2) for 10 min, respectively. To further study the photostability, the real-time temperature change of BRE by irradiating 10 mg/mL solution with an 808 nm laser (1 W/cm^2) for 10 min (laser on) and then cooling to room temperature without irradiation for 10 min (laser off) were recorded. Such heating/cooling processes were repeated four times and used in the calculation of photothermal conversion efficiency. And the details of calculation were given in previous work (Hou et al., 2018).

Live/Dead Staining Assay

4T1 cells were seeded on 96-well plates at a density of 5×10^3 cells/well and incubated with DMEM complete growth medium for 24 h. Then, the cells were treated with different concentrations (0, 2, 5, and 10 mg/mL) of BRE for 24 h. After that, the cells were irradiated with an 808 nm laser (1 W/cm^2) for 5 min and stained using the Live/Dead^{TM} Cell Imaging Kit for 30 min. Then, the cells were washed twice with PBS and the living cells or dead cells

were observed and photographed using a fluorescent microscope (Nikon ECLIPSE Ti-U, Japan).

Inhibition of Cloning Ability

4T1 cells were seeded on 6-well plates at a density of 2000 cells/well and incubated with DMEM medium for 24 h. Then, the cells were treated with different concentrations (0, 0.5, and 1 mg/mL) of BRE and were irradiated for 10 min (808 nm, 1 W/cm^2), and were incubated for another 12 h and 24 h respectively. At fixed time points, the cells were washed twice with PBS and cultured with fresh DMEM medium for another 7 days. Then the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. After that, the cells were washed twice with PBS and stained with 1% crystal violet dissolved in PBS for 30 min. The colony numbers were counted, and photographs were taken using a microscope (Nikon ECLIPSE Ti-U, Japan).

In vivo Anti-tumor Effect

Four-week old female BALB/c mice were purchased from animal laboratory center of Guangdong Province and housed in SPF laboratory animal room. And all animal experiments were approved by Institutional Animal Care and Use Committee (IACUC). The mice were divided into four groups including control group, PBS + NIR, BRE and BRE + NIR group (n = 6). 1 × 10^6 4T1 cells resuspended in 100 μL PBS were subcutaneously injected into the flanks of mice. When tumor size reached 100 mm^3, 50 μL of BRE solution (20 mg/mL) or PBS were intratumorally injected into the tumor region of the mice. The control group received no treatment and the BRE group was only intratumorally injected BRE solution. The mice of PBS + NIR treatment groups and BRE + NIR group were irradiated for 10 min (808 nm, 1 W/cm^2). During irradiation, temperature change and thermal images of these mice were monitored by an infrared thermal imaging camera. After that, the relative tumor volume and body weight were recorded every two days. The survival rate was calculated when all mice were dead or the mice of control, PBS + NIR and BRE group were dead while the mice of BRE + NIR group remained alive 40 days after treatment. To examine the pathological changes of the tumor, one tumor-bearing mouse from each group was sacrificed one day after treatment, and the tumors were dissected and stained with H and E.

Inhibition of Migration Ability

4T1 cells were suspended in serum-free DMEM medium at a density of 5 × 10^5 cells (200 μL) and seeded in the upper chamber of the transwell. The lower cell chamber was added with 500 μL DMEM complete growth medium. Then, the cells were treated with different concentrations (0, 0.5, and 1 mg/mL) of BRE and were irradiated for 10 min (808 nm, 1 W/cm^2). After being cultured for another 24 h, the upper cells were cleaned with cotton swab and transwells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. And the cells were washed twice with PBS and stained with 1% crystal violet dissolved in PBS for 30 min. The membranes were obtained and fixed with neutral gum on the slides overnight. Then the slides were photographed using a microscope (Nikon ECLIPSE Ti-U, Japan).

Inhibition of Invasion Ability

Matrigel (50 μL) was added into the upper chamber of the transwell insert and incubated at 37°C for 2 h. Then 4T1 cells were seeded on the transwell upper chamber at a density of 5 × 10^5 cells/well (200 μL) and incubated with serum-free DMEM medium. The lower chambers were 24-well plates added with 500 μL DMEM complete growth medium. Later, the cells were treated with different concentrations (0, 0.5, and 1 mg/mL) of BRE and irradiated for 10 min (808 nm, 1 W/cm^2). After 72 h incubation, the upper chambers were cleaned with cotton swab and the transwells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. Then the cells were washed twice with PBS and stained with 1% crystal violet dissolved in PBS for 30 min. The membranes were obtained and fixed with neutral gum on the slides overnight. Pictures of the slides were taken using a microscope (Nikon ECLIPSE Ti-U, Japan).

Western Blotting

Western blotting was used to determine the expression level of EMT related proteins. In brief, 4T1 cells were cultured in 6-well culture plates (3 × 10^5 cells/well) overnight. The cells were treated with different concentrations (0, 0.5, and 1 mg/mL) of BRE and were irradiated for 10 min (808 nm, 1 W/cm^2), the cells were incubated for another 24 h. Afterward, the cells were collected and whole-cell extracts were prepared with a RIPA buffer containing 1% protease/phosphatase inhibitor cocktail (100×), and separated by 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE, and transferred to nitrocellulose membranes (Millipore). Antibodies including snail (3879s, CST), vimentin (5741s, CST), N-Cadherin (13116s, CST), β-actin (4967s, CST) were used in this study.

Evaluation of *in vivo* Lung Metastasis Inhibition

Four-week old female BALB/c mice were divided into two groups including control group and BRE + NIR group. 1 × 10^6 4T1 cells resuspended in 100 μL PBS were subcutaneously injected into the flanks of mice. When the tumor size was reached 100 mm^3, 50 μL of BRE solution (20 mg/mL) or PBS were intratumorally injected into the tumor region of the mice. The mice of control groups and BRE + NIR group were irradiated for 10 min (808 nm, 1 W/cm^2). 30 days later, all mice were sacrificed and lungs were obtained and photographed alone. The numbers of lung metastasis were further counted, and lung slides were stained with H and E.

Evaluation of *in vivo* Animal Toxicity

Four-week old female BALB/c mice were divided into two groups including control group and BRE treatment group. The mice of BRE treatment group were intravenously injected with 100 μL BRE solution (20 mg/mL) while the control group received 100 μL PBS solution in the same method. After that, the mice were housed for 30 days until they were sacrificed for toxicity assay. 150 μL whole blood were collected in tubes with spray-coated K_2EDTA for future blood routine assessment including WBC, RBC, HGB, HCT, MCV, MCH, MCHC, and PLT assay.

500 μL whole blood were collected in normal EP tubes for future assessment of hepatic and renal toxicity. Blood samples for biochemical test were temporarily stored in -20°C for 2 h, and were incubated in 37°C for 1 h before centrifuged for 10 min (3000 rpm). After that the serum was collected for AST, ALT, BUN, and SCR assay. Major organs (including heart, liver, spleen, lung, and kidney) of all mice were collected after sacrifice. All major organs for histological analyses were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin, cut into 4-μm-thick sections, and subsequently stained with H and E. H and E staining analysis were used to assess the *in vivo* toxicity.

Statistical Analysis

Quantitative data were expressed as mean ± standard deviation (SD). The statistical differences were assessed using One-way ANOVA analysis. All tests were analyzed using statistical software (SPSS version 20.0). *P*-values of < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Characterization of BRE

Exacted from black rice containing 25% anthocyanin, was derived by serial dilution of BRE powder purchased from TIANXINGJIAN Biochemical Technology Company with PBS (pH = 7.4). First, the vis-NIR absorbance spectrum (700–900 nm) of BRE solutions was assessed. The absorbance of BRE solutions was smoothly decreased from 700 nm to 900 nm, and the relation with different concentrations was close to linear with the R^2 up to 0.97 at 808 nm laser (**Figures 1A,B**), indicating that BRE solutions had a strong absorbance in NIR wavelength. When irradiated for 10 min at 808 nm (1 W/cm^2), the temperature of BRE solution was increased faster and higher with the increasing concentrations of BRE solutions. As a negative control, the same irradiation had little impact on PBS solution. The ΔT of the BRE solution at the concentration of 20 mg/mL was about 35.2°C, indicating the photothermal capability that matches many photothermal materials such as Pd@Au/Ce6/PAH/H-MnO_2 (**Figure 1C**; Liu et al., 2020). In addition, we further showed that the photothermal performance of BRE was dependent on the irradiation power density (**Figure 1D**). The ΔT of the BRE solutions irradiated with the power density at 2 W/cm^2 was about 41.3°C. As shown in **Figure 1E**, the temperature of BRE solutions (10 mg/mL) was increased sharply when the laser was on for 10 min (1 W/cm^2) and decreased when the laser was off. The temperature change was highly reproducible similarly to many photothermal materials such as FA-CuS/DTX@PEI-PpIX-CpG nanocomposites (Chen L. et al., 2019), suggesting the superb photothermal stability of BRE solutions.

Based on the above data, the photothermal conversion efficiency (η) of BRE solutions was calculated according to the previously reported method (Hou et al., 2018). The fitting linear curve of time data versus -lnθ was acquired from the cooling period, and the time constant (τ_s) for heat transfer was calculated to be 182.9 s. The η value of BRE solution was

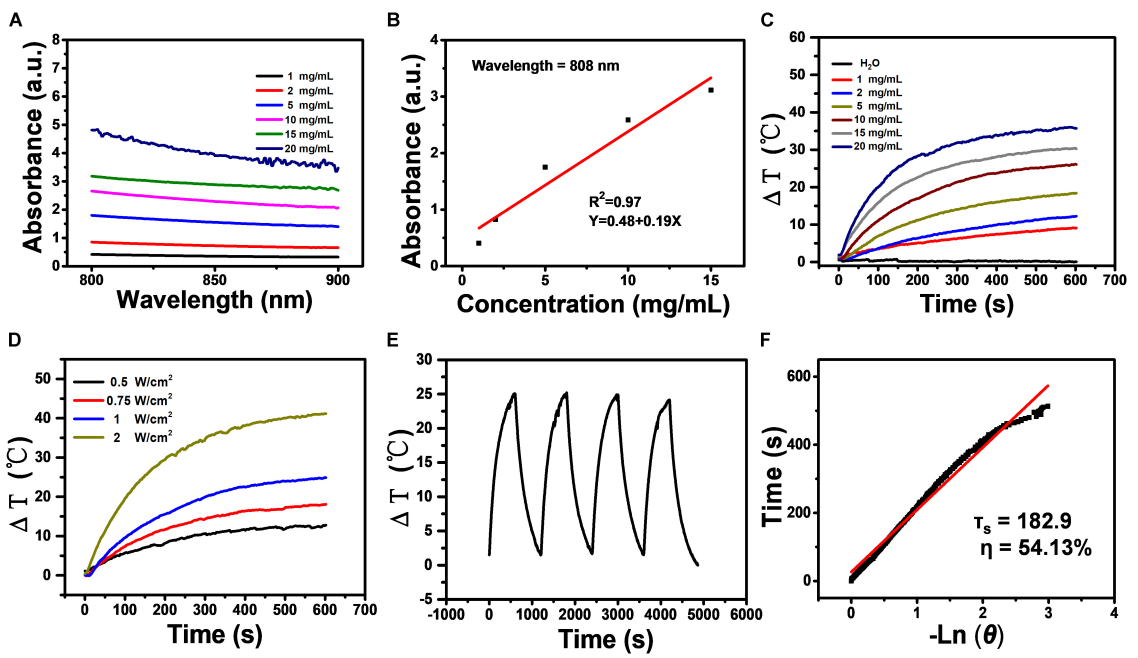


FIGURE 1 | Characterization of BRE. **(A)** The vis-NIR absorbance spectrum (700–900 nm) of BRE with different concentrations. **(B)** The fitting curve of the absorbance of BRE at 808 nm with different concentrations (R^2 = 0.97). **(C)** Photothermal images and corresponding temperature elevation of BRE with different concentrations under an 808 nm laser irradiation (10 min, 1 W/cm^2). **(D)** Temperature elevation of BRE (10 mg/mL) with different power densities under an 808 nm laser irradiation. **(E)** Heating/cooling curves of BRE (10 mg/mL, 1 W/cm^2) for four repeated irradiation cycles. **(F)** The fitting linear curve of time data versus -lnθ acquired from the cooling period, and the time constant (τ_s) for heat transfer was calculated to be 182.9 s.

determined to be 54.13% (**Figure 1F**), which was obviously superior to commercial gold nanorods (21%) (Hessel et al., 2011). In summary, these results indicated that the BRE solution is a promising candidate for PTT.

Evaluation of *in vitro* Anti-tumor Effect

To investigate the photothermal tumor cell killing ability of BRE *in vitro*, Live/Dead staining assay was conducted. While the cells were alive in the BRE group, there was massive cell death in the culture after the treatment with NIR (**Figure 2A**). In further support of the photothermal killing ability of BRE, the colony formation ability of tumor cells after BRE + NIR treatment was assessed. The cloning formation efficiency of tumor cells at 12 and 24 h after BRE (1 mg/mL) + NIR treatment was significantly reduced (**Figures 2B,C**). Therefore, the combinational treatment of tumor cells with BRE and NIR have apparent anti-tumor activity *in vitro*.

Evaluation of *in vivo* Anti-tumor Effect of BRE

To evaluate the *in vivo* anti-tumor effect, BALB/c mice harboring syngeneic 4T1 tumors were divided into four groups including control, PBS + NIR, BRE, and BRE + NIR group (n = 6). During BRE + NIR treatment, the local temperature of 4T1 tumors was increased to 55.4°C, much

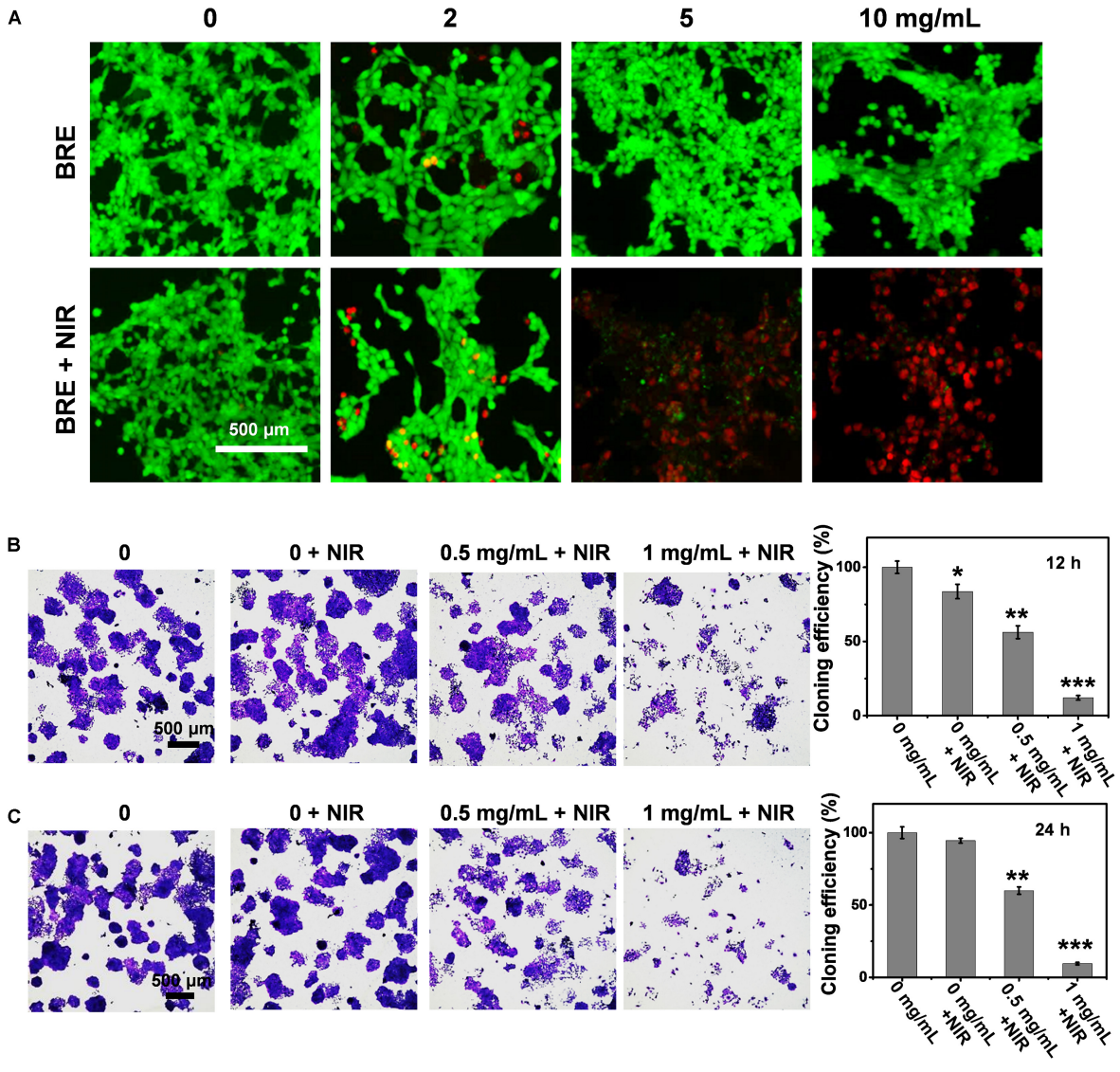


FIGURE 2 | The evaluation of *in vitro* anti-tumor effect. **(A)** The Live/Dead fluorescence images of 4T1 cells treated with BRE at different concentrations for 24 h and placed under an 808 nm laser irradiation (1 W/cm^2) for 5 min. **(B)** Photograph of 4T1 clone forming, quantification of 4T1 cloning efficiency with the BRE and NIR treatment in 12 h. **(C)** Photograph of 4T1 clone forming, quantification of 4T1 cloning efficiency with the BRE and NIR treatment in 24 h. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control.

higher than that of PBS + NIR group (29.1°C), enough to kill tumor cells and ablate subcutaneous tumors *in vivo* (**Figures 3A,B**). In addition, the size of the tumors after BRE + NIR treatment was smaller than that after other three treatments,suggesting that the combinational BRE and NIR treatment could effectively suppress tumors *in vivo* (**Figure 3C**). The survival rate of tumor-bearing mice after BRE + NIR treatment was much higher than that of other three treatments (**Figure 3D**). The body weight of tumor-bearing mice after various treatments showed no apparent difference, suggesting that BRE + NIR treatment confers a low toxicity (**Figure 3E**). Histological analysis of the tumors showed that the tumors after BRE + NIR treatment exhibited extensive necrosis, indicating efficient tumor ablation (**Figure 3F**). These results indicated that the combinational BRE and NIR treatment could efficiently cause local hyperthermia of tumor tissues, and thus presenting a promising treatment for solid tumors with superb killing efficiency.

Mechanisms of *in vitro* and *in vivo* Suppression of Lung Metastasis

Preventing tumor recurrence or inhibiting tumor metastases is as important as the ablation of original tumors (Jin et al., 2018). Therefore, the impact of BRE + NIR treatment on migrational ability and invasive ability was also studied. The migration and invasion of tumor cells were significantly reduced after the BRE (1 mg/mL) + NIR treatment when compared to other three treatments (**Figures 4A,B**).

Because EMT is required for the metastatic behaviors of invasion and migration, we examined the expression of

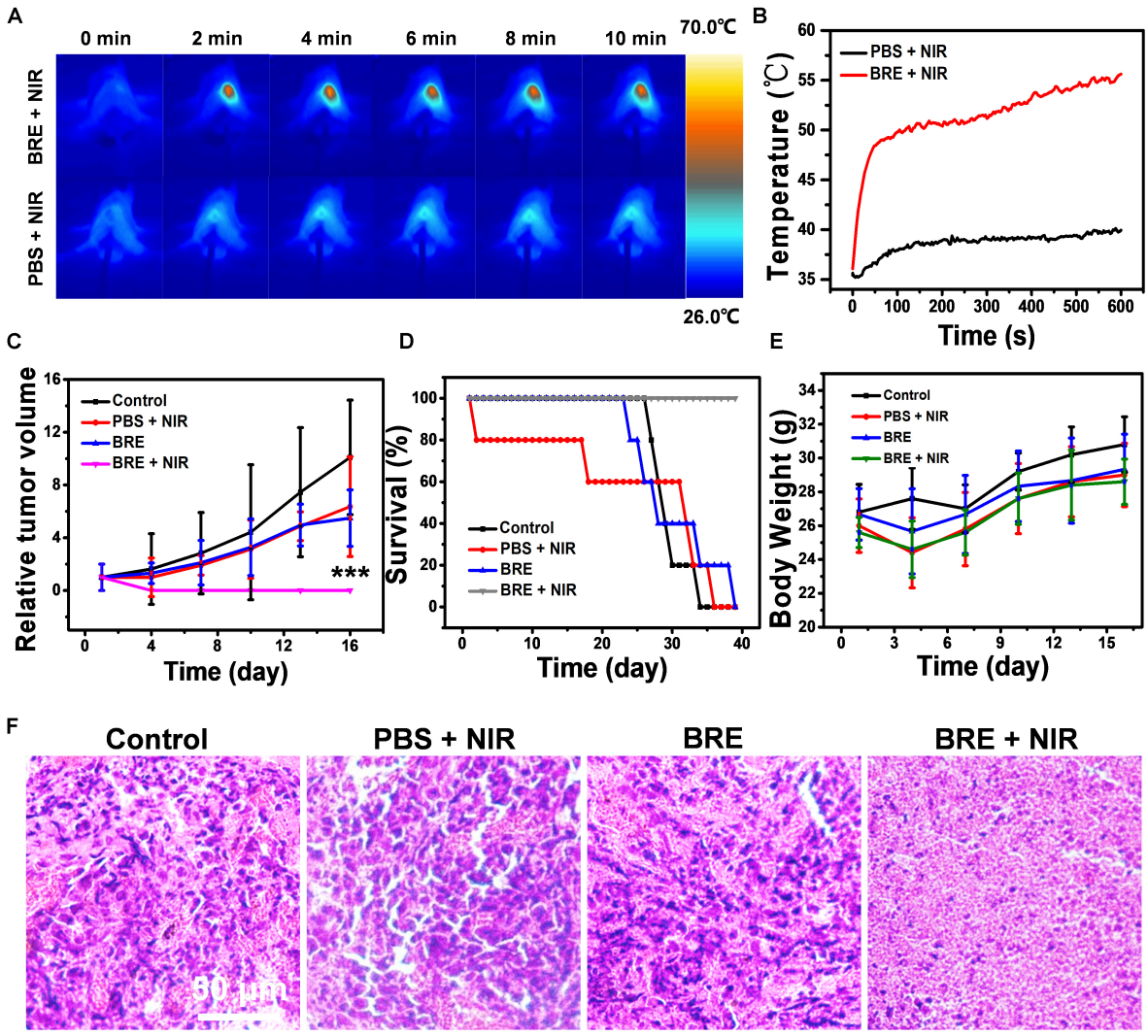


FIGURE 3 | The evaluation of *in vivo* anti-tumor effect. **(A)** Photothermal images and **(B)** temperature curves of BALB/c mice bearing 4T1 tumors irradiated with 808 nm for 10 min (1 W/cm^2). **(C)** The relative tumor volume of different treatment groups. **(D)** The survival rate of different treatment groups. **(E)** The body weight of different treatment groups. **(F)** The tumor HE staining of different treatment groups. Data are presented as mean ± SD. ***$P < 0.001$.

EMT related mesenchymal markers, including N-cadherin, Vimentin, and Snail (Polyak and Weinberg, 2009). Our data showed that BRE treatment reduced the expression of Snail, Vimentin, and N-Cadherin, indicating that BRE treatment exhibits strong anti-EMT effects. In addition, NIR enhanced the anti-EMT effectiveness, suggesting that the combinational BRE and NIR treatment inhibits the transformation of early epithelial cells into mesenchymal stem cells (**Figure 4C**).

Epithelial-mesenchymal transition could contribute to the dissociation of cancer cells from the primary tumor foci and intravasation into blood vessels (Hennessy et al., 2009) leading to distant metastases in other organs (Obenauf and Massagué, 2015). In order to assess the inhibitory ability of BRE + NIR on lung metastasis *in vivo*, lung metastasis model was conducted. Our data indicate that only BRE and BRE + NIR treatments suppressed the lung metastasis (**Figures 5A,B**), In addition, BRE + NIR treatment was more potent than BRE treatment in suppressing lung metastasis and improving survival (**Figures 5A,B**). Therefore, PTT and anti-EMT activities of BRE + NIR treatment contribute to inhibiting the formation of metastatic lung nodules (**Figure 5C**; Chen W. et al., 2015).

Evaluation of *in vivo* Toxicity

To evaluate the *in vivo* toxicity of BRE, the BALB/c mice were injected intravenously with 100 μL BRE solution (20 mg/mL) while the control group received the same volume of PBS solution. 30 days after treatment, all mice were sacrificed for *in vivo* toxicity study. The blood was subjected to the blood routine assessment, hepatic, and renal toxicity assessment. Besides, the HE staining of major organs were also analyzed to detect the toxicity of BRE

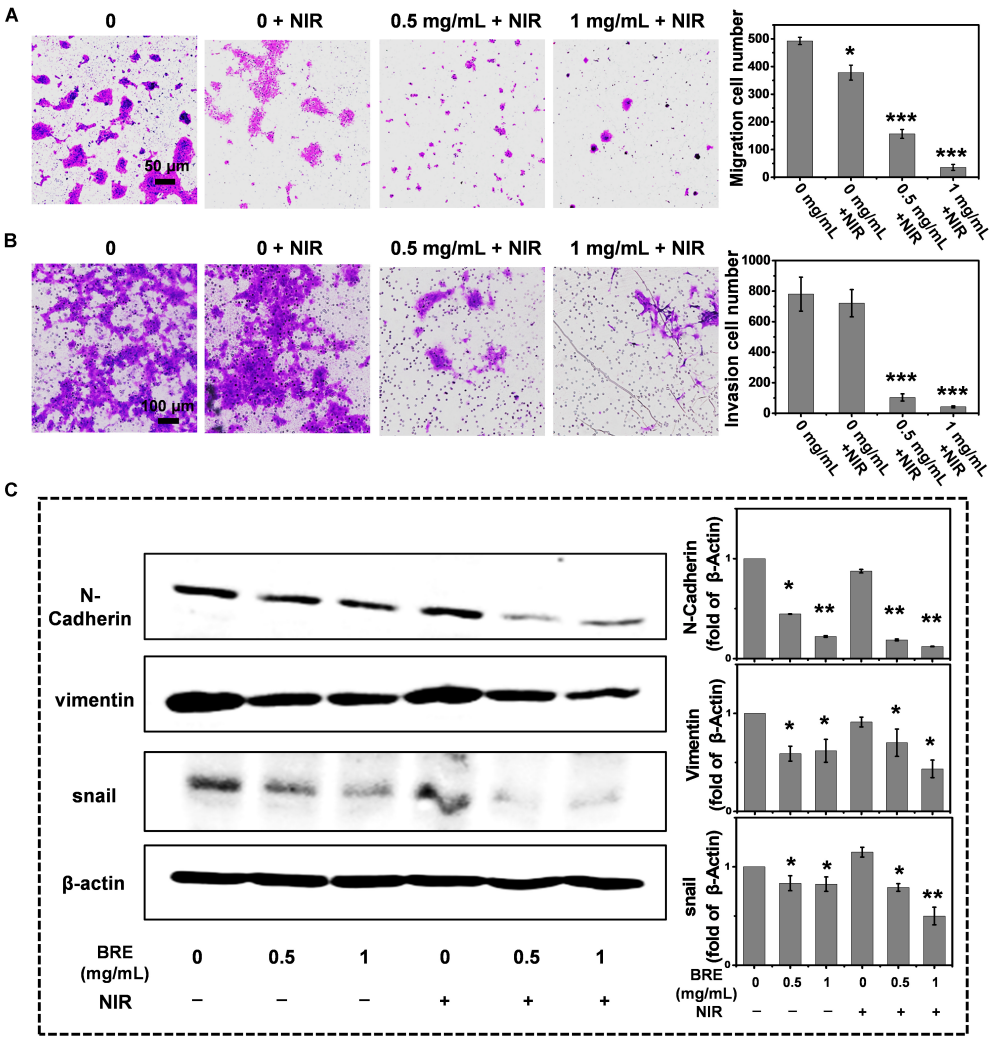


FIGURE 4 | Inhibition of migration, invasion and anti-EMT ability. **(A)** Photograph and quantification of 4T1 migration ability with the BRE and NIR treatment. **(B)** Photograph and quantification of 4T1 invasion ability with the BRE and NIR treatment. **(C)** Western blotting of relative mesenchymal markers including N-cadherin, Vimentin and Snail. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control.

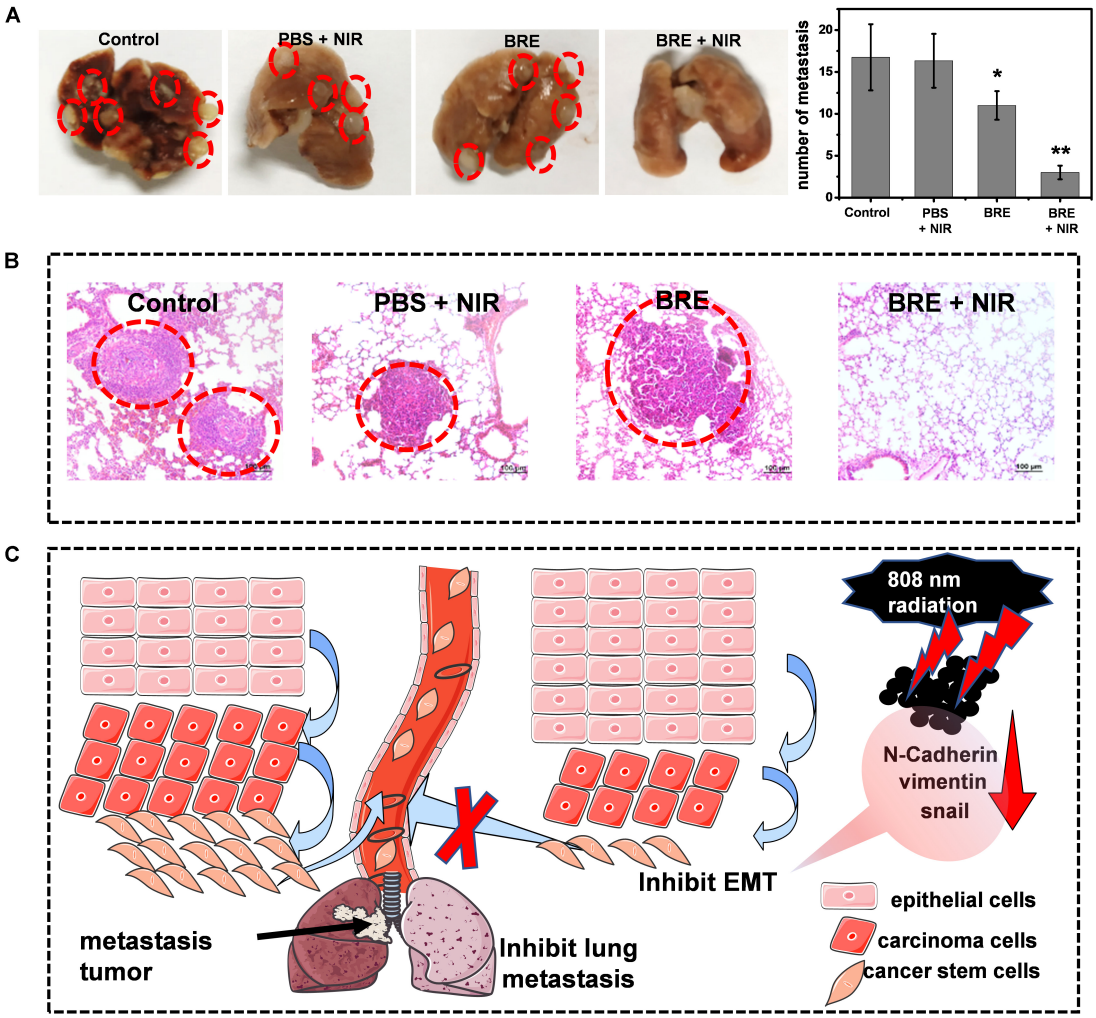


FIGURE 5 | Mechanisms of *in vivo* inhibiting lung metastasis. **(A)** Photographs and numbers of lung metastases in different treatment groups. **(B)** The HE staining of lung in different treatment groups. **(C)** Schematic diagram of BRE and laser radiation for inhibiting lung metastasis and EMT. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

(**Figure 6A**). Blood routine assessment included WBC, RBC, HGB, HCT, MCV, MCH, MCHC, and PLT assays, the indexes showed little difference between the control and BRE treatment group, supporting the notion that BRE have no apparent blood toxicity *in vivo* (**Figure 6B**). Moreover, *in vivo* hepatic and renal toxicity was evaluated by AST, ALT, BUN, and SCR assays respectively. Our results showed that these values were identical between the control and BRE group, indicating that BRE did not induce hepatic and renal toxicity (**Figure 6C**). In addition, the histological analysis indicated that the major organs of BRE treated mice showed little pathological changes, suggesting that BRE did not induce systemic toxicity (**Figure 6D**). Together, these data show that BRE does not induce blood toxicity, hepatic and renal toxicity or even systemic toxicity *in vivo*, and thus representing a safe photothermal agent for *in vivo* application.

CONCLUSION

In summary, BRE, the exaction of the traditional food black rice, has been developed as a novel and low-cost PTT agent with effective anti-tumor and anti-metastasis abilities. With excellent photothermal stability and photothermal conversion efficiency (54.13%), the temperature of BRE could be increased high enough to induce tumor cell death. In the context of anti-tumor and anti-metastasis activities, the results show that the combination of BRE and NIR treatment could significantly inhibit the tumor growth by elevating the local hyperthermia and metastasis by suppressing EMT. In addition, the *in vivo* toxicity data show that BRE causes no obvious systemic toxicity and paved the way for its clinical use. In summary, promising therapeutic effectiveness, low cost, and low toxicity highlight the potential of BRE in tumor therapy.

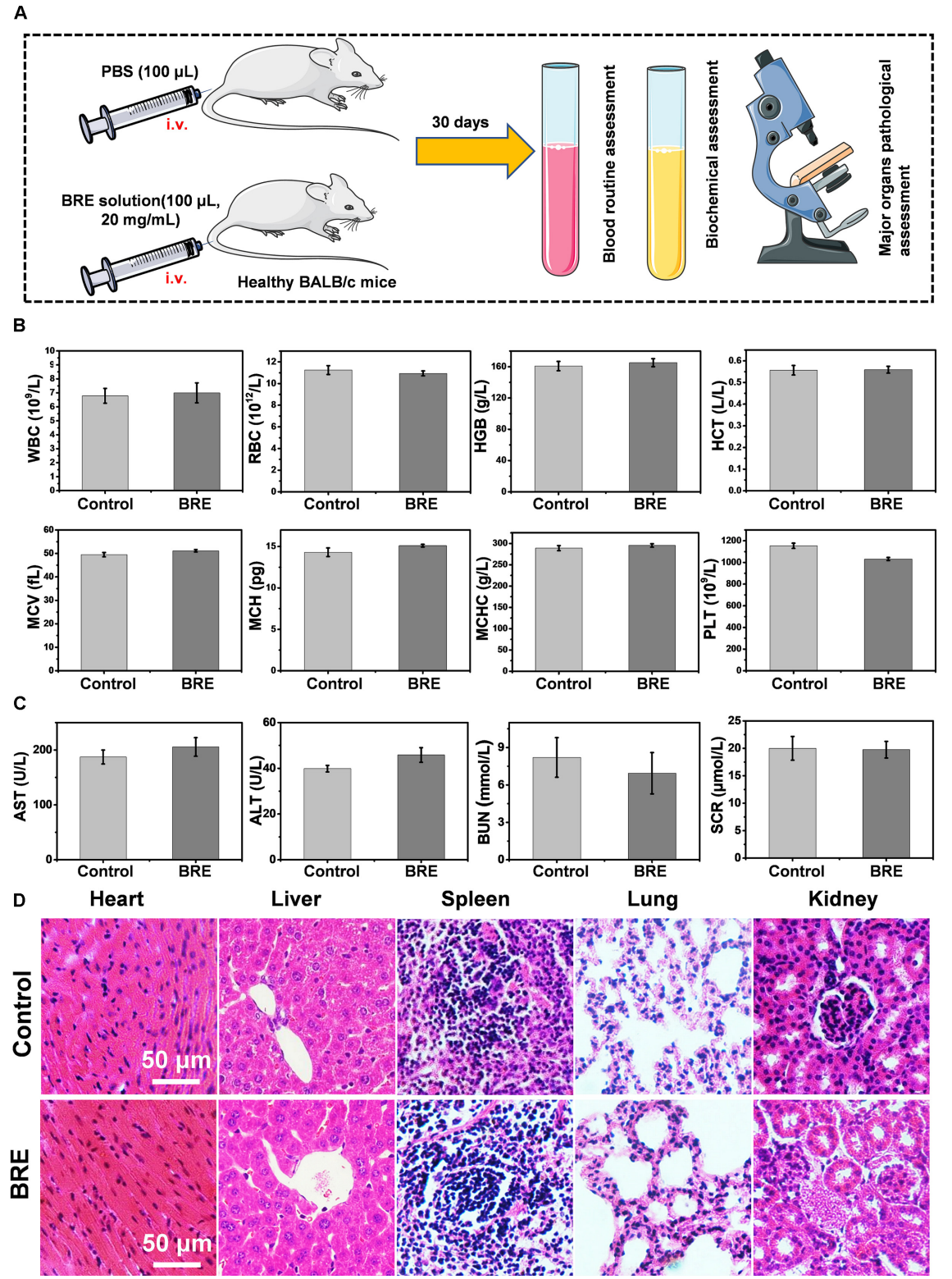


FIGURE 6 | The evaluation of *in vivo* toxicity. **(A)** The scheme of *in vivo* toxicity assay. **(B)** The blood routine assessment of control and the BRE (20 mg/mL) treatment group. **(C)** Physiological function assessment of hepatic and renal toxicity from control and BRE (20 mg/mL) treatment group. **(D)** The HE staining of major organs of control and BRE (20 mg/mL) treatment group. Data are presented as mean ± SD.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC of Southern Medical University.

AUTHOR CONTRIBUTIONS

YX and XF conceived of the original research idea. MT, SZ, and RZ performed the experiments and analyzed the data. YZ helped with the animal experiments. YX, XF, MT, SZ, and RZ prepared the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Nos. 91959204, U1601222, 815300045, and 81871197), the leading talents of Guangdong Province Program (No. 00201516), the Key Research and Development Program of Guangdong Province (810209082089), Science and Technology Innovation Committee of Shenzhen Municipality (JCYJ20180504170301309), Shenzhen "Sanming" Project of Medicine (SZSM201602102), the National High-tech R and D Program (863 Program No. 2015AA020310), and Development and Reform Commission of Shenzhen Municipality (S2016004730009). All funding's contribute to the costs of personnel, supplies, equipment use, core facility use associated with this research.

REFERENCES

Aprodu, I., Milea, ŞA., Anghel, R.-M., Enachi, E., Barbu, V., Crăciunescu, O., et al. (2019). New functional ingredients based on microencapsulation of aqueous anthocyanin-rich extracts derived from black rice (*Oryza sativa* L.). *Mol. (Basel. Switzerland)* 24:3389.doi: 10.3390/molecules24183389

Chen, D., Tang, Y., Zhu, J., Zhang, J., Song, X., Wang, W., et al. (2019). Photothermal-pH-hypoxia responsive multifunctional nanoplatform for cancer photo-chemo therapy with negligible skin phototoxicity. *Biomaterials* 221:119422. doi: 10.1016/j.biomaterials.2019.119422

Chen, L., Zhou, L., Wang, C., Han, Y., Lu, Y., Liu, J., et al. (2019). Tumor-targeted drug and CpG delivery system for phototherapy and docetaxel-enhanced immunotherapy with polarization toward m1-type macrophages on triple negative breast cancers. *Adv. Mater.* 31:1904997. doi: 10.1002/adma.201904997

Chen, W., Wang, X., Zhao, B., Zhang, R., Xie, Z., He, Y., et al. (2019). CuS–MnS2 nano-flowers for magnetic resonance imaging guided photothermal/photodynamic therapy of ovarian cancer through necroptosis. *Nanoscale* 11, 12983–12989. doi: 10.1039/c9nr03114f

Chen, Y., Li, L., Chen, W., Chen, H., and Yin, J. (2019). Near-infrared small molecular fluorescent dyes for photothermal therapy. *Chinese Chem. Lett.* 30, 1353–1360. doi: 10.1016/j.cclet.2019.02.003

Chen, W., Cao, G., Yuan, X., Zhang, X., Zhang, Q., Zhu, Y., et al. (2015). Notch-1 knockdown suppresses proliferation, migration and metastasis of salivary adenoid cystic carcinoma cells. *J. Trans. Med.* 13, 167–167.

Chen, X.-Y., Zhou, J., Luo, L.-P., Han, B., Li, F., Chen, J.-Y., et al. (2015). Black rice anthocyanins suppress metastasis of breast cancer cells by targeting RAS/RAF/MAPK pathway. *BioMed Res. Int.* 2015, 414250–41 4250.

Chen, W., Ouyang, J., Liu, H., Chen, M., Zeng, K., Sheng, J., et al. (2017). Black phosphorus nanosheet-based drug delivery system for synergistic photodynamic/photothermal/chemotherapy of cancer. *Adv. Mater.* 29:1603864. doi: 10.1002/adma.201603864

Deng, R.-H., Zou, M.-Z., Zheng, D., Peng, S.-Y., Liu, W., Bai, X.-F., et al. (2019). Nanoparticles from cuttlefish ink inhibit tumor growth by synergizing immunotherapy and photothermal therapy. *ACS Nano* 13, 8618–8629. doi: 10.1021/acsnano.9b02993

Depciuch, J., Stec, M., Kandler, M., Baran, J., and Parlinska-Wojtan, M. (2020). From spherical to bone-shaped gold nanoparticles—Time factor in the formation of Au NPs, their optical and photothermal properties. *Photodiag. Photody. Ther.* 30:101670. doi: 10.1016/j.pdpdt.2020.101670

Fan, M.-J., Yeh, P.-H., Lin, J.-P., Huang, A.-C., Lien, J.-C., Lin, H.-Y., et al. (2017). Anthocyanins from black rice (Oryza sativa) promote immune responses in leukemia through enhancing phagocytosis of macrophages in vivo. *Exp. Therap. Med.* 14, 59–64. doi: 10.3892/etm.2017.4467

Feng, B., Niu, Z., Hou, B., Zhou, L., Li, Y., and Yu, H. (2020). Enhancing triple negative breast cancer immunotherapy by ICG-templated self-assembly of paclitaxel nanoparticles. *Adv. Funct. Mater.* 30:1906605. doi: 10.1002/adfm.201906605

Georgakopoulos-Soares, I., Chartoumpekis, D. V., Kyriazopoulou, V., and Zaravinos, A. (2020). EMT factors and metabolic pathways in cancer. *Front. Oncol.* 10:499–499.

Hennessy, B. T., Gonzalez-Angulo, A.-M., Stemke-Hale, K., Gilcrease, M. Z., Krishnamurthy, S., Lee, J.-S., et al. (2009). Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. *Cancer Res.* 69, 4116–4124. doi: 10.1158/0008-5472.can-08-3441

Hessel, C. M., Pattani, V. P., Rasch, M., Panthani, M. G., Koo, B., Tunnell, J. W., et al. (2011). Copper selenide nanocrystals for photothermal therapy. *Nano Lett.* 11, 2560–2566. doi: 10.1021/nl201400z

Hou, M., Yan, C., Chen, Z., Zhao, Q., Yuan, M., Xu, Y., et al. (2018). Multifunctional NIR-responsive poly(vinylpyrrolidone)-Cu-Sb-S nanotheranostic agent for photoacoustic imaging and photothermal/photodynamic therapy. *Acta Biomaterialia* 74, 334–343. doi: 10.1016/j.actbio.2018.05.011

Jia, H.-R., Zhu, Y.-X., Liu, X., Pan, G.-Y., Gao, G., Sun, W., et al. (2019). Construction of dually responsive nanotransformers with nanosphere–nanofiber–nanosphere transition for overcoming the size paradox of anticancer nanodrugs. *ACS Nano* 13, 11781–11792. doi: 10.1021/acsnano.9b05749

Jin, L., Han, B., Siegel, E., Cui, Y., Giuliano, A., and Cui, X. (2018). Breast cancer lung metastasis: molecular biology and therapeutic implications. *Cancer Biol. Ther.* 19, 858–868. doi: 10.1080/15384047.2018.1456599

Lamouille, S., Xu, J., and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. Nature reviews. *Mol. Cell Biol.* 15, 178–196. doi: 10.1038/nrm3758

Li, J., Chen, Z., Huang, R., Miao, Z., Cai, L., and Du, Q. (2018). Toxicity assessment and histopathological analysis of nano-ZnO against marine fish (Mugilogobius chulae) embryos. *J. Environ. Sci.* 73, 78–88. doi: 10.1016/j.jes.2018.01.015

Liu, Y., Li, F., Guo, Z., Xiao, Y., Zhang, Y., Sun, X., et al. (2020). Silver nanoparticle-embedded hydrogel as a photothermal platform for combating bacterial infections. *Chem. Eng. J.* 382:122990. doi: 10.1016/j.cej.2019.122990

Miao, Z.-H., Li, K., Liu, P.-Y., Li, Z., Yang, H., Zhao, Q., et al. (2018). Natural humic-acid-based phototheranostic agent. *Adv. Healthcare Mater.* 7:1701202. doi: 10.1002/adhm.201701202

Obenauf, A. C., and Massagué, J. (2015). Surviving at a distance: organ-specific metastasis. *Trends Cancer* 1, 76–91. doi: 10.1016/j.trecan.2015.07.009

Pang, Y., Ahmed, S., Xu, Y., Beta, T., Zhu, Z., Shao, Y., et al. (2018). Bound phenolic compounds and antioxidant properties of whole grain and bran of white, red and black rice. *Food Chem.* 240, 212–221. doi: 10.1016/j.foodchem.2017.07.095

Park, S.-Y., Lee, J.-W., Kim, G.-W., and Kim, H.-Y. (2017). Effect of black rice powder on the quality properties of pork patties. *Korean J. Food Sci. Anim. Resour.* 37, 71–78. doi: 10.5851/kosfa.2017.37.1.71

Peng, C., Liang, Y., Chen, Y., Qian, X., Luo, W., Chen, S., et al. (2020). Hollow mesoporous tantalum oxide based nanospheres for triple sensitization of radiotherapy. *ACS Appl. Mater. Interf.* 12, 5520–5530. doi: 10.1021/acsami.9b20053

Polyak, K., and Weinberg, R. A. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat. Rev. Cancer* 9, 265–273. doi: 10.1038/nrc2620

Samyor, D., Das, A. B., and Deka, S. C. (2017). Pigmented rice a potential source of bioactive compounds: a review. *Int. J. Food Sci. Technol.* 52, 1073–1081. doi: 10.1111/ijfs.13378

Siegel, R. L., Miller, K. D., and Jemal, A. (2019). Cancer statistics, 2019. *Cancer J. Clin.* 69, 7–34.

Wu, C., Sun, J., Zheng, P., Kang, X., Chen, M., Li, Y., et al. (2019). Preparation of an intelligent film based on chitosan/oxidized chitin nanocrystals incorporating black rice bran anthocyanins for seafood spoilage monitoring. *Carbohyd. Polym.* 222:115006. doi: 10.1016/j.carbpol.2019.115006

Wu, R., Wang, H., Hai, L., Wang, T., Hou, M., He, D., et al. (2020). A photosensitizer-loaded zinc oxide-polydopamine core-shell nanotherapeutic agent for photodynamic and photothermal synergistic therapy of cancer cells. *Chinese Chem. Lett.* 31, 189–192. doi: 10.1016/j.cclet.2019.05.004

Xu, F., Li, S., Zhang, J., Wang, L., Wu, X., Wang, J., et al. (2018). Cancer stemness, immune cells, and epithelial–mesenchymal transition cooperatively predict prognosis in colorectal carcinoma. *Clin. Color. Cancer* 17, e579–e592. doi: 10.1016/j.clcc.2018.05.007

Yang, S., Zhou, L., Su, Y., Zhang, R., and Dong, C.-M. (2019). One-pot photoreduction to prepare NIR-absorbing plasmonic gold nanoparticles tethered by amphiphilic polypeptide copolymer for synergistic photothermal-chemotherapy. *Chinese Chem. Lett.* 30, 187–191. doi: 10.1016/j.cclet.2018.02.015

Yuan, M., Xu, S., Zhang, Q., Zhao, B., Feng, B., Ji, K., et al. (2020). Bicompatible porous Co3O4 nanoplates with intrinsic tumor metastasis inhibition for multimodal imaging and DNA damage–mediated tumor synergetic photothermal/photodynamic therapy. *Chem. Eng. J.* 394:124874. doi: 10.1016/j.cej.2020.124874

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 Bioengineering and Biotechnology

REVIEW
published: 15 September 2020
doi: 10.3389/fbioe.2020.575442

The Role of Nutrition in the Prevention and Intervention of Type 2 Diabetes

Yajie Guo, Zihua Huang, Dan Sang, Qiong Gao and Qingjiao Li**

The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, China

Type 2 diabetes (T2D) is a rapidly growing epidemic, which leads to increased mortality rates and health care costs. Nutrients (namely, carbohydrates, fat, protein, mineral substances, and vitamin), sensing, and management are central to metabolic homeostasis, therefore presenting a leading factor contributing to T2D. Understanding the comprehensive effects and the underlying mechanisms of nutrition in regulating glucose metabolism and the interactions of diet with genetics, epigenetics, and gut microbiota is helpful for developing new strategies to prevent and treat T2D. In this review, we discuss different mechanistic pathways contributing to T2D and then summarize the current researches concerning associations between different nutrients intake and glucose homeostasis. We also explore the possible relationship between nutrients and genetic background, epigenetics, and metagenomics in terms of the susceptibility and treatment of T2D. For the specificity of individual, precision nutrition depends on the person's genotype, and microbiota is vital to the prevention and intervention of T2D.

Keywords: nutrition, diet, genetics, epigenetics, gut microbiota, type 2 diabetes

OPEN ACCESS

Edited by:
Yi Cao,
Xiangtan University, China

Reviewed by:
Cristian Del Bo',
University of Milan, Italy
Lu Wang,
Hainan University, China

****Correspondence:***
Yajie Guo
guoyj36@mail.sysu.edu.cn
Qingjiao Li
liqj23@mail.sysu.edu.cn

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *23 June 2020*
Accepted: *17 August 2020*
Published: *15 September 2020*

Citation:
Guo Y, Huang Z, Sang D, Gao Q and Li Q (2020) The Role of Nutrition in the Prevention and Intervention of Type 2 Diabetes. Front. Bioeng. Biotechnol. 8:575442. doi: 10.3389/fbioe.2020.575442

INTRODUCTION

Diabetes mellitus, previously considered as a disease of minor significance to health, is now becoming one of the main threats to human health both in developed and developing countries (Zimmet et al., 2001). There has been an explosive increase in the number of people diagnosed with diabetes in recent decades worldwide (King et al., 1998). According to the ninth edition of the IDF Diabetes Atlas in 2019, 488 million adults aged 20–99 years live with diabetes in the world, and the number will reach 578 million by 2030 and 700 million by 2045. It is estimated that 4.2 million adults aged 20–79 years will die of diabetes, which accounts for 11.3% of all deaths. And this is equivalent to eight deaths every minute.

Diabetes is defined as a metabolic disease characterized by persistent hyperglycemia caused by multiple factors including genetics, nutrition, environment, and physical activity. There are two main forms of diabetes, type 1 diabetes and type 2 diabetes (T2D) (Alberti and Zimmet, 1998). T2D accounts for more than 90% of all diabetes cases (Zimmet et al., 2001), and the diabetes epidemic particularly relates to T2D. Insulin resistance and/or abnormal insulin secretion are the main characters of T2D. Apart from the heightened genetic susceptibility of ethnic groups, environmental and behavioral factors are also very important in the development of T2D. Globalization results in altered dietary and lifestyle habits (Malik et al., 2013), such as taking more high-fat or high-carbohydrate foods and sedentary lifestyles with low energy expenditure

(Zimmet et al., 2001). Diets induce multiple metabolic processes and modify the metabolism homeostasis of the organism (Manore et al., 2017). Therefore, unhealthy dietary habits such as Western diet have been one of the most important drivers of glucose metabolism disorder that leads to diabetes finally (Rico-Campa et al., 2019).

The increase in the prevalence of T2D is associated with a concomitant rise in the incidence of metabolic disorders. Long-term high glucose levels will trigger chronic metabolic syndrome and include obesity (Schwartz and Porte, 2005), cardiovascular disease, retinopathy, nephropathy, dyslipidemia, and hypertension (Moller, 2001). T2D now represents a risk of coronary heart disease, and nearly 80% of diabetic mortality is diabetes-induced cardiovascular disease (Haffner et al., 1998). The life qualities of patients with diabetes decrease largely for the serious diabetes complications.

Diet alone or with hypoglycemic agents is the way to control blood glucose levels in the treatment of T2D (Zimmet et al., 2001; Ley et al., 2014). Different diets with varied nutrient composition result in changes of metabolites and gut microbiome that are responsible for the glucose metabolism of the whole body (Qin et al., 2012; Guasch-Ferre et al., 2016). For example, different amino acid content diets can lead to alterations of plasma branched-chain amino acid (BCAA) concentrations, which are linked to the risk of T2D (Garcia-Perez et al., 2017). Fiber- and protein-enriched diet changed the abundance of *Akkermansia muciniphila*, decreasing fasting glucose levels of participants (Dao et al., 2016). However, the interactions between dietary and glucose metabolism need further study to understand the importance of its actions for glucose management. It is important to identify and make suitable dietary solutions that can diminish the prevalence of diabetes and its related complications (San-Cristobal et al., 2015). These include different kinds of food and also healthy dietary habits.

Genome-wide association studies (GWASs) have revealed many genetic variants related to the susceptibility of complex diseases, and moreover, the interactions between genetic information and nutrition are attracting more attention recently, namely, nutrigenetics. Because of the genetic variability between individuals, the responses to dietary are different. Also, the specific diet and nutrition modify gene expression, epigenetic features, and gut microbiome to personalize the response to interventions. This prompts us to explore more possibilities to understand the pathophysiological mechanisms and precision nutrition solutions to prevent and manage T2D more efficiently.

Herein, first, we introduce the major metabolic pathway related to T2D, namely, insulin signaling pathway, and the compounding factors as well. Then, the roles of macronutrient, micronutrient, and other chemicals in maintaining metabolic homeostasis of the body and their effects on T2D are reviewed in detail. In addition, some nutritional recommendations for T2D are summarized. From the perspective of precision nutrition, we review the diet interactions with genetic background, epigenetics, and gut microbiota contributing to the risk of T2D. Also, responses to dietary interventions mainly aiming at weight loss and management of insulin resistance are screened for their interaction with genetic, epigenetic features, and gut microbiota.

REGULATION OF GLUCOSE METABOLISM

Circulating blood glucose is derived from diet via intestinal absorption, and the process of glucose production is called gluconeogenesis and glycogen breakdown (Rines et al., 2016). Current therapeutic approaches to treat T2D rely on the molecular signaling pathways and targets that impair glucose homeostasis. Insulin signaling pathway dysregulation or insulin resistance is the main reason for T2D. Insulin is an endocrine peptide hormone secreted by the pancreas, and it binds to membrane-bound receptors in target cells of liver, adipose tissue, and skeletal muscle to trigger metabolic responses to numerous stimuli (Petersen and Shulman, 2018). Insulin exerts its low glucose function by binding to the insulin receptor (INSR), and then activated INSR recruits phosphotyrosine-binding scaffold proteins such as the INSR substrate (IRS) family. IRS proteins have NH_2-terminal pleckstrin homology (PH) and PTB domains that target them to activate INSR. Then, the tyrosine phosphorylated IRS proteins recruit PI3K heterodimers that contain a regulatory p85 subunit and a catalytic p110 subunit. PI3K catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) from PIP_2 and PIP_3 and then recruits proteins with PH domains to the plasma membrane, such as pyruvate dehydrogenase kinase 1, which directly phosphorylates AKT. The activated AKT phosphorylates many downstream substrates in various signaling pathways, making it a key node in insulin signaling (Petersen and Shulman, 2018). The activated insulin signaling decreases glucose production, increases glycogen synthesis, and also increases glucose uptake into peripheral tissues such as skeletal muscle and adipose tissue (**Figure 1**).

The dysfunction of insulin signaling will cause insulin resistance, which is a complex metabolic disorder that is closely linked to many pathways including lipid metabolism, energy expenditure, and inflammation (**Figure 2**). Hepatic lipid accumulation is known to cause insulin resistance (Samuel and Shulman, 2012). Diacylglycerol species activate protein kinase C (PKC), which results in impaired insulin signaling (Perry et al., 2014). An excess of lipid accumulation in liver is often accompanied by hepatic inflammation. Kupffer cells and macrophages will decrease insulin sensitivity by secreting proinflammatory molecules, which activate serine/threonine kinases such as c-Jun N-terminal kinase (JNK) and IκB kinase that in turn impair insulin signaling (Lackey and Olefsky, 2016). Moreover, lipid accumulation triggers the unfolded protein response (UPR) pathway, which impairs insulin signaling (Ozcan et al., 2004). UPR may also alter hepatokine secretion and consequently contribute to the development of insulin resistance (Koska et al., 2008). Energy expenditure disorder leads to obesity and insulin resistance, because non-esterified fatty acids impair β-cell functions, reduce PI3K signaling, and enhance gluconeogenic enzyme expressions (Kahn et al., 2006). What is more, increased release of tumor necrosis factor α (TNF-α), interleukin 6 (IL6), and monocyte chemotactic protein 1 are all found to be responsible for the development of insulin resistance (Kahn et al., 2006). In addition, hepatokines, proteins produced from liver and secreted into the circulation, also play

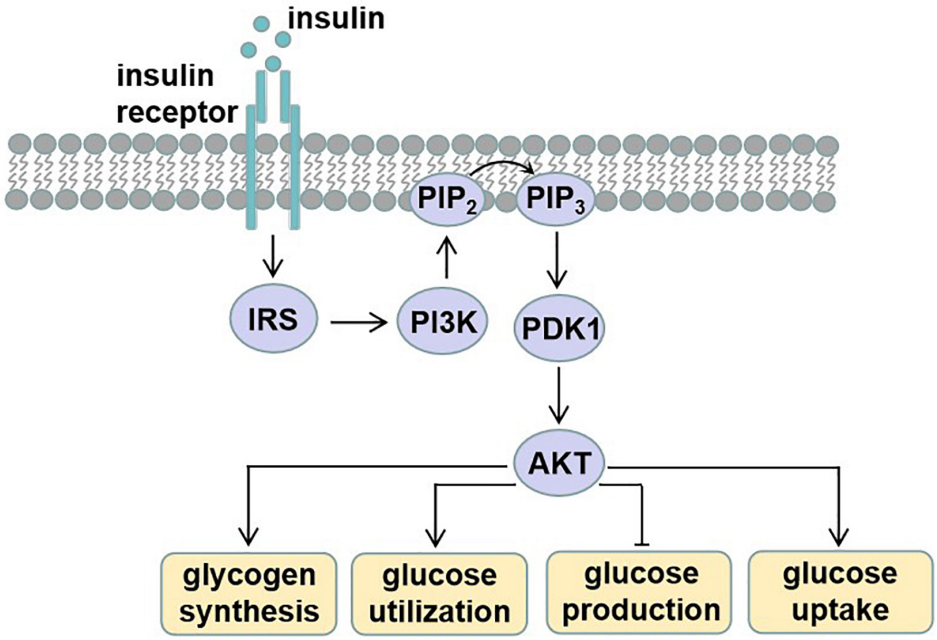


FIGURE 1 | Insulin signaling. Insulin binds and activates insulin receptor (INSR), causing phosphorylation of insulin receptor substrate (IRS). Tyrosine phosphorylated IRS proteins recruit phosphatidylinositide-3 (PI3K), which catalyzes the production of phosphatidylinositol-3,4,5-tris-phosphate (PIP_3) from PIP_2. PIP_3 then recruits proteins with PH domains such as pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates activating protein kinase B (AKT). These effector proteins mediate the effects of insulin on glucose production, utilization, and uptake, as well as glycogen synthesis.

important roles in regulating insulin signaling (Meex and Watt, 2017). Retinol-binding protein 4 (RBP4), α2-macroglobulin (A2M), fetuin A (FETUA), fetuin B (FETUB), hepassocin (FGL1), leukocyte cell–derived chemotaxin 2 (LECT2), and selenoprotein P (SELENOP) are negative regulators of insulin sensitivity, and they will cause insulin resistance, whereas fibroblast growth factor 21 (FGF21), sex hormone–binding globulin (SHBG), adropin, and angiopoietin-like protein 4 (ANGPTL4) are positive regulators (Lai et al., 2008; Meex and Watt, 2017).

MACRONUTRIENT AND T2D

Carbohydrate

It needs a precise control of glucose metabolism to maintain metabolic homeostasis of the body. Hormonal regulation and the related enzyme transcription induced by different metabolites in response to glucose availability are mainly responsible for the control. Insulin induces INSR autophosphorylation and then recruits and phosphorylates IR substrates 1 and 2 (IRS1/2). This results in phosphatidylinositide-3, 4, 5-P3 (PIP3) production, and activating protein kinase B (PKB/AKT) (Saltiel and Kahn, 2001). Thus, it promotes glucose uptake by different tissues, including liver, adipose tissue, and skeletal muscle; inhibits hepatic glucose output; increases glycogen synthesis; and decreases glycogen decompose (Zhang et al., 2009). Insulin induces anabolic responses such as ribosome biogenesis and protein synthesis, which are dependent on nutritional state. The mTOR/S6K1 signaling pathway is also activated by insulin, which plays a vital role in the regulation of glucose homeostasis (Um et al., 2006). Glucose released by diet stimulates the production of PI3P, recruiting proteins to endosomal membranes and finally activating mTOR/S6K1 signaling pathway (Um et al., 2006).

Glucose homeostasis involves different pathways that are carried out in part by the transcriptional control of related genes. Carbohydrate induces the expressions of these enzymes, including pyruvate kinase, glucokinase, ATP citrate lyase, and acetyl CoA carboxylase (Haro et al., 2019). And these genes are regulated by the carbohydrate-responsive element-binding protein (ChREBP) (**Figure 3**), which is a helix–loop–helix leucine zipper transcription factor (Lee and Cha, 2018). It plays a very important role in sugar-induced lipogenesis and glucose homeostasis by regulating carbohydrate digestion and transport (Yamashita et al., 2001). In response to glucose, ChREBP forms a heterodimer and activates the target genes transcriptions, which contain carbohydrate response element motifs. Except for its glucose sensor role, ChREBP is also essential for fructose induced lipogenesis in liver and intestine possibly via the ChREBP-FGF21 signaling axis (Fisher et al., 2017).

Carbohydrate foods that promote sustained but low glucose levels may have benefits to metabolic control of diabetes and its complications. Diets with slow-release carbohydrates lower the glucose and insulin responses throughout the day and improve the capacity for fibrinolysis, which may be a potential therapy to T2D (Russell et al., 2016). When syrup is included in a diabetic diet, it is good to consider sucrose rather than fructose (Wheeler and Pi-Sunyer, 2008). In a short-term trial of T2D patients, scientists showed that isocaloric

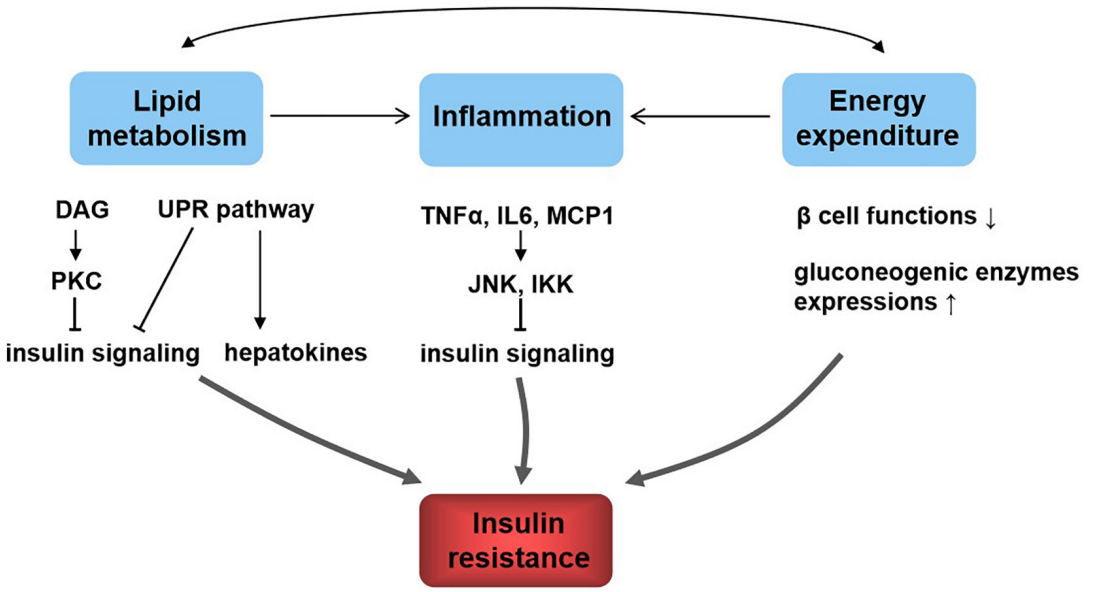


FIGURE 2 | Relationship between lipid metabolism, energy metabolism, inflammation, and insulin resistance. Lipid metabolism and energy metabolism disorder lead to inflammation and affect each other. These all contribute to insulin resistance. The underlying mechanisms include diacylglycerol (DAG), activate protein kinase C (PKC) and lipid accumulation, trigger the unfolded protein response (UPR) pathway, and result in insulin signaling inhibition; UPR affects hepatokine secretion to induce insulin resistance; inflammatory molecules such as tumor necrosis factor α (TNF-α), interleukin 6 (IL6), and monocyte chemotactic protein 1 (MCP1) activate c-Jun N-terminal kinase (JNK) and IκB kinase (IKK), which in turn impair insulin signaling; energy homeostasis disorder impairs β-cell functions, reduces PI3K signaling, and enhances gluconeogenic enzyme expressions, resulting in insulin resistance.

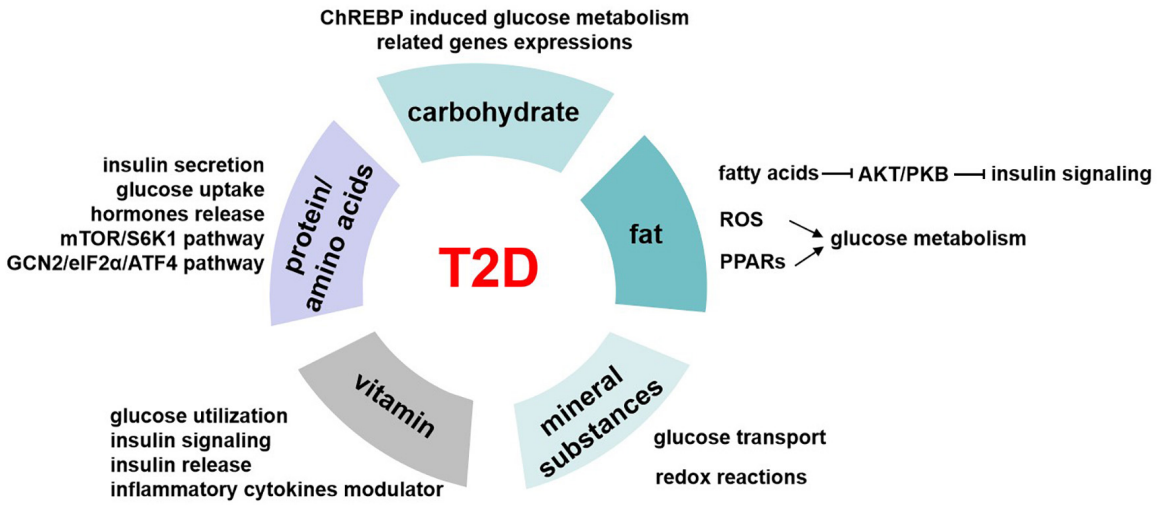


FIGURE 3 | The role of nutrients in T2D. Carbohydrates regulate glucose homeostasis through carbohydrate-responsive element-binding protein (ChREBP) induced glucose metabolism–related genes expressions. Fatty acids inhibit AKT/PKB activation and therefore impair insulin signaling pathway. Besides, fat induces reactive oxygen species (ROS) generation in mitochondrial and activates peroxisome proliferator-activated receptors (PPARs), and all these mediate the regulation of fat on glucose metabolism. The possible underlying pathways or mechanisms of protein/amino acids affecting glucose levels include insulin secretion, glucose uptake, hormone release, mTOR/S6K1 signaling pathway, and GCN2/eIF2α/ATF4 transduction pathway. Mineral substances are activating cofactors and coenzymes for metabolism control, oxidative stress, and genetic transcription. This makes them play roles in glucose transport and redox reactions, which finally affect glucose homeostasis. Vitamin has a role in regulating glucose utilization, insulin signaling, and insulin release from β cells to maintain blood glucose levels, and it is also the modulator of inflammatory cytokines related to glucose metabolism.

fructose replacement of other carbohydrates such as sucrose and starch improved glycemic control and had no effects on insulin signaling (Cooper et al., 2012). However, it should take more account to the point that high sucrose or fructose diet is not recommended to diabetic individuals and others who have impaired glucose metabolism. Besides, certain diet

components may affect the regulation role of foods on glucose levels. For example, diet with fiber, certain proteins, or lipids to mix may influence the rates of carbohydrate digestion and absorption, which may be beneficial to T2D patients (Russell et al., 2016). Polyols have been used as sugar replacers for about 80 years. Clinical trial showed that polyols had a role in lowering serum glucose levels in T2D patients (Mohsenpour et al., 2019), which may provide a new strategy to manage T2D. Dietary fibers, which are mainly found in cereals, fruits, vegetables, or legumes, showed close associations with T2D. Increased fiber intake, especially soluble fiber, played a beneficial role in improving glycemic control in patients with T2D (Chandalia et al., 2000).

Fat

Our body obtains kinds of lipid metabolites from diet intake directly or generated intracellularly by liver and adipose tissue in different pathways. Lipidomics help us better understand the circulating lipid species. Among these, some are considered as biomarkers related to insulin resistance, such as stearic acid and deoxysphingolipids, and saturation and chain length of fatty acids (Meikle and Summers, 2017). High-fat diet–induced insulin resistance and T2D have been largely known since 20 years ago. High-fat diet increases lipid accumulation in cells and leads to obesity. Excess of fat increases proinflammatory cytokines and other hormones or factors involved in insulin resistance (Kahn et al., 2006). Free fatty acids inhibit Akt/PKB activation, thus impairing the insulin signaling pathway (Bruce and Febbraio, 2007). Besides, the reactive oxygen species generation in mitochondrial is increased, which also affects the glucose homeostasis (Bruce and Febbraio, 2007). Peroxisome proliferator-activated receptors (PPARs) function as lipid sensors that can be activated by both dietary fatty acids and their derivatives. PPARs regulate the expression of genes involved in a variety of processes including glucose and lipid metabolism, immune response, and cell growth (Evans et al., 2004; **Figure 3**). PPARα is vital in regulating fatty acid oxidation and therefore has indirect effects on improving glucose metabolism (Evans et al., 2004). Besides, PPARα activates tribbles pseudokinase 3 (TRB3), a direct target, to inhibit AKT activation and impairs insulin sensitivity (Du et al., 2003). PPARγ is an effector of adipogenesis via C/EBP and is responsible for the glucose regulation.

Scientists investigated the impact of fatty acid intake on blood glucose and insulin in the diet of adults with T2D and found that replacement of saturated fats with monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids may improve their glucose or insulin tolerance (Russell et al., 2016). And *in vitro* experiments have also confirmed that MUFAs or oleate rather than palmitate prevents insulin resistance (Gao et al., 2009). Postprandial hyperlipidemia is common in T2D patients, and it was shown that omega-3 fatty acids could reduce postprandial lipids but may not correct them completely (Tomlinson et al., 2020). However, the role of *trans*-fats in regulating glucose control is still controversial. Meta-analysis showed cholesterol-rich diet had a positive relationship with T2D risk (Tajima et al., 2014). Besides, supplementation of plant sterols or stanols lowered serum cholesterol levels (Derosa et al., 2018) that may be indirectly beneficial to glucose metabolism.

Protein

Dietary proteins are vital to life for its important role in acquiring essential amino acids to maintain protein synthesis and degradation and supporting cellular processes such as cell growth and development (Tremblay et al., 2007). In recent years, more and more studies have shown that proteins had different effects on glucose homeostasis by affecting insulin action and secretion except for body weight and feeding behavior. In normal or diabetic humans, dietary proteins stimulate insulin secretion so as to reduce glycemia (Spiller et al., 1987). High-protein diets seem to have beneficial effects on weight loss and glucose metabolism, significantly increase insulin sensitivity, and decrease inflammation in the short term (Russell et al., 2016). But long-term high-protein intake seems to result in insulin resistance in the whole body, by increasing mTOR/S6K1 signaling pathway and stimulating gluconeogenesis and high glucagon turnover (Linn et al., 2000). Studies showed that a 6-month high-protein diet (1.87 ± 0.26 g protein/kg body weight per day) in healthy individuals increased fasting glucose levels, impaired hepatic glucose output suppression by insulin, and enhanced gluconeogenesis (Linn et al., 2000). On the other hand, low-protein diets (5%–10% protein calories) suggested improved insulin sensitivity that is beneficial to T2D, and this may be realized through the general control non-derepressible 2 (GCN2)/transcription factor 4 (ATF4)/FGF21 signaling pathway (Haro et al., 2019; **Figure 3**).

Soy protein is one kind of protein that is good for its hypolipidemic and hypocholesterolemic benefits in humans (Anderson et al., 1995). Studies showed that soy protein intake can positively affect glucose metabolism in addition to its effect on decreasing serum lipids. In comparison with casein, soy protein reduced fasting glucose and insulin levels in animals and prevented insulin resistance induced by a high-sucrose diet. Moreover, in humans, it was also revealed that soy protein decreased glucose levels compared to casein (Hubbard et al., 1989). And this function might be explained by the differential hormonal response. Besides, soy protein can also stimulate INSR mRNA expression and thereby increase insulin signaling in fat and liver finally improve insulin sensitivity in these tissues (Iritani et al., 1997).

Fish protein is another protein and widely known protein for years, as Alaska and Greenland populations have a low incidence of T2D for taking large amounts of fish. In lean fish, protein is the most abundant nutrient; consumption of fish protein showed improved cholesterol transport via high-density lipoprotein and reduced triglycerides via very low-density lipoprotein (Chen et al., 2020). Meanwhile, compared to casein-fed animals, cod protein–fed rats were protected against insulin resistance induced by sucrose or in saturated fat (Lavigne et al., 2000) by stimulating glucose uptake by skeletal muscle (Lavigne et al., 2001). Cod protein activated PI3K/AKT signaling pathway and selectively improved GLUT4 translocation to the T tubules, improving glucose transport in response to insulin (Tremblay et al., 2003). Moreover, human

studies also showed cod protein exerted beneficial effects to T2D. Cod protein induced a lower insulin-to-glucose ratio compared with milk protein (von Post-Skagegard et al., 2006) and increased postmeal plasma insulin concentrations compared with beef protein (Tremblay et al., 2007).

Protein breakdown or synthesis leads to the change of amino acids levels. There are eight amino acids that cannot be produced inside the body but must come from food. Amino acids are considered as gene expression regulators such as CHOP, which is important to glucose metabolism (Tremblay and Marette, 2001). Amino acids activate the mTOR/S6K1 pathway, and the activation of mTOR inhibits PI3K that results in insulin resistance (Kimball and Jefferson, 2006). BCAAs are kind of important amino acids in regulating homeostasis. BCAAs regulate the release of hormones, including leptin (LEP), GLP-1, and ghrelin, which affects glucose control (Potier et al., 2009). Besides, BCAAs regulate glucose metabolism partly through activating the mTORC1/PKC signaling pathway (Vary and Lynch, 2007).

A healthy and balanced diet should meet all the requirements in amino acids and proteins from varied sources in appropriate proportions. The canonical pathway to respond to amino acid deficiency is amino acid response (Chou et al., 2012). When the essential amino acids decrease, it would cause the deacetylation of the corresponding tRNAs. Uncharged tRNAs bind and activate the GCN2 kinase, and then the activated GCN2 phosphorylates the eukaryotic initiation factor 2α (eIF2α), and induces ATF4 activation (Hao et al., 2005). Numerous studies have shown that increasing dietary levels of BCAAs had a positive effect on T2D (Lynch and Adams, 2014), whereas others suggested that deficiency of BCAAs was beneficial for improving insulin sensitivity and glucose tolerance. Leucine deprivation or methionine deficiency all showed improved insulin sensitivity, energy expenditure, and thermogenesis via GCN2/eIF2α/ATF4/FGF21 transduction pathway (Haro et al., 2019).

MICRONUTRIENT AND T2D

Mineral Substances

As micronutrient, mineral substances are required at very low concentrations for the normal growth but play important roles in maintaining metabolism homeostasis (Shenkin, 2006). Some of the mineral substances are activating cofactors and coenzymes for metabolism control, oxidative stress, and genetic transcription. The deficiency of mineral substances was shown to have relationship with T2D (**Figure 3**).

Selenium is a vital component of enzymes for redox reactions such as glutathione peroxidase and thioredoxin reductase in human body, and importantly, the dose range to toxicity is very narrow (Sun et al., 2013). The main dietary sources of selenium are cereals, black tea, milk, mushrooms, soybeans, bamboo shoots, nuts, and broccoli (Rayman et al., 2008). Appropriate concentration of selenium intake can act as an insulin minetic to attenuate diabetes, with the role of decreasing glucose and insulin tolerance, thus preventing hepatic insulin resistance (Zhou et al., 2013). However, high selenium concentration will result in gluconeogenesis, and fasting blood glucose (FBG) levels increased and therefore have a risk to diabetes (Ogawa-Wong et al., 2016).

Vanadium is common in nature but appears at very low concentrations in humans. It occurs with proteins such as transferrin, albumin, and hemoglobin that are vital to the physiological processes (Pessoa and Tomaz, 2010). *In vitro* and *in vivo* researches suggested that vanadium had insulin-mimetic properties and may be a potential therapeutic agent to T2D (Domingo and Gomez, 2016). Oral administration of 1 mg/kg per day of vanadyl sulfate for 4 weeks significantly decreased glucose levels in diabetes patients. The possible mechanism underlying this might be through increasing GLUT translocation to plasma membrane and then resulting in glucose transport increase (Cohen et al., 1995).

Chromium plays an important role in glucose metabolism by enhancing the binding of insulin to INSR (Cefalu and Hu, 2004). Clinical trials suggested 4 months' supplementation of chromium significantly decreased postprandial and also fasting glucose levels. Mechanisms underlying this beneficial function of chromium may partly be explained by the increase of GLUT2 expression and the activation of PI3K/AKT pathway in skeletal muscle (Panchal et al., 2017).

Zinc is an important component of enzymes that play vital roles in regulating insulin sensitivity and glucose homeostasis. Researches showed that, in patients with T2D, the concentrations of zinc in plasma and tissues are lower (Russell et al., 2016). Zinc supplementation improved insulin sensitivity and glucose tolerance in diabetic mice models (Chen et al., 2000) and was found to have similar functions in humans (Russell et al., 2016).

High sodium intake leads to a higher risk of hypertension and cardiovascular diseases in patients with diabetes mellitus. Sodium intake increases natriuresis via PPARδ/SGLT2 pathway and subsequently regulates glucose metabolism of type 2 diabetic patients (Zhao et al., 2016). In contrast, another substance, magnesium, was suggested to decrease the risk of cardiovascular diseases in T2D patients. Magnesium deficiency was associated with diabetes risk, whereas magnesium supplementation could attenuate insulin resistance and improve glycemic control in T2D patients (Wa et al., 2018).

Vitamin

In recent years, vitamin has received increased attention because of its roles in regulating the development of T2D by modulating insulin resistance and pancreatic β-cell functions (**Figure 3**). Among these, vitamins D and E are the two most popular types. Vitamin D was used to be a regulator of bone metabolism but was found to have various clinical functions. It is a key hormone involved in calcium and phosphorous balance with several derivatives (Muscogiuri et al., 2017). Vitamin D receptor (VDR) is found in the pancreatic β cells and insulin response tissues such as skeletal muscle and adipose tissue (Fan et al., 2016). Studies showed that

vitamin D affected glucose utilization in VDR-dependent manner in muscle and adipose tissue and activated PPARδ, which is a transcription factor involved in fatty acid metabolism (Grammatiki et al., 2017). Besides, vitamins modulated insulin action and insulin sensitivity by directly stimulating INSR gene expressions (Maestro et al., 2000) or altered calcium flux to influence insulin release of β cells (Muscogiuri et al., 2017). Moreover, vitamin is a negative modulator of inflammatory cytokine such as TNF-α and IL6, which are closely related to insulin resistance (Garbossa and Folli, 2017).

Insulin resistance is the main diagnosis in most T2D patients, and vitamin D deficiency was found to result in insulin resistance and metabolic syndrome such as hypogonadotrophic, renal diseases and cardiovascular complications (Garbossa and Folli, 2017). And some beneficial effects of vitamin D supplementation have been reported. In several clinical trials, vitamin D administration decreased serum fasting glucose levels and improved Homeostatic Model Assessment of Insulin Resistance index in T2D patients (Talaei et al., 2013; Grammatiki et al., 2017). Vitamin E is a fat-soluble vitamin, which is well known for its antioxidant capacity. Besides, it also functions on cell cycle, cell signaling, lipid metabolism, and inflammation (Gray et al., 2011). Several years ago, vitamin E has been reported to have a role in regulation of insulin sensitivity (Galmes et al., 2018). Vitamin E supplementation significantly decreased plasma glucose and hemoglobin A_{1c} (HbA_{1c}) levels (Paolisso et al., 1993). The underlying mechanisms may include several pathways. For its antioxidant capacity, vitamin E alters IRS1 phosphorylation, thus affecting insulin signaling (Gray et al., 2011). Besides, vitamin E was shown to directly regulate gene expression such as PPARγ, which plays important roles in insulin sensitivity (Landrier et al., 2009).

OTHER CHEMICALS AND T2D

Besides macronutrients and micronutrients, others such as phytochemicals and bioactives that are widely distributed in diets or chemicals (such as alcohol) also have potential effects on T2D. Phytochemicals or bioactives exist in fruits, flowers, wood, seeds, bark, and stems, and some of them are found in traditional Chinese medicine (Zhao et al., 2019). They have been reported for their beneficial and therapeutic roles on diabetes in various studies. Phytochemical compounds such as lignans or flavonoids protect against oxidative stress and help diabetic wound healing (Bacanli et al., 2019). Bioactives, such as curcumin, capsaicin, berberine, celastrol, or artemisinin, were shown to improve insulin sensitivity to combat diabetes (Zhao et al., 2019). Despite the promising benefits, the molecular activity and toxicity of these numerous phytochemicals and bioactives need to be explored in further studies.

Alcohol is closely related to diseases such as fatty liver, cardiovascular diseases, and also T2D. Recently, a dose-response meta-analysis suggested that light and moderate alcohol intake may reduce the risk of T2D, whereas heavy alcohol intake showed inconclusive association (Knott et al., 2015).

TABLE 1 | SNPs–diet interactions increase the risk of type 2 diabetes.

Genes	Polymorphisms	Alleles	Diet interaction	References
FTO	rs9939609	T	Low adherence to Mediterranean diet	Ortega-Azorin et al., 2012
MC4R	rs17782313	T	Low adherence to Mediterranean diet	Ortega-Azorin et al., 2012
TCF7L2	rs7903146	T	High dessert and milk	Ouhaibi-Djellouli et al., 2014
TCF7L2	rs7903146	T	High fiber	Hindy et al., 2012
IRS1	rs2943641	T	Low carbohydrate intake	Ericson et al., 2013
Adiponectin gene	SNP276G > T	G > T	Carbohydrate	Hwang et al., 2013
TCF7L2	rs12255372	T	Coffee	InterAct Consortium, 2016
PGC-1α	rs10517030/ rs10517032	C/T	Low-energy diet	Park et al., 2017
ACE gene	rs4343	I/D	High-fat diet	Schuler et al., 2017

FTO, FTO α-ketoglutarate–dependent dioxygenase; MC4R, melanocortin 4 receptor; TCF7L2, transcription factor 7–like 2.

NUTRITIONAL RECOMMENDATIONS FOR T2D

Prospective studies and clinical trials suggest different nutritional recommendations for the prevention and management of T2D. And they all highlight the importance of dietary habits and lifestyles. For example, calorie restriction and exercise are helpful to reduce the risk of T2D. From the perspective of nutrients, the quality is more important than the quantity. To better improve glucose control in T2D patients, diets rich in fruits, vegetables, legumes, and whole grains are recommended. Low-carbohydrate, low-GI (glycemic index), and high-protein diet patterns will protect us from hyperglycemia incidence. Moreover, moderate consumption of nuts and alcohol is also beneficial (Ley et al., 2014). Different populations or individuals have different foods, dietary habits, and disease susceptibility as well, so nutritional strategies should vary according to their cultures and genetic background.

DIET WITH GENETICS, EPIGENETICS, AND METAGENOMICS INVOLVED IN THE RISK OF T2D

Genetic backgrounds and environments (e.g., high-fat and high-energy dietary habits, and a sedentary lifestyle) are major factors that contribute to high susceptibility of T2D. The impressive progress of next-generation sequencing (NGS) technology has enabled genome sequencing to be obtained in a cheap and reliable large-scale manner, which provides a comprehensive description of genetic variants including single-nucleotide polymorphisms

(SNPs), copy number variations, and other structural variants. Various technologies combined with NGS are developed to explore an increasingly diverse range of biological problems extensively for transcriptome, epigenome, and microbiome. Genetic variants account for only 5–10% for the observed heritability of T2D (Schwenk et al., 2013). Recent advances in precision nutrition have recognized that an individual's diet may increase the disease risk of T2D by interacting with specific gene variants, affecting the expression of genes, modifying the epigenetic features, or altering microbial composition involved in critical metabolic pathways.

Genetic Variants and Diet Interactions

Genetic variants are the most widely studied features in the field of precision nutrition, and the GWASs have generated extensive knowledge about the genetic background of T2D (**Table 1**; Hindy et al., 2012; Ortega-Azorin et al., 2012; Ericson et al., 2013; Hwang et al., 2013; Ouhaibi-Djellouli et al., 2014; InterAct Consortium, 2016; Park et al., 2017; Schuler et al., 2017). For example, The α-ketoglutarate–dependent dioxygenase (FTO) and melanocortin-4 receptor (MC4R) genes were confirmed to be obesity-associated loci, which promotes researchers to study the association of these variants with T2D. GWASs showed these two genes were not significantly associated with diabetes, and conversely, SNP–diet interactions were found to play an important role in the risk of T2D (Ortega-Azorin et al., 2012). FTO rs9939609 and MC4R rs17782313 polymorphisms conferred a higher risk of T2D in subjects with low adherence to the Mediterranean diet. Transcription factor 7–like 2 protein (TCF7L2) was reported to play an important role in the pathogenesis of T2D, and the rs7903146 polymorphism was associated with a high risk of T2D in an Algerian population (Ouhaibi-Djellouli et al., 2014). In addition, the risk was increased in the subjects with both rs7903146 SNP and high dessert and milk intakes, which had higher fasting plasma glucose concentration.

In order to better understand the cumulative effect of known T2D-related genetic variants, genetic risk score (GRS) has been developed. For example, 22 T2D-related SNPs identified by GWAS were chosen, where 15 SNPs affect β-cell function, and 7 SNPs affect insulin response, and the number of risk alleles present for each SNP was summed as a GRS for each individual (Layton et al., 2018). The GRSs were found to be significantly related to the risk of T2D in African Americans. There were also several studies using GRS to examine the effect of SNPs on diet interactions and disease risk. In the Malmö Diet and Cancer cohort (1991–1996) in Sweden (Ericson et al., 2018), GRS and dietary risk score (DRS) were found to be associated with risk of T2D independently, and the individuals with both high GRS and DRS have the highest risk of T2D. However, no interaction was observed between GRS and dietary intakes in terms of disease risk. Likewise, the same observation was reported in the EPIC-InterAct case-cohort study restricted to Mediterranean diet (Langenberg et al., 2014). However, in United States men, a Western dietary pattern, characterized by a high intake of processed meat, red meat, refined cereals, butter, eggs and high-fat dairy products, showed a significant interaction with the GRS based on 10 T2D-associated SNPs, to increase the risk of T2D (Qi et al., 2009).

TABLE 2 | Dietary intakes changing gene expression and epigenetics increase the risk of type 2 diabetes.

Dietary factors	Target genes	Modification	References
Low protein	NR1H3	Low expression	Vo et al., 2013
Low protein	HSD11B1, G6PC	High expression	Vo et al., 2013
Vitamin D deficiency	NFKBIA	Low expression	Zhang et al., 2014
Chromium deficiency	Insulin signaling genes	Low expression	Zhang et al., 2017
Low protein	NR1H3	Histone acetylation decrease	Vo et al., 2013
Vitamin D deficiency	NFKBIA	DNA methylation	Zhang et al., 2014
Chromium deficiency	Insulin signaling genes	DNA methylation	Zhang et al., 2017
Magnesium deficiency	HSD11B2	DNA unmethylation	Takaya et al., 2011
Calcium deficiency	HSD11B1	DNA methylation	Takaya et al., 2013

NR1H3, nuclear receptor subfamily 1 group H member 3; HSD11B1, hydroxysteroid 11-β dehydrogenase 1; G6PC, glucose-6-phosphatase; NFKB1, nuclear factor κB subunit 1; HSD11B2, hydroxysteroid 11-β dehydrogenase 2.

The Effect of Dietary Intakes on Gene Expression and Epigenetic Modification

Besides interacting with genetic background, diet styles have been shown to change transcriptions related to T2D and increase the disease risk (**Table 2**). There are two types of transcriptome studies focusing on the gene expression change response to long-term dietary interventions or differentially expressed transcripts comparing the conditions from different habitual dietary exposures. Dietary intervention studies were usually carried out in rat or mice, and the effects on the metabolism were tested in both maternal and offspring. Low-protein diets in rat model down-regulated the expression of NR1H3 and then increased the expression of hepatic gluconeogenic genes (including G6PC and HSD11B1) and consequently resulted in glucose intolerance in adult offspring (Vo et al., 2013). Vitamin D deficiency in pregnant rat induced the down-regulation of nuclear factor κB inhibitor α (Iκbα) and resulted in insulin resistance in the offspring, which was associated with persistently increased inflammation (Zhang et al., 2014). Chromium was reported to regulate blood glucose first in 1959 (Mertz and Schwarz, 1959), a recent study using a mouse model found that chromium deficiency increased T2D susceptibility by downregulating insulin signaling genes to result in glucose intolerance (Zhang et al., 2017).

Epigenetics, including DNA methylation, histone modification, non-coding RNAs, chromatin structure, and so on, can regulate gene expression without changing the DNA coding sequence. Epigenetics are inheritable and reversible processes and involved in every aspect of life, for example, cell differentiation, embryogenesis, and development. In recent years, researches have recovered that epigenetic changes play

an important role in various diseases including cancers, mental disorders, immune disease, diabetes, and cardiovascular diseases. In several population studies (Chambers et al., 2015; Dayeh et al., 2016; Wahl et al., 2017), DNA methylation markers were reported to be significantly associated with T2D incidence, and the DNA methylation risk score was able to predict the risk of T2D. The environment and lifestyle can directly interact with the genome to modify the epigenetics, and their influence can even be passed to the next generation. In the previous study, low-protein diet decreased the acetylation of histone H3 surrounding NR1H3 promoter to silence its expression and increased the risk of T2D in the offspring (Vo et al., 2013). Iκbα expression was found to be repressed potentially by Iκbα methylation when vitamin D was deficient (Zhang et al., 2014). DNA methylation profiling of the maternal liver tissue with chromium restriction diet revealed hypermethylated genes mainly involved in insulin signaling pathway; these genes were downregulated and consequently promoted T2D (Zhang et al., 2017). Likewise, magnesium and calcium deficiency increased the risk of T2D by inducing DNA methylation aberrations in genes related to glucocorticoid metabolism (Takaya et al., 2011, 2013).

The Effect of Dietary Intakes on Gut Microbiota

Gut microbiome is related to the pathogenesis of most chronic diseases, for example, controlling body weight and regulating insulin resistance. Among the environmental factors contributing to T2D, diet plays an important role through changing the gut microbiome. With the technology advances recently, 16S rRNA gene amplicon sequencing, shotgun metagenomic, and metatranscriptomic sequencing have been well established and widely used for comprehensive mapping of gut microbes. In a recent review (Gurung et al., 2020), the authors summarized 42 observational studies about bacterial microbiome and T2D and reported that five genera (including *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Akkermansia*, and *Roseburia*) were negatively associated with T2D, whereas the genera of *Ruminococcus*, *Fusobacterium*, and *Blautia* were positively associated with T2D. It is known that gut microbiota influences the nutrition absorption, and correspondingly, nutrition modulates the composition of gut microbiota. Several literatures have studied how food intakes change the gut microbiome and then promote T2D. From 59 T2D patients, high-carbohydrate, high-fat, and high-protein diets were found to increase counts of *Clostridium* clusters IV and XI and decreased counts of *Bifidobacterium* species, order *Lactobacillales*, and *Clostridium* cluster IV in gut; therefore, fecal short-chain fatty acid (SCFA) production was decreased subsequently, leading to metabolic disorders, which increased the blood insulin levels and insulin resistance (Yamaguchi et al., 2016).

These scientific advances allow us to predict individual risk by taking into account the genetic, epigenetic information, and dietary habits, thus enabling personalized prevention of the disease by formulating dietary recommendations.

DIET WITH GENETICS, EPIGENETICS, AND METAGENOMICS INVOLVED IN THE INTERVENTION OF T2D

Dietary intervention is an important way to control blood glucose levels in the treatment of T2D. There have been more recognitions that nutrition adjustment for T2D, which mainly aim at adjusting the metabolic disorders (i.e., insulin resistance), has different responses, given the individuals' genetic features. Dietary interventions can also change the expression and epigenetic feature of genes involved in the important metabolic pathway, whereas the expression profiles and epigenetic markers can be used to predict personalized response. Moreover, the gut microbiota compositions can be modulated directly by nutrition during dietary interventions.

SNPs–Diet Interactions Showing Differential Responses to Dietary Intervention

FTO rs1558902 polymorphisms with high-fat diet were reported to improve the insulin sensitivity differently rather than low-fat diet from a randomized weight-loss dietary interventional trial (Zheng et al., 2015). Similarly, food interventions aimed at restricting caloric intake or modifying energy derived from fat, protein, or carbohydrates were screened with several SNPs, showing that different SNPs–diet interactions resulted in varied response in terms of weight loss, fasting insulin, and HOMA-IR (Grau et al., 2010; Qi et al., 2011, 2012, 2015; Xu et al., 2013; Huang T. et al., 2015). In a POUNDS LOST trial (Huang et al., 2016), a 2-year low-protein weight-loss diet for individuals with low diabetes GRS was found to significantly improve β-cell function and insulin resistance, whereas a high-protein diet might be more beneficial for patients with high GRS.

Epigenetic Modification After Dietary Intervention

Epigenetics play an important role in the metabolic disorders contributing to T2D, whereas lifestyle interventions aiming at diet and physical activity can reversely change the epigenetics and metabolic pathways. The current nutritional recommendations for diabetes management mostly aim to achieve modest weight loss and maintenance. There are not much direct studies about dietary intervention for T2D patients; however, several weight loss programs studied the interactions between DNA methylation and diet intervention. In a trial using 27 obese women with an 8-week low-calorie diet to study the interindividual difference (Cordero et al., 2011), good responders with a successful weight loss showed lower methylation of LEP and TNF-α promoter in adipose tissue and improved the lipid profile and fat mass percentage after the dietary intervention. This observation indicates the potential to predict the efficiency of weight loss by dietary intervention using DNA methylation of LEP and TNF-α promoter. Similarly, differential methylation of five regions located in or near AQP9, DUSP22, HIPK3, TNNT1, and TNNI3 genes was discovered between high and low responders to a weight loss intervention (Moleres et al., 2013). Interestingly, a

pilot study reported that DNA methylation patterns of RYR1, TUBA3C, and BDNF in peripheral blood mononuclear cell were changed after weight loss intervention, and the DNA methylation pattern in the successful weight loss maintainers for up to 3 years after intervention was similar to normal-weight individuals rather than obese participants (Huang Y.T. et al., 2015). Therefore, DNA methylation markers might be used to predict body weight maintenance after weight loss.

Modulation of Gut Microbiota After Dietary Intervention

Unhealthy food intakes, e.g., Western diet, might change gut microbiota to increase the risk of T2D; conversely, the gut microbiome can be used as a target for the treatment of T2D. Several studies have shown that dietary intervention can modulate gut microbiota composition to treat T2D (**Table 3**). Deficiency in SCFA production has been associated with T2D (Puddu et al., 2014). A randomized clinical study using fecal shotgun metagenomic sequencing uncovered that high-fiber diet increased the abundance of SCFA-producing microbiota in TD2 patients to alleviate their phenotype (Zhao L. et al., 2018). Similarly, fiber-rich macrobiotic Ma-Pi 2 diet or a recommended control diet for T2D treatment (Candela et al., 2016) was found to have a positive impact on modulating gut microbe dysbiosis, especially recovering the SCFA-producing microbiome such as *Faecalibacterium*, *Roseburia*, *Lachnospira*, *Bacteroides*, and *Akkermansia*. Moreover, the Ma-Pi 2 diet showed the potential to reverse proinflammatory dysbiosis in T2D by counteracting the increase in the proinflammatory groups, such as *Collinsella* and *Streptococcus*. Low-calorie formula diet was proven to have a favorable impact on gut microbiome in a standardized three-phase weight loss program for T2D patients (Taheri et al., 2019). The result showed that all of the participants lost their weight and accompanied by a significant improvement of glucose metabolism indicated by a reduction of HbA_{1c}, fasting glucose, and insulin. Meanwhile, both the phylogenetic diversity and β diversity markedly shifted during the end of the low-calorie formula diet. Based on the epidemiological studies, increased circulating BCAAs are associated with insulin resistance and T2D. A randomized crossover trial performed on T2D patients identified that decreased intake of BCAAs was negatively relevant to postprandial insulin secretion (Karusheva et al., 2019). Meanwhile, the analysis of fecal microbiome showed enrichment in *Bacteroidetes* but decrease of *Firmicutes*.

There were also many researches using animal models to investigate dietary intervention for T2D. A report demonstrated that pumpkin polysaccharide had the ability to ease the phenotype of T2D in rat model induced by high-fat diet and streptozotocin and selectively enriched the butyric acid–producing microbiota in rat gut, which is the potential mechanism (Liu et al., 2018). In a non-obese type 2 diabetic animal model, the pancreatectomized rats were provided diets supplemented with aronia, red ginseng, shiitake mushroom, and nattokinase (AGM) (Yang et al., 2018). After 12 weeks' feed, the experimental group showed enhanced insulin secretion and reduced insulin resistance, as well as improved the gut microbiome dysbiosis. According to traditional Chinese medicine, oil tea containing green tea and ginger has potential to treat various ailments (Lin et al., 2018). Lin et al. (2018) orally gavaged the *db/db* mice with oil tea for 8 weeks and tested FBG, oral glucose tolerance test, and lipid levels. The result showed that oil tea can effectively suppress the blood glucose elevation, and meanwhile the gut microbiota was markedly enriched with Lachnospiraceae. It was also reported that the high-fat diet–fed mice showed reduced insulin resistance after oral gavage red pitaya β-cyanins for 14 weeks (Song et al., 2016). 16S rRNA sequencing analysis found the structure of gut microbiota was modulated especially with the decreased ratio of *Firmicutes* and *Bacteroidetes* and increased relative abundance of *Akkermansia*. In another streptozotocin-induced type 2 diabetic mice model, *Lessonia nigrescens* ethanolic extract was shown to decrease FBG levels (Zhao C. et al., 2018). The gene and protein of PI3K in liver were upregulated, whereas JNK was significantly downregulated. Meanwhile, the gut microbiota was enriched with beneficial

TABLE 3 | Dietary interventions modulate gut microbiota.

Dietary factors	Microbiota modulation	Metabolism	References
Fiber-rich diet	Higher counts of SCFA-producing gut microbiota	Improving SCFA production	Zhao L. et al., 2018
Ma-Pi 2 diet	Higher counts of SCFA-producing microbiota	Improving SCFA production	Candela et al., 2016
Low-calorie diet	Increased gut microbiota diversity, reducing levels of *Collinsella*		Taheri et al., 2019
Branched-chain amino acids reduced diet	Enrichment in *Bacteroidetes* but decrease of *Firmicutes*	Reducing meal-induced insulin secretion	Karusheva et al., 2019
Pumpkin polysaccharide	Enrichment of the butyric acid–producing gut microbiota	Improving SCFA production	Liu et al., 2018
Combination of aronia, red ginseng, shiitake mushroom, and nattokinase	Improving gut microbiome dysbiosis	Enhancing insulin secretion and reducing insulin resistance	Yang et al., 2018
Oil tea	Higher counts of Lachnospiraceae		Lin et al., 2018
Red pitaya β-cyanins	Decreasing the ratio of *Firmicutes* and *Bacteroidetes*, increasing relative abundance of *Akkermansia*	Improving insulin resistance	Song et al., 2016
Lessonia nigrescens ethanolic extract	Enrichment of beneficial bacteria, *Barnesiella*, decrease of *Clostridium* and *Alistipes*	Higher expression of PI3K and lower expression of JNK in liver	Zhao C. et al., 2018

PI3K, phosphatidylinositol 3-kinase; JNK, c-Jun N-terminal kinase.

bacteria, *Barnesiella*, and had less abundances of *Clostridium* and *Alistipes*.

In summary, previous studies have shown that the specific dietary (e.g., high-fiber, low-fat, or low-calorie formula diet) can regulate insulin secretion and resistance through modulating the gut microbiota. Therefore, modification of the gut bacteria composition by dietary intervention might be a feasible method to alleviate the symptom of T2D. However, concrete conclusion remains to be obtained by future well-designed and long-term studies. Especially, more effort is expected to study the role of individual food compounds or nutrients in regulating the metabolism to prevent and treat T2D.

CONCLUSION

Type 2 diabetes is a metabolic disease characterized by insulin resistance and/or abnormal insulin secretion that is caused by multiple factors including genetics, nutrition, and physical activity. Insulin signaling dysregulation in glucose metabolism is the major mechanism contributing to T2D, and the factors involved in this pathway can be targets for prevention and intervention of T2D. Growing evidence suggest the important role of nutrition in developing T2D, and the mechanisms behind are explored, respectively, in terms of five main nutrients, namely, proteins, carbohydrates, fats, vitamins, and minerals. Previous suggestions for management of T2D are usually made based on average population; however, with the advance of precision medicine, precision nutrition has attracted increasing attention in T2D. Genetic predisposition combined with diet specifically influences the risk of developing T2D for individuals, and food intakes change gene expression, epigenetic features, and gut microbiota to characterize individuals' response to prevention and treatment by adjusting dietary patterns. However, precision nutrition is still in its infancy, and the studies performed are not comprehensive and sometimes have contradicted conclusions, possibly due to the limited sample size, varied population, and unstandardized study design. In conclusion, nutrition plays a big role in the prevention and intervention of T2D, and precision nutrition holds promise for future therapeutic strategies.

AUTHOR CONTRIBUTIONS

YG and QL conceived the idea and wrote the manuscript with input from ZH, DS, and QG. YG prepared the figures. QL and ZH prepared the tables. All authors edited and approved the final manuscript.

FUNDING

This review was supported by the National Natural Science Foundation of China (No. 81700750), Guangdong Natural Science Foundation (No. 2019A1515111174), Shenzhen Outbound post-doctoral research funding (CZBSHKYJJ002), the Fundamental Research Funds for the Central Universities, Sun Yat-sen University (19ykpy02), the Basic Research Start-up Project of The Eighth Affiliated Hospital of Sun Yat-sen University (GCCRCYJ022), and The Eighth Affiliated Hospital of Sun Yat-sen University Outstanding Youth Reserve Talent Science Fund (FBJQ2019004).

REFERENCES

Alberti, K. G., and Zimmet, P. Z. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med.* 15, 539–553. doi: 10.1002/(sici)1096-9136(199807)15:7<539::aid-dia668>3.0.co;2-s

Anderson, J. W., Johnstone, B. M., and Cook-Newell, M. E. (1995). Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* 333, 276–282. doi: 10.1056/NEJM199508033330502

Bacanli, M., Dilsiz, S. A., Basaran, N., and Basaran, A. A. (2019). Effects of phytochemicals against diabetes. *Adv. Food Nutr. Res.* 89, 209–238. doi: 10.1016/bs.afnr.2019.02.006

Bruce, C. R., and Febbraio, M. A. (2007). It's what you do with the fat that matters! *Nat. Med.* 13, 1137–1138. doi: 10.1038/nm1007-1137

Candela, M., Biagi, E., Soverini, M., Consolandi, C., Quercia, S., Severgnini, M., et al. (2016). Modulation of gut microbiota dysbioses in type 2 diabetic patients by macrobiotic Ma-Pi 2 diet. *Br. J. Nutr.* 116, 80–93. doi: 10.1017/S0007114516001045

Cefalu, W. T., and Hu, F. B. (2004). Role of chromium in human health and in diabetes. *Diabetes Care* 27, 2741–2751. doi: 10.2337/diacare.27.11.2741

Chambers, J. C., Loh, M., Lehne, B., Drong, A., Kriebel, J., Motta, V., et al. (2015). Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: a nested case-control study. *Lancet Diabetes Endocrinol.* 3, 526–534. doi: 10.1016/S2213-8587(15)00127-8

Chandalia, M., Garg, A., Lutjohann, D., von Bergmann, K., Grundy, S. M., and Brinkley, L. J. (2000). Beneficial effects of high dietary fiber intake in patients with type 2 diabetes mellitus. *N. Engl. J. Med.* 342, 1392–1398. doi: 10.1056/NEJM200005113421903

Chen, M. D., Song, Y. M., and Lin, P. Y. (2000). Zinc effects on hyperglycemia and hypoleptinemia in streptozotocin-induced diabetic mice. *Horm. Metab. Res.* 32, 107–109. doi: 10.1055/s-2007-978600

Chen, Z., Franco, O. H., Lamballais, S., Ikram, M. A., Schoufour, J. D., Muka, T., et al. (2020). Associations of specific dietary protein with longitudinal insulin resistance, prediabetes and type 2 diabetes: the rotterdam study. *Clin. Nutr.* 39, 242–249. doi: 10.1016/j.clnu.2019.01.021

Chou, C. J., Affolter, M., and Kussmann, M. (2012). A nutrigenomics view of protein intake: macronutrient, bioactive peptides, and protein turnover. *Prog. Mol. Biol. Transl. Sci.* 108, 51–74. doi: 10.1016/B978-0-12-398397-8.00003-4

Cohen, N., Halberstam, M., Shlimovich, P., Chang, C. J., Shamoon, H., and Rossetti, L. (1995). Oral vanadyl sulfate improves hepatic and peripheral insulin sensitivity in patients with non-insulin-dependent diabetes mellitus. *J. Clin. Invest.* 95, 2501–2509. doi: 10.1172/JCI117951

Cooper, A. J., Forouhi, N. G., Ye, Z., Buijsse, B., Arriola, L., Balkau, B., et al. (2012). Fruit and vegetable intake and type 2 diabetes: ePIC-InterAct prospective study and meta-analysis. *Eur. J. Clin. Nutr.* 66, 1082–1092. doi: 10.1038/ejcn.2012.85

Cordero, P., Campion, J., Milagro, F. I., Goyenechea, E., Steemburgo, T., Javierre, B. M., et al. (2011). Leptin and TNF-alpha promoter methylation levels measured by MSP could predict the response to a low-calorie diet. *J. Physiol. Biochem.* 67, 463–470. doi: 10.1007/s13105-011-0084-4

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., et al. (2016). Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut* 65, 426–436. doi: 10.1136/gutjnl-2014-308778

Dayeh, T., Tuomi, T., Almgren, P., Perfilyev, A., Jansson, P. A., de Mello, V. D., et al. (2016). DNA methylation of loci within ABCG1 and PHOSPHO1 in blood

DNA is associated with future type 2 diabetes risk. *Epigenetics* 11, 482–488. doi: 10.1080/15592294.2016.1178418

Derosa, G., Catena, G., Raddino, R., Gaudio, G., Maggi, A., D'Angelo, A., et al. (2018). Effects on oral fat load of a nutraceutical combination of fermented red rice, sterol esters and stanols, curcumin, and olive polyphenols: a randomized, placebo controlled trial. *Phytomedicine* 42, 75–82. doi: 10.1016/j.phymed.2018.01.014

Domingo, J. L., and Gomez, M. (2016). Vanadium compounds for the treatment of human diabetes mellitus: a scientific curiosity? A review of thirty years of research. *Food Chem. Toxicol.* 95, 137–141. doi: 10.1016/j.fct.2016.07.005

Du, K., Herzig, S., Kulkarni, R. N., and Montminy, M. (2003). TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science* 300, 1574–1577. doi: 10.1126/science.1079817

Ericson, U., Hindy, G., Drake, I., Schulz, C. A., Brunkwall, L., Hellstrand, S., et al. (2018). Dietary and genetic risk scores and incidence of type 2 diabetes. *Genes Nutr.* 13:13. doi: 10.1186/s12263-018-0599-1

Ericson, U., Rukh, G., Stojkovic, I., Sonestedt, E., Gullberg, B., Wirfält, E., et al. (2013). Sex-specific interactions between the IRS1 polymorphism and intakes of carbohydrates and fat on incident type 2 diabetes. *Am. J. Clin. Nutr.* 97, 208–216. doi: 10.3945/ajcn.112.046474

Evans, R. M., Barish, G. D., and Wang, Y. X. (2004). PPARs and the complex journey to obesity. *Nat. Med.* 10, 355–361. doi: 10.1038/nm1025

Fan, Y., Futawaka, K., Koyama, R., Fukuda, Y., Hayashi, M., Imamoto, M., et al. (2016). Vitamin D3/VDR resists diet-induced obesity by modulating UCP3 expression in muscles. *J. Biomed. Sci.* 23:56. doi: 10.1186/s12929-016-0271-2

Fisher, F. M., Kim, M., Doridot, L., Cunniff, J. C., Parker, T. S., Levine, D. M., et al. (2017). A critical role for ChREBP-mediated FGF21 secretion in hepatic fructose metabolism. *Mol. Metab.* 6, 14–21. doi: 10.1016/j.molmet.2016.11.008

Galmes, S., Serra, F., and Palou, A. (2018). Vitamin E metabolic effects and genetic variants: a challenge for precision nutrition in obesity and associated disturbances. *Nutrients* 10:1919. doi: 10.3390/nu10121919

Gao, D., Griffiths, H. R., and Bailey, C. J. (2009). Oleate protects against palmitate-induced insulin resistance in L6 myotubes. *Br. J. Nutr.* 102, 1557–1563. doi: 10.1017/S0007114509990948

Garbossa, S. G., and Folli, F. (2017). Vitamin D, sub-inflammation and insulin resistance. A window on a potential role for the interaction between bone and glucose metabolism. *Rev. Endocr. Metab. Disord* 18, 243–258. doi: 10.1007/s11154-017-9423-2

Garcia-Perez, I., Posma, J. M., Gibson, R., Chambers, E. S., Hansen, T. H., Vestergaard, H., et al. (2017). Objective assessment of dietary patterns by use of metabolic phenotyping: a randomised, controlled, crossover trial. *Lancet Diabetes Endocrinol.* 5, 184–195. doi: 10.1016/S2213-8587(16)30419-3

Grammatiki, M., Rapti, E., Karras, S., Ajjan, R. A., and Kotsa, K. (2017). Vitamin D and diabetes mellitus: causal or casual association? *Rev. Endocr. Metab. Disord.* 18, 227–241. doi: 10.1007/s11154-016-9403-y

Grau, K., Cauchi, S., Holst, C., Astrup, A., Martinez, J. A., Saris, W. H., et al. (2010). TCF7L2 rs7903146-macronutrient interaction in obese individuals' responses to a 10-wk randomized hypoenergetic diet. *Am. J. Clin. Nutr.* 91, 472–479. doi: 10.3945/ajcn.2009.27947

Gray, B., Swick, J., and Ronnenberg, A. G. (2011). Vitamin E and adiponectin: proposed mechanism for vitamin E-induced improvement in insulin sensitivity. *Nutr. Rev.* 69, 155–161. doi: 10.1111/j.1753-4887.2011.00377.x

Guasch-Ferre, M., Hruby, A., Toledo, E., Clish, C. B., Martinez-Gonzalez, M. A., Salas-Salvado, J., et al. (2016). Metabolomics in prediabetes and diabetes: a systematic review and meta-analysis. *Diabetes Care* 39, 833–846. doi: 10.2337/dc15-2251

Gurung, M., Li, Z., You, H., Rodrigues, R., Jump, D. B., Morgun, A., et al. (2020). Role of gut microbiota in type 2 diabetes pathophysiology. *EBioMedicine* 51:102590. doi: 10.1016/j.ebiom.2019.11.051

Haffner, S. M., Lehto, S., Ronnemaa, T., Pyorala, K., and Laakso, M. (1998). Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N. Engl. J. Med.* 339, 229–234. doi: 10.1056/NEJM199807233390404

Hao, S., Sharp, J. W., Ross-Inta, C. M., McDaniel, B. J., Anthony, T. G., Wek, R. C., et al. (2005). Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* 307, 1776–1778. doi: 10.1126/science.1104882

Haro, D., Marrero, P. F., and Relat, J. (2019). Nutritional Regulation of Gene Expression: carbohydrate-, Fat- and Amino Acid-Dependent Modulation of Transcriptional Activity. *Int. J. Mol. Sci.* 20:1386. doi: 10.3390/ijms20061386

Hindy, G., Sonestedt, E., Ericson, U., Jing, X. J., Zhou, Y., Hansson, O., et al. (2012). Role of TCF7L2 risk variant and dietary fibre intake on incident type 2 diabetes. *Diabetologia* 55, 2646–2654. doi: 10.1007/s00125-012-2634-x

Huang, T., Huang, J., Qi, Q., Li, Y., Bray, G. A., Rood, J., et al. (2015). PCSK7 genotype modifies effect of a weight-loss diet on 2-year changes of insulin resistance: the POUNDS LOST trial. *Diabetes Care* 38, 439–444. doi: 10.2337/dc14-0473

Huang, T., Ley, S. H., Zheng, Y., Wang, T., Bray, G. A., Sacks, F. M., et al. (2016). Genetic susceptibility to diabetes and long-term improvement of insulin resistance and beta cell function during weight loss: the Preventing Overweight Using Novel Dietary Strategies (POUNDS LOST) trial. *Am. J. Clin. Nutr.* 104, 198–204. doi: 10.3945/ajcn.115.121186

Huang, Y. T., Maccani, J. Z. J., Hawley, N. L., Wing, R. R., Kelsey, K. T., and McCaffery, J. M. (2015). Epigenetic patterns in successful weight loss maintainers: a pilot study. *Int. J. Obes.* 39, 865–868. doi: 10.1038/ijo.2014.213

Hubbard, R., Kosch, C. L., Sanchez, A., Sabate, J., Berk, L., and Shavlik, G. (1989). Effect of dietary protein on serum insulin and glucagon levels in hyper- and normocholesterolemic men. *Atherosclerosis* 76, 55–61. doi: 10.1016/0021-9150(89)90193-7

Hwang, J. Y., Park, J. E., Choi, Y. J., Huh, K. B., Chang, N., and Kim, W. Y. (2013). Carbohydrate intake interacts with SNP276G>T polymorphism in the adiponectin gene to affect fasting blood glucose, HbA1C, and HDL cholesterol in Korean patients with type 2 diabetes. *J. Am. Coll. Nutr.* 32, 143–150. doi: 10.1080/07315724.2013.791795

InterAct Consortium (2016). Investigation of gene-diet interactions in the incretin system and risk of type 2 diabetes: the EPIC-InterAct study. *Diabetologia* 59, 2613–2621. doi: 10.1007/s00125-016-4090-5

Iritani, N., Sugimoto, T., Fukuda, H., Komiya, M., and Ikeda, H. (1997). Dietary soybean protein increases insulin receptor gene expression in Wistar fatty rats when dietary polyunsaturated fatty acid level is low. *J. Nutr.* 127, 1077–1083. doi: 10.1093/jn/127.6.1077

Kahn, S. E., Hull, R. L., and Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444, 840–846. doi: 10.1038/nature05482

Karusheva, Y., Koessler, T., Strassburger, K., Markgraf, D., Mastrototaro, L., Jelenik, T., et al. (2019). Short-term dietary reduction of branched-chain amino acids reduces meal-induced insulin secretion and modifies microbiome composition in type 2 diabetes: a randomized controlled crossover trial. *Am. J. Clin. Nutr.* 110, 1098–1107. doi: 10.1093/ajcn/nqz191

Kimball, S. R., and Jefferson, L. S. (2006). New functions for amino acids: effects on gene transcription and translation. *Am. J. Clin. Nutr.* 83, 500S–507S. doi: 10.1093/ajcn/83.2.500S

King, H., Aubert, R. E., and Herman, W. H. (1998). Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 21, 1414–1431. doi: 10.2337/diacare.21.9.1414

Knott, C., Bell, S., and Britton, A. (2015). Alcohol consumption and the risk of type 2 diabetes . a systematic review and dose-response meta-analysis of more than 1.9 million individuals from 38 observational studies. *Diabetes Care* 38, 1804–1812. doi: 10.2337/dc15-0710

Koska, J., Stefan, N., Permana, P. A., Weyer, C., Sonoda, M., Bogardus, C., et al. (2008). Increased fat accumulation in liver may link insulin resistance with subcutaneous abdominal adipocyte enlargement, visceral adiposity, and hypoadiponectinemia in obese individuals. *Am. J. Clin. Nutr.* 87, 295–302. doi: 10.1093/ajcn/87.2.295

Lackey, D. E., and Olefsky, J. M. (2016). Regulation of metabolism by the innate immune system. *Nat. Rev. Endocrinol.* 12, 15–28. doi: 10.1038/nrendo.2015.189

Lai, K. K., Kolippakkam, D., and Beretta, L. (2008). Comprehensive and quantitative proteome profiling of the mouse liver and plasma. *Hepatology* 47, 1043–1051. doi: 10.1002/hep.22123

Landrier, J. F., Gouranton, E., El Yazidi, C., Malezet, C., Balaguer, P., Borel, P., et al. (2009). Adiponectin expression is induced by vitamin E via a peroxisome proliferator-activated receptor gamma-dependent mechanism. *Endocrinology* 150, 5318–5325. doi: 10.1210/en.2009-0506

Langenberg, C., Sharp, S. J., Franks, P. W., Scott, R. A., Deloukas, P., Forouhi, N. G., et al. (2014). Gene-lifestyle interaction and type 2 diabetes: the EPIC interact case-cohort study. *PLoS Med.* 11:e1001647. doi: 10.1371/journal.pmed.1001647

Lavigne, C., Marette, A., and Jacques, H. (2000). Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. *Am. J. Physiol. Endocrinol. Metab.* 278, E491–E500. doi: 10.1152/ajpendo.2000.278.3.E491

Lavigne, C., Tremblay, F., Asselin, G., Jacques, H., and Marette, A. (2001). Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. *Am. J. Physiol. Endocrinol. Metab.* 281, E62–E71. doi: 10.1152/ajpendo.2001.281.1.E62

Layton, J., Li, X., Shen, C., de Groot, M., Lange, L., Correa, A., et al. (2018). Type 2 diabetes genetic risk scores are associated with increased type 2 diabetes risk among african americans by cardiometabolic status. *Clin. Med. Insights Endocrinol. Diabetes* 11:1179551417748942. doi: 10.1177/1179551417748942

Lee, H. J., and Cha, J. Y. (2018). Recent insights into the role of ChREBP in intestinal fructose absorption and metabolism. *BMB Rep.* 51, 429–436. doi: 10.5483/bmbrep.2018.51.9.197

Ley, S. H., Hamdy, O., Mohan, V., and Hu, F. B. (2014). Prevention and management of type 2 diabetes: dietary components and nutritional strategies. *Lancet* 383, 1999–2007. doi: 10.1016/S0140-6736(14)60613-9

Lin, R., He, X., Chen, H., He, Q., Yao, Z., Li, Y., et al. (2018). Oil tea improves glucose and lipid levels and alters gut microbiota in type 2 diabetic mice. *Nutr. Res.* 57, 67–77. doi: 10.1016/j.nutres.2018.05.004

Linn, T., Santosa, B., Gronemeyer, D., Aygen, S., Scholz, N., Busch, M., et al. (2000). Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia* 43, 1257–1265. doi: 10.1007/s001250051521

Liu, G., Liang, L., Yu, G., and Li, Q. (2018). Pumpkin polysaccharide modifies the gut microbiota during alleviation of type 2 diabetes in rats. *Int. J. Biol. Macromol.* 115, 711–717. doi: 10.1016/j.ijbiomac.2018.04.127

Lynch, C. J., and Adams, S. H. (2014). Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat. Rev. Endocrinol.* 10, 723–736. doi: 10.1038/nrendo.2014.171

Maestro, B., Campion, J., Davila, N., and Calle, C. (2000). Stimulation by 1,25-dihydroxyvitamin D3 of insulin receptor expression and insulin responsiveness for glucose transport in U-937 human promonocytic cells. *Endocr. J.* 47, 383–391. doi: 10.1507/endocrj.47.383

Malik, V. S., Willett, W. C., and Hu, F. B. (2013). Global obesity: trends, risk factors and policy implications. *Nat. Rev. Endocrinol.* 9, 13–27. doi: 10.1038/nrendo.2012.199

Manore, M. M., Larson-Meyer, D. E., Lindsay, A. R., Hongu, N., and Houtkooper, L. (2017). Dynamic energy balance: an integrated framework for discussing diet and physical activity in obesity prevention-is it more than eating less and exercising more?. *Nutrients* 9:905. doi: 10.3390/nu9080905

Meex, R. C. R., and Watt, M. J. (2017). Hepatokines linking nonalcoholic fatty liver disease and insulin resistance. *Nat. Rev. Endocrinol.* 13, 509–520. doi: 10.1038/nrendo.2017.56

Meikle, P. J., and Summers, S. A. (2017). Sphingolipids and phospholipids in insulin resistance and related metabolic disorders. *Nat. Rev. Endocrinol.* 13, 79–91. doi: 10.1038/nrendo.2016.169

Mertz, W., and Schwarz, K. (1959). Relation of glucose tolerance factor to impaired intravenous glucose tolerance of rats on stock diets. *Am. J. Physiol.* 196, 614–618. doi: 10.1152/ajplegacy.1959.196.3.614

Mohsenpour, M. A., Kaseb, F., Nazemian, R., Mozaffari-Khosravi, H., Fallahzadeh, H., and Salehi-Abargouei, A. (2019). The effect of a new mixture of sugar and sugar-alcohols compared to sucrose and glucose on blood glucose increase and the possible adverse reactions: a phase I double-blind, three-way randomized cross-over clinical trial. *Endocrinol. Diabetes Nutr.* 66, 647–653. doi: 10.1016/j.endinu.2018.12.008

Moleres, A., Campion, J., Milagro, F. I., Marcos, A., Campoy, C., Garagorri, J. M., et al. (2013). Differential DNA methylation patterns between high and low responders to a weight loss intervention in overweight or obese adolescents: the EVASYON study. *FASEB J.* 27, 2504–2512. doi: 10.1096/fj.12-215566

Moller, D. E. (2001). New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* 414, 821–827. doi: 10.1038/414821a

Muscogiuri, G., Altieri, B., Annweiler, C., Balercia, G., Pal, H. B., Boucher, B. J., et al. (2017). Vitamin D and chronic diseases: the current state of the art. *Arch. Toxicol.* 91, 97–107. doi: 10.1007/s00204-016-1804-x

Ogawa-Wong, A. N., Berry, M. J., and Seale, L. A. (2016). Selenium and metabolic disorders: an emphasis on type 2 diabetes risk. *Nutrients* 8:80. doi: 10.3390/nu8020080

Ortega-Azorin, C., Sorli, J. V., Asensio, E. M., Coltell, O., Martinez-Gonzalez, M. A., Salas-Salvado, J., et al. (2012). Associations of the FTO rs9939609 and the MC4R rs17782313 polymorphisms with type 2 diabetes are modulated by diet, being higher when adherence to the Mediterranean diet pattern is low. *Cardiovasc Diabetol* 11:137. doi: 10.1186/1475-2840-11-137

Ouhaibi-Djellouli, H., Mediene-Benchekor, S., Lardjam-Hetraf, S. A., Hamani-Medjaoui, I., Meroufel, D. N., Boulenouar, H., et al. (2014). The TCF7L2 rs7903146 polymorphism, dietary intakes and type 2 diabetes risk in an Algerian population. *BMC Genet.* 15:134. doi: 10.1186/s12863-014-0134-3

Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., et al. (2004). Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306, 457–461. doi: 10.1126/science.1103160

Panchal, S. K., Wanyonyi, S., and Brown, L. (2017). Selenium, vanadium, and chromium as micronutrients to improve metabolic syndrome. *Curr. Hypertens. Rep.* 19:10. doi: 10.1007/s11906-017-0701-x

Paolisso, G., D'Amore, A., Giugliano, D., Ceriello, A., Varricchio, M., and D'Onofrio, F. (1993). Pharmacologic doses of vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetic patients. *Am. J. Clin. Nutr.* 57, 650–656. doi: 10.1093/ajcn/57.5.650

Park, S., Kim, B. C., and Kang, S. (2017). Interaction effect of PGC-1α rs10517030 variants and energy intake in the risk of type 2 diabetes in middle-aged adults. *Eur. J. Clin. Nutr.* 71, 1442–1448. doi: 10.1038/ejcn.2017.68

Perry, R. J., Samuel, V. T., Petersen, K. F., and Shulman, G. I. (2014). The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. *Nature* 510, 84–91. doi: 10.1038/nature13478

Pessoa, J. C., and Tomaz, I. (2010). Transport of therapeutic vanadium and ruthenium complexes by blood plasma components. *Curr. Med. Chem.* 17, 3701–3738. doi: 10.2174/092986710793213742

Petersen, M. C., and Shulman, G. I. (2018). Mechanisms of insulin action and insulin resistance. *Physiol. Rev.* 98, 2133–2223. doi: 10.1152/physrev.00063.2017

Potier, M., Darcel, N., and Tome, D. (2009). Protein, amino acids and the control of food intake. *Curr. Opin. Clin. Nutr. Metab. Care* 12, 54–58. doi: 10.1097/MCO.0b013e32831b9e01

Puddu, A., Sanguineti, R., Montecucco, F., and Viviani, G. L. (2014). Evidence for the gut microbiota short-chain fatty acids as key pathophysiological molecules improving diabetes. *Mediators Inflamm.* 2014:162021. doi: 10.1155/2014/162021

Qi, L., Cornelis, M. C., Zhang, C., van Dam, R. M., and Hu, F. B. (2009). Genetic predisposition, Western dietary pattern, and the risk of type 2 diabetes in men. *Am. J. Clin. Nutr.* 89, 1453–1458. doi: 10.3945/ajcn.2008.27249

Qi, Q., Bray, G. A., Hu, F. B., Sacks, F. M., and Qi, L. (2012). Weight-loss diets modify glucose-dependent insulinotropic polypeptide receptor rs2287019 genotype effects on changes in body weight, fasting glucose, and insulin resistance: the preventing overweight using novel dietary strategies trial. *Am. J. Clin. Nutr.* 95, 506–513. doi: 10.3945/ajcn.111.025270

Qi, Q., Bray, G. A., Smith, S. R., Hu, F. B., Sacks, F. M., and Qi, L. (2011). Insulin receptor substrate 1 gene variation modifies insulin resistance response to weight-loss diets in a 2-year randomized trial: the Preventing Overweight Using Novel Dietary Strategies (POUNDS LOST) trial. *Circulation* 124, 563–571. doi: 10.1161/CIRCULATIONAHA.111.025767

Qi, Q., Zheng, Y., Huang, T., Rood, J., Bray, G. A., Sacks, F. M., et al. (2015). Vitamin D metabolism-related genetic variants, dietary protein intake and improvement of insulin resistance in a 2 year weight-loss trial: pOUNDS Lost. *Diabetologia* 58, 2791–2799. doi: 10.1007/s00125-015-3750-1

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490, 55–60. doi: 10.1038/nature11450

Rayman, M. P., Infante, H. G., and Sargent, M. (2008). Food-chain selenium and human health: spotlight on speciation. *Br. J. Nutr.* 100, 238–253. doi: 10.1017/S0007114508922522

Rico-Campa, A., Martinez-Gonzalez, M. A., Alvarez-Alvarez, I., Mendonca, R. D., de la Fuente-Arrillaga, C., Gomez-Donoso, C., et al. (2019). Association between consumption of ultra-processed foods and all cause mortality: sUN prospective cohort study. *BMJ* 365:l1949. doi: 10.1136/bmj.l1949

Rines, A. K., Sharabi, K., Tavares, C. D., and Puigserver, P. (2016). Targeting hepatic glucose metabolism in the treatment of type 2 diabetes. *Nat. Rev. Drug Discov.* 15, 786–804. doi: 10.1038/nrd.2016.151

Russell, W. R., Baka, A., Bjorck, I., Delzenne, N., Gao, D., Griffiths, H. R., et al. (2016). Impact of diet composition on blood glucose regulation. *Crit. Rev. Food Sci. Nutr.* 56, 541–590. doi: 10.1080/10408398.2013.792772

Saltiel, A. R., and Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414, 799–806. doi: 10.1038/414799a

Samuel, V. T., and Shulman, G. I. (2012). Mechanisms for insulin resistance: common threads and missing links. *Cell* 148, 852–871. doi: 10.1016/j.cell.2012.02.017

San-Cristobal, R., Navas-Carretero, S., Celis-Morales, C., Brennan, L., Walsh, M., Lovegrove, J. A., et al. (2015). Analysis of dietary pattern impact on weight status for personalised nutrition through on-line advice: the food4Me spanish cohort. *Nutrients* 7, 9523–9537. doi: 10.3390/nu7115482

Schuler, R., Osterhoff, M. A., Frahnow, T., Mohlig, M., Spranger, J., Stefanovski, D., et al. (2017). Dietary fat intake modulates effects of a frequent ace gene variant on glucose tolerance with association to type 2 diabetes. *Sci. Rep.* 7:9234. doi: 10.1038/s41598-017-08300-7

Schwartz, M. W., and Porte, D. Jr. (2005). Diabetes, obesity, and the brain. *Science* 307, 375–379. doi: 10.1126/science.1104344

Schwenk, R. W., Vogel, H., and Schurmann, A. (2013). Genetic and epigenetic control of metabolic health. *Mol. Metab.* 2, 337–347. doi: 10.1016/j.molmet.2013.09.002

Shenkin, A. (2006). Micronutrients in health and disease. *Postgrad. Med. J.* 82, 559–567. doi: 10.1136/pgmj.2006.047670

Song, H., Chu, Q., Yan, F., Yang, Y., Han, W., and Zheng, X. (2016). Red pitaya betacyanins protects from diet-induced obesity, liver steatosis and insulin resistance in association with modulation of gut microbiota in mice. *J. Gastroenterol. Hepatol.* 31, 1462–1469. doi: 10.1111/jgh.13278

Spiller, G. A., Jensen, C. D., Pattison, T. S., Chuck, C. S., Whittam, J. H., and Scala, J. (1987). Effect of protein dose on serum glucose and insulin response to sugars. *Am. J. Clin. Nutr.* 46, 474–480. doi: 10.1093/ajcn/46.3.474

Sun, M., Liu, G., and Wu, Q. (2013). Speciation of organic and inorganic selenium in selenium-enriched rice by graphite furnace atomic absorption spectrometry after cloud point extraction. *Food Chem.* 141, 66–71. doi: 10.1016/j.foodchem.2013.03.002

Taheri, S., Frost, F., Storck, L. J., Kacprowski, T., Gärtner, S., Rühlemann, M., et al. (2019). A structured weight loss program increases gut microbiota phylogenetic diversity and reduces levels of Collinsella in obese type 2 diabetics: a pilot study. *PLoS One* 14:e0219489. doi: 10.1371/journal.pone.0219489

Tajima, R., Kodama, S., Hirata, M., Horikawa, C., Fujihara, K., Yachi, Y., et al. (2014). High cholesterol intake is associated with elevated risk of type 2 diabetes mellitus - a meta-analysis. *Clin. Nutr.* 33, 946–950. doi: 10.1016/j.clnu.2014.03.001

Takaya, J., Iharada, A., Okihana, H., and Kaneko, K. (2011). Magnesium deficiency in pregnant rats alters methylation of specific cytosines in the hepatic hydroxysteroid dehydrogenase-2 promoter of the offspring. *Epigenetics* 6, 573–578. doi: 10.4161/epi.6.5.15220

Takaya, J., Iharada, A., Okihana, H., and Kaneko, K. (2013). A calcium-deficient diet in pregnant, nursing rats induces hypomethylation of specific cytosines in the 11beta-hydroxysteroid dehydrogenase-1 promoter in pup liver. *Nutr. Res.* 33, 961–970. doi: 10.1016/j.nutres.2013.07.015

Talaei, A., Mohamadi, M., and Adgi, Z. (2013). The effect of vitamin D on insulin resistance in patients with type 2 diabetes. *Diabetol. Metab. Syndr.* 5:8. doi: 10.1186/1758-5996-5-8

Tomlinson, B., Chan, P., and Lam, C. W. K. (2020). Postprandial hyperlipidemia as a risk factor in patients with type 2 diabetes. *Expert Rev Endocrinol Metab* 15, 147–157. doi: 10.1080/17446651.2020.1750949

Tremblay, F., Lavigne, C., Jacques, H., and Marette, A. (2003). Dietary cod protein restores insulin-induced activation of phosphatidylinositol 3-kinase/Akt and GLUT4 translocation to the T-tubules in skeletal muscle of high-fat-fed obese rats. *Diabetes* 52, 29–37. doi: 10.2337/diabetes.52.1.29

Tremblay, F., Lavigne, C., Jacques, H., and Marette, A. (2007). Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu. Rev. Nutr.* 27, 293–310. doi: 10.1146/annurev.nutr.25.050304.092545

Tremblay, F., and Marette, A. (2001). Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J. Biol. Chem.* 276, 38052–38060. doi: 10.1074/jbc.M106703200

Um, S. H., D'Alessio, D., and Thomas, G. (2006). Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1. S6K1. *Cell Metab.* 3, 393–402. doi: 10.1016/j.cmet.2006.05.003

Vary, T. C., and Lynch, C. J. (2007). Nutrient signaling components controlling protein synthesis in striated muscle. *J. Nutr.* 137, 1835–1843. doi: 10.1093/jn/137.8.1835

Vo, T. X., Revesz, A., Sohi, G., Ma, N., and Hardy, D. B. (2013). Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver X receptor. *J. Endocrinol.* 218, 85–97. doi: 10.1530/JOE-13-0055

von Post-Skagegard, M., Vessby, B., and Karlstrom, B. (2006). Glucose and insulin responses in healthy women after intake of composite meals containing cod-, milk-, and soy protein. *Eur. J. Clin. Nutr.* 60, 949–954. doi: 10.1038/sj.ejcn.1602404

Wa, E. L. I, Naser, A., Taleb, M. H., and Abutair, A. S. (2018). The effects of oral magnesium supplementation on glycemic response among type 2 diabetes patients. *Nutrients* 11;44. doi: 10.3390/nu11010044

Wahl, S., Drong, A., Lehne, B., Loh, M., Scott, W. R., Kunze, S., et al. (2017). Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. *Nature* 541, 81–86. doi: 10.1038/nature20784

Wheeler, M. L., and Pi-Sunyer, F. X. (2008). Carbohydrate issues: type and amount. *J. Am. Diet. Assoc.* 108(4 Suppl. 1), S34–S39. doi: 10.1016/j.jada.2008.01.024

Xu, M., Qi, Q., Liang, J., Bray, G. A., Hu, F. B., Sacks, F. M., et al. (2013). Genetic determinant for amino acid metabolites and changes in body weight and insulin resistance in response to weight-loss diets: the preventing overweight using novel dietary strategies (POUNDS LOST) trial. *Circulation* 127, 1283–1289. doi: 10.1161/CIRCULATIONAHA.112.000586

Yamaguchi, Y., Adachi, K., Sugiyama, T., Shimozato, A., Ebi, M., Ogasawara, N., et al. (2016). Association of Intestinal Microbiota with Metabolic Markers and Dietary Habits in Patients with Type 2 Diabetes. *Digestion* 94, 66–72. doi: 10.1159/000447690

Yamashita, H., Takenoshita, M., Sakurai, M., Bruick, R. K., Henzel, W. J., Shillinglaw, W., et al. (2001). A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9116–9121. doi: 10.1073/pnas.161284298

Yang, H., Kim, M., Kwon, D., Kim, D., Zhang, T., Ha, C., et al. (2018). Combination of aronia, red ginseng, shiitake mushroom and nattokinase potentiated insulin secretion and reduced insulin resistance with improving gut microbiome dysbiosis in insulin deficient type 2 diabetic rats. *Nutrients* 10:948. doi: 10.3390/nu10070948

Zhang, B. B., Zhou, G., and Li, C. (2009). AMPK: an emerging drug target for diabetes and the metabolic syndrome. *Cell Metab.* 9, 407–416. doi: 10.1016/j.cmet.2009.03.012

Zhang, H., Chu, X., Huang, Y., Li, G., Wang, Y., Li, Y., et al. (2014). Maternal vitamin D deficiency during pregnancy results in insulin resistance in rat offspring, which is associated with inflammation and Ikappabalpha methylation. *Diabetologia* 57, 2165–2172. doi: 10.1007/s00125-014-3316-7

Zhang, Q., Sun, X., Xiao, X., Zheng, J., Li, M., Yu, M., et al. (2017). Dietary chromium restriction of pregnant mice changes the methylation status of hepatic genes involved with insulin signaling in adult male offspring. *PLoS One* 12:e0169889. doi: 10.1371/journal.pone.0169889

Zhao, C., Yang, C., Chen, M., Lv, X., Liu, B., Yi, L., et al. (2018). Regulatory efficacy of brown seaweed lessonia nigrescens extract on the gene expression profile and intestinal microflora in type 2 diabetic mice. *Mol. Nutr. Food Res.* 62:1700730. doi: 10.1002/mnfr.201700730

Zhao, C., Yang, C., Wai, S. T. C., Zhang, Y., Paoli, P., Wu, Y., et al. (2019). Regulation of glucose metabolism by bioactive phytochemicals for the management of type 2 diabetes mellitus. *Crit. Rev. Food Sci. Nutr.* 59, 830–847. doi: 10.1080/10408398.2018.1501658

Zhao, L., Zhang, F., Ding, X., Wu, G., Lam, Y. Y., Wang, X., et al. (2018). Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. *Science* 359, 1151–1156. doi: 10.1126/science.aao5774

Zhao, Y., Gao, P., Sun, F., Li, Q., Chen, J., Yu, H., et al. (2016). sodium intake regulates glucose homeostasis through the PPARdelta/adiponectin-mediated SGLT2 pathway. *Cell Metab.* 23, 699–711. doi: 10.1016/j.cmet.2016.02.019

Zheng, Y., Huang, T., Zhang, X., Rood, J., Bray, G. A., Sacks, F. M., et al. (2015). dietary fat modifies the effects of FTO genotype on changes in insulin sensitivity. *J. Nutr.* 145, 977–982. doi: 10.3945/jn.115.210005

Zhou, J., Huang, K., and Lei, X. G. (2013). Selenium and diabetes–evidence from animal studies. *Free Radic. Biol. Med.* 65, 1548–1556. doi: 10.1016/j.freeradbiomed.2013.07.012

Zimmet, P., Alberti, K. G., and Shaw, J. (2001). Global and societal implications of the diabetes epidemic. *Nature* 414, 782–787. doi: 10.1038/414782a

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 Bioengineering and Biotechnology

REVIEW
published: 05 November 2020
doi: 10.3389/fbioe.2020.590869

Exploring the Preventive Effect and Mechanism of Senile Sarcopenia Based on "Gut–Muscle Axis"

*Xiaoshan Liao†, Mengting Wu†, Yuting Hao and Hong Deng**

Guangdong Provincial Key Laboratory of Tropical Disease Research, Department of Nutrition and Food Hygiene, School of Public Health, Southern Medical University, Guangzhou, China

Age-related sarcopenia probably leads to chronic systemic inflammation and plays a vital role in the development of the complications of the disease. Gut microbiota, an environmental factor, is the medium of nutritional support to muscle cells, having significant impact on sarcopenia. Consequently, a significant amount of studies explored and showed the presence of gut microbiome–muscle axis (gut–muscle axis for short), which was possibly considered as the disease interventional target of age-related sarcopenia. However, a variety of nutrients probably affect the changes of the gut–muscle axis so as to affect the healthy balance of skeletal muscle. Therefore, it is necessary to study the mechanism of intestinal–muscle axis, and nutrients play a role in the treatment of senile sarcopenia through this mechanism. This review summarizes the available literature on mechanisms and specific pathways of gut–muscle axis and discusses the potential role and therapeutic feasibility of gut microbiota in age-related sarcopenia to understand the development of age-related sarcopenia and figure out the novel perspective of the potential therapeutic interventional targets.

Keywords: age-related sarcopenia, gut microbiota, gut-muscle axis, mechanisms, therapy

OPEN ACCESS

Edited by:
Yi Cao,
Xiangtan University, China

Reviewed by:
Min Gu,
Louisiana State University, United States
Yongsheng Chen,
Jinan University, China
Jan Jacek Kaczor,
Gdansk University of Physical Education and Sport, Poland

***Correspondence:**
Hong Deng
hongd@smu.edu.cn;
echoliaoxiaoshan@163.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *03 August 2020*
Accepted: *09 October 2020*
Published: *05 November 2020*

Citation:
Liao X, Wu M, Hao Y and Deng H (2020) Exploring the Preventive Effect and Mechanism of Senile Sarcopenia Based on "Gut–Muscle Axis". Front. Bioeng. Biotechnol. 8:590869. doi: 10.3389/fbioe.2020.590869

INTRODUCTION

With the global improvement of life quality and medical care, average human life expectancy has be prolonged dramatically and it will prolong continuously (Kontis et al., 2017; Ben-Haim et al., 2018; Partridge et al., 2018). However, age-related diseases are thriving (Carmona and Michan, 2016), which means longer human lives result in a global burden of late-life disease (Partridge et al., 2018). It is imperative to obtain more understanding about the aging process, since healthy aging has become a popular topic (Lu et al., 2019). In recent years, more and more studies suggest that the muscle mass and function significantly and inexorably decline with age (Blau et al., 2015; Curtis et al., 2015; Brook et al., 2016; Cartee et al., 2016; Giallauria et al., 2016; Francis et al., 2017; Tezze et al., 2017; Tieland et al., 2018; Wilkinson et al., 2018; Larsson et al., 2019), which is termed as *sarcopenia* (Larsson et al., 2019). Sarcopenia is strictly defined as an age-related syndrome, which is characterized by progressive and generalized loss of skeletal muscle mass and strength. Moreover, sarcopenic obesity, a new concept that emerged recently, reflects a combination of sarcopenia and obesity (Choi, 2016). Sarcopenia may reduce mobility lead to fall-related injuries, such as bone fracture, diminish health-related quality of life and lead to premature death (Curtis et al., 2015; Shaw et al., 2017; Larsson et al., 2019). A systematic review and meta-analysis study shows that a substantial proportion of the old people have sarcopenia, and the overall estimate of prevalence in both men

and women is 10% (Shafiee et al., 2017). The cause of sarcopenia or sarcopenia obesity is complex. The lack of exercise, age-related decreases in hormone concentrations and low vitamin D status are considered as the risk factors inducing sarcopenia presently (Dhillon and Hasni, 2017; Petroni et al., 2019). Interestingly, an increasing number of studies suggest gut microbiota is closely associated with sarcopenia in aging (Ticinesi et al., 2019b).

It is not difficult to find that the relation between the gut microbiota and human health is being increasingly recognized, so the concept that the human gut microbiota is involved in multiple interactions influencing host health during the host's entire lifespan is well known. Notably, the composition of human gut microbiota changes with age, and some transition points and patterns in the changes of composition in gut microbiota with age have been indicated (Odamaki et al., 2016). A separate phylogenetic study finds out the core intestinal flora of people in different age groups: young people (22–48 years old), elderly (65–75 years old), centenarians (99–104 years old), and half-centenarians (105–109 years old; Biagi et al., 2016). The core intestinal flora is composed of *Bacteroides*, Rumen bacteria, and Spirulina, implying unhealthy intestine status probably due to changes of composition of the core intestinal flora or the risk of unhealthy intestines with age. Actually, as the biological age increases, the overall abundance of gut microbiome decreases, with the addition of some microbial classifications related to unhealthy aging (Kim and Jazwinski, 2018). Compared with younger individuals, the frail elderly have a greater change in the intestinal flora, with an observed change that dominated population of *Bacteroides* in the microbial community (Mangiola et al., 2018). The transition between the adults and the elderly is mainly characterized by a decrease in bacterial diversity, the transition between the adults and the elderly is mainly characterized by a decrease in bacterial diversity and a decrease in bifidobacteria and an increase in *Clostridium*, *Lactobacillus*, *Enterobacteriaceae*, and *Enterococcus* (Mitsuoka, 2014). A study shows that the fecal samples of individuals from 0 to 104 years old were analyzed by 16S rRNA sequencing, whose results support that the gut microbiome changes with age (Odamaki et al., 2016).

Specifically speaking, the composition of gut microbiota is proved to be dynamic throughout the lifespan attributing to the factors such as dietary changes, antibiotic intake, age, disease, and so on (Manuel Marti et al., 2017). The host and the microbiota are extremely related, owing to the functions mediated or affected by bacteria of host. The regulatory effect of the microbiota for the balance of body health includes fiber catabolism, host immune system regulation and resistance to pathogens, vitamin and amino acid biosynthesis, xenobiotic detoxification, etc. (Kundu et al., 2017). Because of the regulatory effect of gut microbiome on human metabolism and immunology, the gut microbiome is considered as a possible determinant of healthy aging (Claesson et al., 2012; Candela et al., 2014). The maintenance of host microbial homeostasis probably counteracts inflammation (Biagi et al., 2010), intestinal barrier permeability (Nicoletti, 2015), and so on. However, the composition of gut microbiota changes rapidly again, leading to gastrointestinal dysbiosis, which is associated with increasing biological age (Maffei et al., 2017; Salazar et al., 2017). An increasing number of evidence suggest that dysbiosis in the microbiome is associated with a variety of diseases, including atherosclerosis, hypertension, obesity, diabetes (types 1 and 2), cancer, sarcopenia, and so on (Lloyd-Price et al., 2016; Cigarran Guldris et al., 2017; Lau et al., 2017; Li et al., 2017; Rajagopala et al., 2017; Tang et al., 2017; Weiss and Hennet, 2017; Picca et al., 2018a; Durazzo et al., 2019).

Interestingly, although the dysbiosis in the microbiome can induce a lot of diseases, the health effects of regulating gut microbiota in the body obtained more and more attention in recent years. As for sarcopenia, it is remarkable that an increasing number of studies suggest the presence of gut–muscle axis, indicating the gut microbiome may affect the health of skeletal muscle and vice versa (Picca et al., 2018a). A Chinese cohort survey finds that different dietary habits attributed to different geographical locations lead to significant differences in the composition of the gut microbiome (Zhang et al., 2015). Because aging is often accompanied by a decrease in the amount and diversity of fiber-containing foods intake, and a risk of malnutrition and lifestyle, especially diet, plays a vital role on aging gut health (Claesson et al., 2012). Therefore, changes in dietary patterns, supplementation of nutrients, and intervention of active substances improve the incidence of sarcopenia via the gut–muscle axis and have become some of the new treatment methods in recent years.

This review summarized the available literature on evidence and mechanisms of gut–muscle axis and aimed at coming up with promising preventive and therapeutic measures targeting gut microbiome on sarcopenia in order to improve the quality of life in aging and decrease global burden of late-life disease.

SKELETAL MUSCLE AND MICROBIOME

In recent years, more and more studies have shown that gut microbes are related to skeletal muscle metabolism. The state of gut microbes possibly affects the content and function of skeletal muscle. Intestinal microbial disorders cause health loss to patients and even the elderly, affecting the quality of life. Therefore, it is urgent for us to efficiently find out intervention approach via clarifying the role of the gut microbiota in skeletal muscle metabolism.

Effect of Gut Microbiome on Skeletal Muscle: Animal Studies

Skeletal muscle descending size and function are related to metabolic disorders (Kelley et al., 1999) and osteoporosis (Nair, 2005), and animal studies show that intestinal microbial metabolism can regulate skeletal muscle function. The study by Honglin Yan et al. finds that, compared with lean Yorkshire pigs, obese Rongchang pigs (RP) have a different composition of gut microbes. The intestinal microbes of RP were transplanted to germ-free (GF) mice, and the muscle characteristics of GF mice are highly similar to those of RP, which indicated gut microbiome plays a potential role in the skeletal muscle development (Yan et al., 2016). Similarly, it is generally believed that the physiological metabolism of gut microbiome, muscle, and immunity is related to age. Combined with

the analysis of intestinal microbiome, biochemical indicators of muscle metabolism, and serum proteomics and liposome profile of elderly rats with age, the results show that old rats have a higher inflammation/immune status compared with adult rats; the intestinal flora probably participated in the metabolic processes of musculoskeletal system, nutrition, and inflammation/immunity through various complex mechanisms (Siddharth et al., 2017). The antibiotic metronidazole-treated mice have a significant increase of the bacterial phylum *Proteobacteria* in fecal pellets, accompanied by a decrease in muscle weight of the hind limbs, resulting in smaller tibial anterior muscle fibers. In the gastrocnemius muscle, metronidazole treatment leads to the increasing expression of neurogenic atrophy-related biochemical indicators of skeletal muscle, including the up-regulation of HDAC4, myogenin, MuRF1, and avergin1 (Manickam et al., 2018).

Based on the physiological analytic results, intestinal microbial composition is closely related to skeletal muscle content and related biochemical and metabolic indicators (Manickam et al., 2018; Lahiri et al., 2019); some researchers have tried to apply intestinal flora transplantation in skeletal muscle improvement. Certain gut microbiomes are able to produce intestinal metabolites that promote skeletal muscle anabolism. Feces transplanted from unhealthy children (or malnourished children) changed the growth of mice (Ticinesi et al., 2017). Having a gut microbial composition similar to that of the donor, the sterile mice transplanted with feces from the elderly show high (with higher lean meat and lower fat mass) or low function (Fielding et al., 2019). The high function transplanted mice show higher grip strength and a higher proportion of microorganisms. GF mice had lower muscle mass and fewer muscle fibers, while muscle atrophy markers increased compared to pathogen-free mice; however, these are mostly reversed after fecal transplantation and short-chain fatty acid (SCFA) treatment, surprisingly (Lahiri et al., 2019). Researchers continuously find from animal models that change of the composition of gut microbiome regulates the metabolic function of skeletal muscle. Therefore, it is of significance to understand the complex relationship between gut microbiome and host physiology to determine approaches of lifestyle/nutrition/pharmaceutical intervention in maintaining the potential range of skeletal muscle health.

Effect of Gut Microbiome on Skeletal Muscle: Human Studies

The relationship between the composition of the gut microbiome of the elderly and the function of skeletal muscle has also been found in human studies. Changes in gut microbiome that occur when the elderly were transferred to long-term care facilities may eventually change bones and body composition, leading to sarcopenia, osteoporosis, and obesity, increasing the risk of fractures (Inglis and Ilich, 2015). According to the potential close relationship between intestinal population composition and skeletal muscle metabolism, some researchers have explored its possibility as a clinical diagnostic indicator in the clinic. The Sequential and Orthogonalized Covariance Selection (SO-CovSel) prediction model was established by a multimarker method of labeling biomarkers of gut microbial profile, systemic inflammation, and metabolic characteristics in the available cohort of elderly people, and the model correctly distinguished 91.7% of the elderly in the physical frailty and sarcopenia (PF&S) group and 87.5% of the non-PF&S control group (Picca et al., 2020), suggesting close relationship between the gut microbiome, inflammation, nutritional, metabolic status and muscle rebuilding.

Simultaneously, gut microbiome has been explored its possibility as a therapeutic intervention target of skeletal muscle metabolism in the clinic. Sarcopenia, as a common complication in patients with chronic kidney disease (CKD), is related to the activation of protein breakdown signal pathways. The dynamic balance symbiosis system composed of gut microbiome and the human body is destroyed in CKD state, and the resulting intestinal microecological imbalance can accelerate the progress of sarcopenia (Tang et al., 2020). Therefore, the dynamic balance of gut microbiome is considered as the novel and effective intervention target. Clinically, non-target comprehensive metabolomics analysis was performed on middle-aged men with metabolic syndrome treated with resveratrol (RSV). Among men treated with RSV, muscle renewal biochemical markers increase; lipid metabolism is affected; and the urinary derivatives of aromatic amino acids, that mainly reflect the changes of the composition of gut microbiome, have been altered, which may be owing to the change of metabolic function of gut microbiome (Korsholm et al., 2017). The curative effect of the treatment of sarcopenia that targets gut microbiome for intervention has begun to be explored. We will describe the mechanism of action of "gut–muscle axis" and intervention treatment in detail in the following sections.

MECHANISM OF GUT-MUSCLE AXIS

At present, the role of the gut–muscle axis in regulating age-related muscle health has been confirmed by animal models and human studies, while its mechanisms have not been systematically understood. We will systematically introduce the mechanisms probably involved, including protein metabolism, systemic chronic inflammation and metabolic resistance, mitochondrial dysfunction, and modulation of host gene expression (**Figure 1**).

Protein Metabolism

In fact, gut microbiota can be regarded as a highly significant metabolic organ or endocrine organ that generates bioactive metabolites and impacts physiological processes those are vital to host health such as regulation of immune mediators, energy homeostasis, neurobehavioral development, and gut epithelial health (Marchesi et al., 2016; Takiishi et al., 2017; Tang et al., 2017; Barko et al., 2018). Besides, gut microbiota can alter the bioavailability of amino acids through utilizing several amino acids that originate from both alimentary and endogenous proteins, influencing muscle protein synthesis and breakdown, and having an effect on host muscle (Neis et al., 2015; Karlund

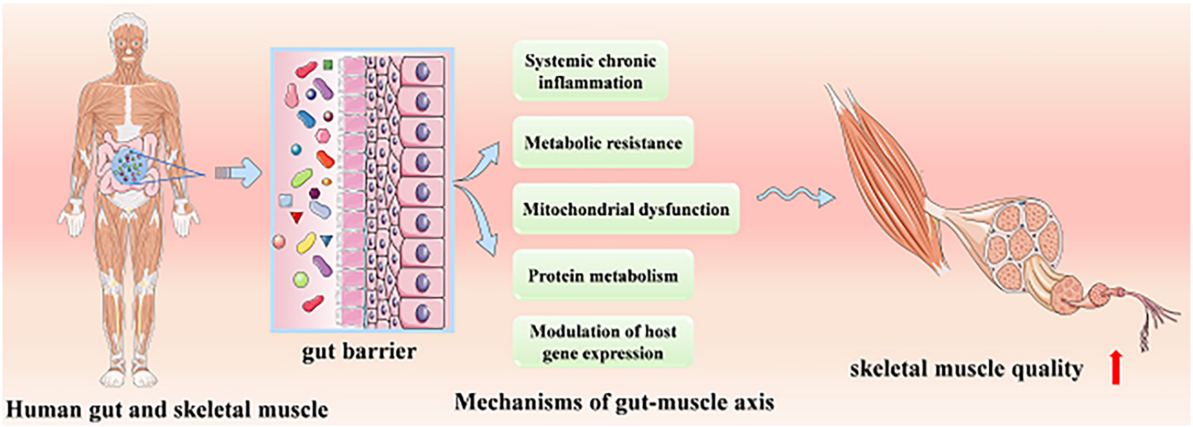


FIGURE 1 | The possible mechanisms of the skeletal muscular improving quality via gut–muscle axis.

et al., 2019). As we all know, protein is vital for skeleton muscle. In addition, the gut microbiota is capable of synthesizing some nutritionally essential amino acids *de novo*, such as tryptophan, which represents the fundamental substrates for muscle protein anabolism (Lin et al., 2017). Tryptophan probably plays a role of stimulating the insulin-like growth factor 1/p70s6k/mTOR pathway in muscle cells and promoting the expression of genes involved in myofibrillar synthesis (Dukes et al., 2015). However, it is also suggested that protein-enriched diets may switch bacterial metabolism toward amino acids degradation and fermentation (Picca et al., 2020).

Systemic Chronic Inflammation and Metabolic Resistance

It is recognized that age-related sarcopenia can be caused by systemic chronic inflammation, as well as metabolic resistance in aging, and the mechanism is gradually understood by people.

Systemic Chronic Inflammation and Gut Barrier Function

Studies suggest that age-related systemic chronic inflammation ("inflaming") is involved in the development of sarcopenia (Boirie, 2009; Bindels and Delzenne, 2013; Ogawa et al., 2016; Steves et al., 2016; Ticinesi et al., 2017; Grosicki et al., 2018; Liguori et al., 2018; Ni Lochlainn et al., 2018). The changes in the gut microbiome could alter the inflammatory state of the individual and consequently result in sarcopenia (Biagi et al., 2010; Bindels and Delzenne, 2013; Quigley, 2013; Steves et al., 2016; Ni Lochlainn et al., 2018; Picca et al., 2018a). It is reported that modulation of the gut microbiota can influence the gut's barrier function, thereby playing an important role in maintaining the balance of proinflammatory and anti-inflammatory responses (Ni Lochlainn et al., 2018; Picca et al., 2018a). The healthy gut microbiome induces a big variety of host responses within the intestinal mucosa and thereby strengthens the gut barrier function, exerting immunomodulatory actions within the gut and beyond (Bindels and Delzenne, 2013; Lin and Zhang, 2017; Ni Lochlainn et al., 2018). In addition, work in animal models shows evidence of gut barrier dysfunction in association with age-associated microbial dysbiosis, increasing intestinal permeability (Grosicki et al., 2018; Ni Lochlainn et al., 2018; Thevaranjan et al., 2018). This could contribute to facilitating translocation of microbial byproducts into the circulation. Microbial byproducts include endotoxins such as lipopolysaccharide (LPS); they can induce systemic chronic inflammation as well as insulin resistance which is one form of metabolic resistance and finally lead to sarcopenia (Bindels and Delzenne, 2013; Ni Lochlainn et al., 2018). A low representation of SCFAs, producers in gut microbiota, are proved involved in increasing subclinical chronic inflammation and then resulted in sarcopenia (den Besten et al., 2013; Ticinesi et al., 2017). In other words, SCFAs produced by gut microbiota can help reduce inflammation and thereby prevent sarcopenia. From a skeletal muscle perspective, one of the most studied mediators among SCFAs is butyrate. Butyrate also plays a vital role in intestinal barrier function and therefore may be associated in intestinal permeability (Peng et al., 2009; Ni Lochlainn et al., 2018). Besides, its anti-inflammatory properties are demonstrated in inflammatory bowel diseases (Leonel and Alvarez-Leite, 2012; Ticinesi et al., 2017). Interestingly, lack of SCFAs can also lead to metabolic resistance (Sonnenburg and Backhed, 2016; Ticinesi et al., 2017; Vaiserman et al., 2017; Ni Lochlainn et al., 2018).

Metabolic Resistance

It is suggested that age-related sarcopenia is closely associated with metabolic resistance (**Figure 2**; Ticinesi et al., 2017; Ni Lochlainn et al., 2018). There is evidence showing "anabolic resistance" in older adults, which means a higher dose of protein is needed to achieve the same myofibrillar protein synthesis response as a younger person (Dillon, 2013; Welch, 2014; Moore et al., 2015; Mitchell et al., 2017). The gut microbiota is involved in many of the postulated mechanisms for anabolic resistance in older people, either directly or indirectly, and it is probably to be a complex interaction between these postulated processes (Ni Lochlainn et al., 2018). The changes in gut microbiome composition and/or diversity result in changes in

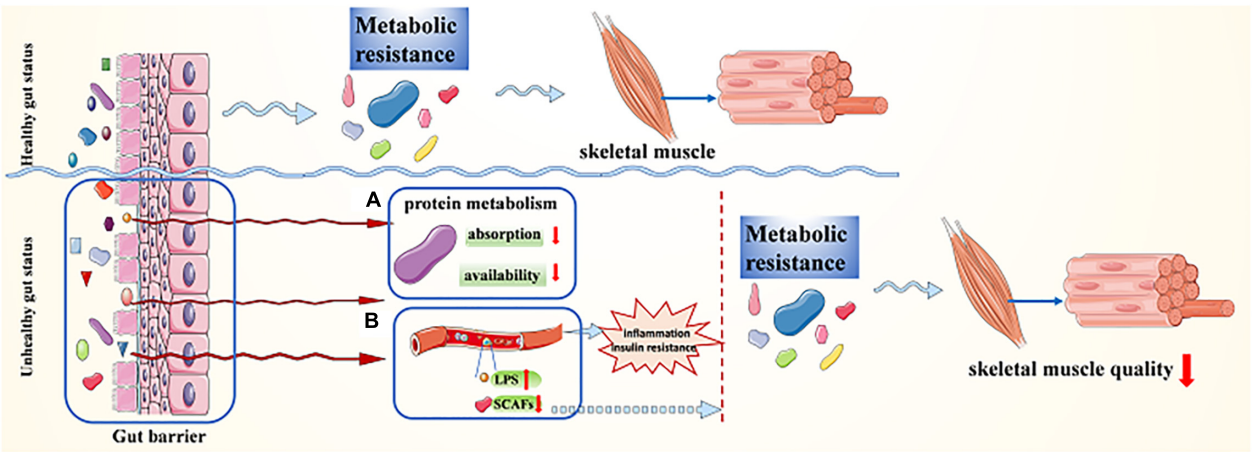


FIGURE 2 | Transmutation of gut status maintains sarcopenia via metabolic resistance. **(A)** The changes in gut microbiome composition and/or diversity result in changes in protein metabolism; **(B)** gut microbial dysbiosis leads to gut barrier dysfunction, which causes the concentration of LPS in the blood to rise and/or SCFAs to decline.

protein metabolism (**Figure 2A**), including absorption as well as availability reduction and increased hydrolysis, leading to anabolic resistance, reduction of muscle protein synthesis, and the development of sarcopenia (Rampelli et al., 2013; Mitchell et al., 2017). Besides, gut microbial dysbiosis can lead to gut barrier dysfunction (**Figure 2B**), which causes the concentration of LPS in the blood to rise. In addition, gut microbial dysbiosis can also result in reduced production of SCFAs. Both the rise of LPS concentration in the blood and the reduction of SCFAs can bring about inflammation and insulin resistance first and then lead to metabolic resistance, or they can also result in metabolic resistance without causing inflammation or insulin resistance first (Ni Lochlainn et al., 2018; Mesinovic et al., 2019). Anabolic resistance should not be limited to one or two mechanisms but must be regarded as a complex and multidimensional construct. The etiologies and mechanisms implicated are not clear; thus, further investigation is required to understand the potential role of the gut microbiota in a variety of postulated mechanisms for anabolic resistance.

Mitochondrial Dysfunction

In skeletal muscle, primary aging leads to mitochondrial energetics deficiency and muscle mass reduction (Cartee et al., 2016). It is suggested that ultrastructural modifications in the number and functionality of mitochondria caused reduced muscle protein synthesis (Marzetti et al., 2013, 2016; St-Jean-Pelletier et al., 2017). Ibebunjo et al. suggest that aged rats lose muscle mass and function gradually (i.e., sarcopenia) through mechanisms involving mitochondrial dysfunction (Ibebunjo et al., 2013; Siddharth et al., 2017). As for human, a study finds that sedentary but not active humans show an age-related decline in optic atrophy 1 (OPA1), a mitochondrial protein, leading to mitochondrial dysfunction; finally, it results in muscle loss. Specifically speaking, the ablation of Opa1 results in endoplasmic reticulum stress, which signals via the FoxOs and unfolded protein response, inducing a catabolic program of systemic aging and muscle loss (Tezze et al., 2017). Interestingly, evidence shows that there is a strict connection between microbiota and mitochondrial function. There is a regulatory relationship between the gut microbiota and mitochondria; therefore, a disruption of the relationship leads to dysfunctional mitochondria. A network analysis shows that *Atopobium parvulum* was the most prominent microbe associated with mitochondrial dysfunction and the relative abundance of *A. parvulum* correlates negatively with both the level of butyrate producers and the mitochondrial protein expression. Besides, the loss of butyrate producers will cause decreased butyrate production that in turn will impair mitochondrial functions (Mottawea et al., 2016). What is more, SCFAs are the putative mediators of the effect of gut microbiome on skeletal muscle, whose main host targets are skeletal muscle mitochondria; it also suggests a close connection between microbiota and mitochondrial function (Kimura et al., 2014; Clark and Mach, 2017).

It is worth noting that mitochondrial dysfunction may play a vital role to link the relation between chronic inflammation and age-related sarcopenia, and the gut microbiota dysbiosis may be a key role in the gut–muscle crosstalk (Picca et al., 2018a). Nucleoids or oxidized cell free-mtDNA extruded from damaged mitochondria could trigger inflammation in sarcopenia (**Figure 3B**); they would activate the innate immune system as well as induce body to produce inflammatory mediators. The release of the latter would maintain a vicious circle in myocytes, leading to further mitochondrial impairment, finally resulting in sarcopenia (Picca et al., 2018b). Moreover, mitochondrial dysfunction is suggested as one of the possible mechanisms for anabolic resistance (Ni Lochlainn et al., 2018).

Modulation of Host Gene Expression

In fact, gut microbiota is able to produce a variety of metabolites that reach the muscle to give proanabolic signals to the

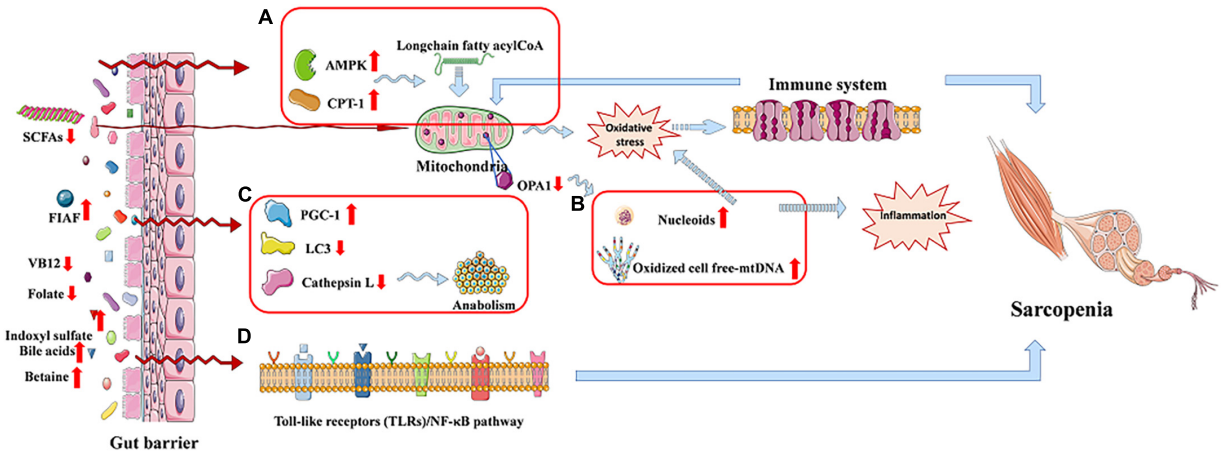


FIGURE 3 | The gut–muscle axis led to sarcopenia via regulating the processes of mitochondrial dysfunction and modulation of host gene expression. **(A)** AMP-activated protein kinase (AMPK) and carnitine palmitoyl transferase-1 (CPT-1) are increased. **(B)** Mitochondrial dysfunction. **(C)** Autophagy-lysosomal pathway related proteins reduced. **(D)** Cell signaling pathway regulation process.

host, modulating gene expression of the host and enhancing antioxidant activity.

Bäckhed et al. show that the activities of AMP-activated protein kinase, which monitor cellular energy status, as well as carnitine palmitoyl transferase-1, are increased in the muscle of GF mice compared to mice with gut microbiota, promoting long-chain fatty acyl-CoA to enter into mitochondria, where they will be oxidized, which indicates an increased oxidative capacity (**Figure 3A**). Besides, GF mice show increased intestinal levels of fasting-induced adipocyte factor that is associated with an increased expression of proliferator-activated receptor-γ coactivator-1 in the gastrocnemius muscle. These increased activities counteract the effect of denervation and fasting on muscle atrophy (Backhed et al., 2007; Bindels and Delzenne, 2013; Picca et al., 2018a). A study shows a reduction of LC3 and cathepsin L in tibialis and gastrocnemius muscles, and they are two markers of the autophagy–lysosomal pathway, which is a major system, in skeletal muscle, of protein breakdown (**Figure 3C**; Mammucari et al., 2007; Bindels and Delzenne, 2013). It is reported that a healthy gut microbiota can produce a mass of complex cobalamin (or vitamin B_{12}) and folate, which may prevent reduction of muscle function through improving muscle anabolism, as well as preventing both hyperhomocysteinemia-induced oxidative stress and endothelial damage (Kuo et al., 2007; Guy LeBlanc et al., 2013). Besides, indoxyl sulfate, gut microbiota-specific metabolite, can lead C2C12 myoblasts to increase the activity of the pentose phosphate pathway and show an up-regulation of glycolysis, inducing sarcopenia (Wikoff et al., 2009; Sato et al., 2016; Grosicki et al., 2018). In addition, bile acids produced by gut microbiota can be signaling molecules in regulating G-protein–coupled bile acid receptor-1 (also known as TGR5) in the muscle, promoting intracellular thyroid hormone activation, and thereby elevate energy expenditure in human skeletal muscle cells (Watanabe et al., 2006; Di Ciaula et al., 2017). Except this, bile acids also prevent muscle fat deposition by activating the nuclear farnesoid X receptor (Watanabe et al., 2006; Cipriani et al., 2010; Di Ciaula et al., 2017). Nuclear factor κB (NF-κB), the transcription factor of muscle-specific activation, causes sarcopenia, which calls toll-like receptors/NF-κB pathway (**Figure 3D**), and may be associated with the gut microbiota-muscle axis (Bindels and Delzenne, 2013). What is more, betaine is a microbial metabolite that activates cytosolic calcium influx, synthesis of IGF-1, and extracellular signal–regulated kinase in human osteoblast cultures, which may have effects on skeletal muscle (Villa et al., 2017; Grosicki et al., 2018). The induction of Klf15 gene expression and activation of the branched-chain amino acid pathway in GF mice may be another possible mechanism of gut–muscle axis, which is called HPA-glucocorticoid–driven atrophy of skeletal muscle mass (Lahiri et al., 2019).

Others

Interestingly, gut microbiota could also affect muscle function via the mediation of the central nervous system (Ticinesi et al., 2017), Lahiri et al. observed reduced serum choline, which is the precursor of acetylcholine and the key neurotransmitter that signals between muscle and nerve at neuromuscular junctions (NMJs) in GF mice, which suggests that the communication between muscle and nerve cells at NMJs may be impaired under GF conditions. Besides, gut microbiota is observed to alter the expression of genes encoding Rapsyn and Lrp4, two proteins important for NMJ assembly and function expression of NMJ-associated genes in GF mouse muscle alterations (Lahiri et al., 2019). However, further experiments are needed to reach a definitive conclusion.

TREATMENT

On the basis of mechanisms of gut–muscle axis, a significant amount of studies suggest that gut microbiota modulation may potentially be a future therapeutic target in age-related

sarcopenia. In this review, we will summarize the possible ways to modulate gut microbiota and thereby come up with potential treatments of sarcopenia.

Modulate Gut Microbiota Directly

Supplement of Probiotics and Prebiotics and Dietary Fibers

More and more animal and human studies suggest that the use of prebiotics and/or probiotics has beneficial effects on skeletal muscle. Prebiotics, fermented in the lower part of the gut and selectively stimulating the growth and/or activity(ies) of a limited number of bacteria, are non-digestible carbohydrates and therefore have healthy effects on the host (Delzenne and Cani, 2011). There are two substances, inulin and *trans*-galacto-oligosaccharides, meeting the criteria for classification as a prebiotic (Steves et al., 2016; Vandeputte et al., 2017), and more investigated prebiotics anticipated as standard in recent years (Jäger et al., 2019; Khuituan et al., 2019). Cani et al. (2009) demonstrate that the supplement of prebiotics has beneficial efficacy on skeletal muscle of mice. Besides, an increase in muscle strength and endurance can be observed after prebiotic supplementation (inulin plus fructo-oligosaccharides) in older people (Buigues et al., 2016). Probiotics are live microorganisms, such as *Lactobacillus* species and so on, which confer a health benefit on the host when administered in adequate amount (Delzenne and Cani, 2011). Sarcopenia is attenuated through oral supplementation with specific *Lactobacillus* species in a mice model of acute leukemia (Bindels et al., 2012). Besides, the muscle mass and function increased by supplementation with *Lactobacillus plantarum* (Chen et al., 2016). Treatment with probiotic formulations, containing *Faecalibacterium prausnitzii*, which is one of the main SCFA producers, is associated with reduced systemic inflammation in mice (Munukka et al., 2017). As for human studies, two probiotic trials show an increase in skeletal muscle function in elite athletes (Salarkia et al., 2013; Shing et al., 2014). Interestingly, it is suggested that supplement of prebiotic and/or probiotic elevates the abundance of butyrate producers, as well as *Bifidobacterium*, and thereby improves the muscle mass and function in older people (Vulevic et al., 2008; Eloe-Fadrosh et al., 2015). However, most of the evidence for a benefit of probiotics is in rodents. In humans, probiotic studies have only shown evidence in randomized controlled trials in young populations or very old or the severely ill (Steves et al., 2016). Moreover, dietary fibers play a key role in gut microbiome diversity and composition, and its supplementation may be a significant approach for increasing gut bacterial SCFA production (Lustgarten, 2020), which may have positive effects on skeletal muscle mass and physical function in humans (Lustgarten, 2019).

Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) is the infusion of a solution of donor feces to patients (van Nood et al., 2013). Many studies suggest that FMT could potentially be a method to improve skeletal muscle mass and function. Yan et al. (2016) transfer gut microbiota from obese pigs to GF mice, and the result is that the metabolic profile and fiber characteristics of the skeletal muscle are replicated in the recipients, which indicates the beneficial effect of FMT on skeletal muscle performance. Compared to supplementation with prebiotic and/or probiotic, FMT is a more radical treatment option (Steves et al., 2016).

Diet and Nutrition

During aging, most of gut microbiota alterations due to diet, in other words, gut microbiota composition, depends heavily on diet composition (Claesson et al., 2012). It is suggested that long-term dietary habits have important effects on gut microbiome (Fielding et al., 2019); according to the report, increased levels of *Prevotella*, some *Firmicutes*, and SCFAs are significantly associated with consumption of a Mediterranean diet (De Filippis et al., 2016). In fact, except changing the composition of gut microbiota, nutrition may also influence metabolic process of it, such as modulating the host gene expression via various mediators, because most of the mediators produced by gut bacteria originate from dietary intake (Salazar et al., 2017). In this context, new nutritional therapeutic avenues have been proposed to relieve age-related sarcopenia (Siddharth et al., 2017). We will summarize the balance effects of different nutrients supplements on the gut and/or skeletal muscle health of animal models or patients in **Table 1**.

High-Protein Diets

A study shows that protein consumption is correlated positively with gut microbiota diversity (Singh et al., 2017), and the source of protein seemed influential. In an animal study, compared to animal protein feeding, hamsters show higher microbial diversity in those fed soy protein (An et al., 2014; Butteiger et al., 2016). As is known to all, supplementation with protein can improve skeletal muscle mass and function, and it has been reported that a high-protein diet (HPD) adhering to the recommended acceptable macronutrient distribution ranges might help reduce sarcopenia (Ford et al., 2020). Moreover, studies suggest that whey protein and leucine supplementation appeared to improve muscle mass and function (Bechshoft et al., 2016; Kobayashi, 2018). However, further study is needed to prove that the increase in muscle mass and function through HPDs is involved in the modulation of gut microbiota.

Supplement of Other Nutrients and Plant Active Substances

Recently, an animal study shows that feeding a mixture of SCFAs to GF mice was able to improve muscle strength (Lahiri et al., 2019). In addition, there is evidence showing that butyrate treatment may be a promising approach to counteract age-related sarcopenia; by the way, butyrate can be produced by *Bifidobacterium* (Walsh et al., 2015). Melatonin is proved to have the activity of effectively improving age-related skeletal muscle disorders (Stacchiotti et al., 2020). It possibly targets mitochondria through the gut–muscle axis, effectively maintaining mitochondrial function, scavenging free radicals, reducing oxidative damage, and achieving the purpose of maintaining age-related sarcopenia. Notably, plant active

TABLE 1 | The balance effects of different nutrients supplements and exercise on the gut and/or skeletal muscle health of animal models or human.

Methods	Objects	Effects	Possible mechanisms involved	References
Prebiotics supplement: *oligofructose*	Mice	Reduction of intestinal permeability and improvement of tight-junction integrity	Improved gut barrier functions through glucagon-like peptide-2-dependent mechanism (GLP-2) and reduced inflammatory tone	Cani et al., 2009
Prebiotics supplement: *a mixture of inulin plus fructo-oligosaccharides*	Older people	Improvement of muscle strength	Reduced proinflammatory cytokines in blood and inflammation	Buigues et al., 2016
Prebiotics supplement: *GOS mixture (B-GOS)*	Older people	Decrease of less beneficial bacteria and an increase of beneficial bacteria	Improved NK cell activity and phagocytosis, increased secretion of the anti-inflammatory cytokine interleukin 10 (IL-10), and decreased secretion of proinflammatory cytokines [IL-6, IL-1β, and tumor necrosis factor (TNF-α)]	Vulevic et al., 2008
Probiotics supplement: *L. reuteri100-23 and L. gasseri 311476*	Mice	Increase of tibialis muscle weight	Decreased the plasma level of inflammatory cytokines (IL-4, monocyte chemoattractant protein 1, IL-6, granulocyte colony-stimulating factor) and maintained the plasma level of the anti-inflammatory IL-10, to modulate inflammation and systemic immunity	Bindels et al., 2012
Probiotics supplement: *L. plantarum* TWK10 (LP10)	Mice	Increase of muscle mass, strength and endurance	Reduced inflammation; enhanced glucose utilization by increasing the number of gastrocnemius type I muscle fibers	Chen et al., 2016
Probiotics supplement: *Faecalibacterium prausnitzii*	Mice	Increase of muscle mass	Enhanced mitochondrial respiration; improved intestinal integrity and reduced inflammation; improved insulin sensitivity	Munukka et al., 2017
Probiotics supplement: probiotics capsule	Athletes	Increase of muscle endurance	Reduction in gastrointestinal permeability, serum LPS concentrations and inflammation	Shing et al., 2014
Probiotics supplement: *Lactobacillus rhamnosus GG ATCC 53103 (LGG)*	Older people	Modified the resident microbiota, producers of the short-chain fatty acid (SCFA) butyrate increased	Reducing intestinal permeability and inflammation by SCFA butyrate	Eloe-Fadrosh et al., 2015
Fecal microbiota transplantation (FMT)	Pig and mice	Transfer of fiber characteristics and lipid metabolic profiles of skeletal muscle from pigs to germ-free mice through gut microbiota	Specific gut microbiome could inhibit ectopic fat deposition in skeletal muscle and enhances muscle growth	Yan et al., 2016
Protein supplement	Human	Improvement of muscle mass and function	Increase of overall diversity of gut microbiota that can alter the bioavailability of amino acids, influencing muscle protein synthesis and breakdown	Bechshoft et al., 2016; Singh et al., 2017; Karlund et al., 2019
Dietary fiber supplement	Mice	Increase of muscle mass and strength	Fecal SCFAs increased, improving intestinal barrier function and reducing inflammation	Lustgarten, 2019, 2020 13,14
SCFAs (butyrate) supplement	Mice	Increase of muscle fiber cross-sectional area and prevention of intramuscular fat accumulation	Butyrate is a histone deacetylase (HDAC) inhibitor; HDACs regulate myogenesis via the transcription factor myocyte enhancer factor−2	Walsh et al., 2015
Exercise	Human	Increase of ability and capacity of skeletal muscle to synthesize proteins	Increased gut microbiota biodiversity and representation of taxa with beneficial metabolic functions, and gut microbiota could conversely have influence on muscle through modulating the inflammatory response, changing bioavailability of dietary proteins and producing substances that have beneficial and proanabolic effects in skeletal muscle cells; fecal SCFAs increased, improving intestinal barrier function and reducing inflammation	Dhillon and Hasni, 2017; Lustgarten, 2019; Ticinesi et al., 2019a

substances have also been confirmed to have an impact on gut and skeletal muscle health. A recent study shows that lycopene has a dose-dependent health effect on the human intestine (Wiese et al., 2019). The relative abundance of *Bifidobacterium* in the adolescent group and *Bifidobacterium longum* in the lycopene-treated middle-aged group increases and is accompanied by dose-dependent changes in blood, liver metabolism, and skeletal muscle and skin parameters. Additionally, RSV, a polyphenol found in walnuts, berries, and grapes, is capable of improving muscle performance in mature aged mice (Rodriguez-Bies et al., 2016). After 4 months of RSV treatment for middle-aged men with metabolic syndrome, it was found that the composition of the intestinal microflora is changed, accompanied by an increase in biochemical indicators of skeletal muscle turnover process (Korsholm et al., 2017). Dietary nutrients and natural plant active substances commonly have thriving biological activity and biocompatibility, while they are often required for the gut microbiome as a medium for health function. Therefore, it is of great significance to investigate their interventional and improvement effects in diseases, such as age-related sarcopenia, through the gut–muscle axis. However, further studies are needed to prove the effect of the supplement of these nutrients and plant active substances.

Exercise

There are no pharmacologic agents for the treatment of sarcopenia, and the main treatment of sarcopenia is physical therapy for muscle strengthening and gait training (Dhillon and Hasni, 2017). Exercise training (particularly resistance training) has long been regarded as the most prospective method for improving muscle mass and strength in older people (Giallauria et al., 2016), and almost all clinical trials have proved the beneficial effects of exercise in preventing sarcopenia (Beaudart et al., 2017). Exercise has significant impact on intestinal microbiome, because some investigations show that exercise is associated with increased biodiversity, as well as representation of taxa with beneficial metabolic functions (Clarke et al., 2014; Bressa et al., 2017; Ticinesi et al., 2019a). It is suggested that there is a bidirectional relationship between skeletal muscle and the gut microbiome (Ni Lochlainn et al., 2018). Interestingly, a large number of studies suggest that there is a synergistic effect in improving muscle performance, between exercise and supplementation with protein (Bechshoft et al., 2016; Kobayashi, 2018; Ni Lochlainn et al., 2018). However, the findings of using a combined approach of exercise and dietary protein supplements were inconsistent among various populations; thus, further studies are needed (Dhillon and Hasni, 2017).

DISCUSSION

In conclusion, gut microbiota plays a highly important role in skeletal muscle, and chronic inflammation is the main pathogenic mechanism of sarcopenia. In fact, systemic chronic inflammation also represents the substrate of aging and a significantly important risk factor for both morbidity and mortality in older people (Picca et al., 2018a). However, inflammation-independent changes in skeletal muscle cell metabolism have also been observed, such as autophagy–lysosomal pathway, which illustrates that gut microbiota may affect the skeletal muscle mass and strength through multitude of ways. The potential therapeutic methods targeting gut microbiota are various accordingly, but not all methods work in all situations. For example, the treatment of exercise is not suitable for someone bedridden, such as paralyzed old people. The good news is that more and more studies are exploring the mechanisms of gut–muscle axis, and the new potential therapeutic approaches will be put forward accompanied by the breakthrough of the discovery of mechanism of gut–muscle axis, highlighting the multitude of ways in which gut microbiota may influence the metabolic function of skeletal muscle. Notably, nutrient supplementation is one of the effective treatment methods due to its simpleness and easy realization; it has become one of the potential and promising therapeutic methods. Nutrients are capable of changing the composition of the gut microbiome and affecting the metabolic process, because most of the mediators produced by intestinal bacteria come from dietary intake (Salazar et al., 2017). The intervention of nutrients and plant active extracts on age-related sarcopenia has been considered as a new research field.

AUTHOR CONTRIBUTIONS

All the authors listed have made a rational and substantial contribution to the work and approved the publication.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant number 81373008) and by the Nutritional Science Foundation of By-Health Inc., Guangdong, China (grant code TY0110101). The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

REFERENCES

An, C., Kuda, T., Yazaki, T., Takahashi, H., and Kimura, B. (2014). Caecal fermentation, putrefaction and microbiotas in rats fed milk casein, soy protein or fish meal. *Appl. Microbiol. Biotechnol.* 98, 2779–2787. doi: 10.1007/s00253-013-5271-5

Backhed, F., Manchester, J. K., Semenkovich, C. F., and Gordon, J. I. (2007). Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 979–984. doi: 10.1073/pnas.0605374104

Barko, P. C., Mcmichael, M. A., Swanson, K. S., and Williams, D. A. (2018). The gastrointestinal microbiome: a review. *J. Vet. Intern. Med.* 32, 9–25.

Beaudart, C., Dawson, A., Shaw, S. C., Harvey, N. C., Kanis, J. A., Binkley, N., et al. (2017). Nutrition and physical activity in the prevention and treatment of sarcopenia: systematic review. *Osteoporosis Int.* 28, 1817–1833. doi: 10.1007/s00198-017-3980-9

Bechshoft, R. L., Reitelseder, S., Hojfeldt, G., Castro-Mejia, J. L., Khakimov, B., Bin Ahmad, H. F., et al. (2016). Counteracting age-related loss of skeletal muscle mass: a clinical and ethnological trial on the role of protein supplementation

and training load (CALM Intervention Study): study protocol for a randomized controlled trial. *Trials* 17:397.

Ben-Haim, M. S., Kanfi, Y., Mitchell, S. J., Maoz, N., Vaughan, K. L., Amariglio, N., et al. (2018). Breaking the ceiling of human maximal life span. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 73, 1465–1471. doi: 10.1093/gerona/glx219

Biagi, E., Franceschi, C., Rampelli, S., Severgnini, M., Ostan, R., Turroni, S., et al. (2016). Gut microbiota and extreme longevity. *Curr. Biol.* 26, 1480–1485.

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., et al. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One* 5:e10667. doi: 10.1371/journal.pone.0010667

Bindels, L. B., Beck, R., Schakman, O., Martin, J. C., De Backer, F., Sohet, F. M., et al. (2012). Restoring specific lactobacilli levels decreases inflammation and muscle atrophy markers in an acute leukemia mouse model. *PLoS One* 7:e37971. doi: 10.1371/journal.pone.0037971

Bindels, L. B., and Delzenne, N. M. (2013). Muscle wasting: the gut microbiota as a new therapeutic target? *Int. J. Biochem. Cell Biol.* 45, 2186–2190. doi: 10.1016/j.biocel.2013.06.021

Blau, H. M., Cosgrove, B. D., and Ho, A. T. V. (2015). The central role of muscle stem cells in regenerative failure with aging. *Nat. Med.* 21, 854–862. doi: 10.1038/nm.3918

Boirie, Y. (2009). Physiopathological mechanism of sarcopenia. *J. Nutr. Health Aging* 13, 717–723. doi: 10.1007/s12603-009-0203-x

Bressa, C., Bailen-Andrino, M., Perez-Santiago, J., Gonzalez-Soltero, R., Perez, M., Gregoria Montalvo-Lominchar, M., et al. (2017). Differences in gut microbiota profile between women with active lifestyle and sedentary women. *PLoS One* 12:e0171352. doi: 10.1371/journal.pone.0171352

Brook, M. S., Wilkinson, D. J., Phillips, B. E., Perez-Schindler, J., Philp, A., Smith, K., et al. (2016). Skeletal muscle homeostasis and plasticity in youth and ageing: impact of nutrition and exercise. *Acta Physiol.* 216, 15–41. doi: 10.1111/apha.12532

Buigues, C., Fernandez-Garrido, J., Pruimboom, L., Hoogland, A. J., Navarro-Martinez, R., Martinez-Martinez, M., et al. (2016). Effect of a prebiotic formulation on frailty syndrome: a randomized, double-blind clinical trial. *Int. J. Mol. Sci.* 17:932. doi: 10.3390/ijms17060932

Butteiger, D. N., Hibberd, A. A., Mcgraw, N. J., Napawan, N., Hall-Porter, J. M., and Krul, E. S. (2016). Soy protein compared with milk protein in a western diet increases gut microbial diversity and reduces serum lipids in golden syrian hamsters. *J. Nutr.* 146, 697–705. doi: 10.3945/jn.115.224196

Candela, M., Biagi, E., Brigidi, P., O'toole, P. W., and De Vos, W. M. (2014). Maintenance of a healthy trajectory of the intestinal microbiome during aging: a dietary approach. *Mech. of Age. Dev.* 136, 70–75. doi: 10.1016/j.mad.2013.12.004

Cani, P. D., Possemiers, S., Van De Wiele, T., Guiot, Y., Everard, A., Rottier, O., et al. (2009). Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* 58, 1091–1103. doi: 10.1136/gut.2008.165886

Carmona, J. J., and Michan, S. (2016). Biology of healthy aging and longevity. *Rev. Investig. Clin. Clin. Transl. Investig.* 68, 7–16.

Cartee, G. D., Hepple, R. T., Bamman, M. M., and Zierath, J. R. (2016). Exercise promotes healthy aging of skeletal muscle. *Cell Metab.* 23, 1034–1047. doi: 10.1016/j.cmet.2016.05.007

Chen, Y.-M., Wei, L., Chiu, Y.-S., Hsu, Y.-J., Tsai, T.-Y., Wang, M.-F., et al. (2016). *Lactobacillus plantarum* TWK10 supplementation improves exercise performance and increases muscle mass in mice. *Nutrients* 8:205. doi: 10.3390/nu8040205

Choi, K. M. (2016). Sarcopenia and sarcopenic obesity. *Korean J. Intern. Med.* 31, 1054–1060.

Cigarran Guldris, S., Gonzalez Parra, E., and Cases Amenos, A. (2017). Gut microbiota in chronic kidney disease. *Nefrologia* 37, 9–19. doi: 10.1016/j.nefroe.2017.01.017

Cipriani, S., Mencarelli, A., Palladino, G., and Fiorucci, S. (2010). FXR activation reverses insulin resistance and lipid abnormalities and protects against liver steatosis in Zucker (fa/fa) obese rats. *J. Lipid Res.* 51, 771–784. doi: 10.1194/jlr.m001602

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'connor, E. M., Cusack, S., et al. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature* 488, 178–184.

Clark, A., and Mach, N. (2017). The crosstalk between the gut microbiota and mitochondria during exercise. *Front. Physiol.* 8:319. doi: 10.3389/fphys.2017.00319

Clarke, S. F., Murphy, E. F., O'sullivan, O., Lucey, A. J., Humphreys, M., Hogan, A., et al. (2014). Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* 63, 1913–1920. doi: 10.1136/gutjnl-2013-306541

Curtis, E., Litwic, A., Cooper, C., and Dennison, E. (2015). Determinants of muscle and bone aging. *J. Cell. Physiol.* 230, 2618–2625. doi: 10.1002/jcp.25001

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., et al. (2016). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut* 65, 1812–1821. doi: 10.1136/gutjnl-2015-309957

Delzenne, N. M., and Cani, P. D. (2011). Interaction between obesity and the gut microbiota: relevance in nutrition. *Annu. Rev. Nutr.* 31, 15–31. doi: 10.1146/annurev-nutr-072610-145146

den Besten, G., Lange, K., Havinga, R., Van Dijk, T. H., Gerding, A., Van Eunen, K., et al. (2013). Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *Am. J. Physiol. Gastrointest. Liver Physiol.* 305, G900–G910.

Dhillon, R. J. S., and Hasni, S. (2017). Pathogenesis and management of sarcopenia. *Clin. Geriatr. Med.* 33, 17–26. doi: 10.1016/j.cger.2016.08.002

Di Ciaula, A., Garruti, G., Baccetto, R. L., Molina-Molina, E., Bonfrate, L., Wang, D. Q. H., et al. (2017). Bile acid physiology. *Ann. Hepatol.* 16, S4–S14.

Dillon, E. L. (2013). Nutritionally essential amino acids and metabolic signaling in aging. *Amino Acids* 45, 431–441. doi: 10.1007/s00726-012-1438-0

Dukes, A., Davis, C., El Refaey, M., Upadhyay, S., Mork, S., Arounleut, P., et al. (2015). The aromatic amino acid tryptophan stimulates skeletal muscle IGF1/p70s6k/mTor signaling in vivo and the expression of myogenic genes in vitro. *Nutrition* 31, 1018–1024. doi: 10.1016/j.nut.2015.02.011

Durazzo, M., Ferro, A., and Gruden, G. (2019). Gastrointestinal microbiota and type 1 diabetes mellitus: the state of art. *J. Clin. Med.* 8:1843. doi: 10.3390/jcm8111843

Eloe-Fadrosh, E. A., Brady, A., Crabtree, J., Drabek, E. F., Ma, B., Mahurkar, A., et al. (2015). Functional dynamics of the gut microbiome in elderly people during probiotic consumption. *mBio* 6:e00231-15.

Fielding, R. A., Reeves, A. R., Jasuja, R., Liu, C., Barrett, B. B., and Lustgarten, M. S. (2019). Muscle strength is increased in mice that are colonized with microbiota from high-functioning older adults. *Exp. Gerontol.* 127:110722. doi: 10.1016/j.exger.2019.110722

Ford, A. L., Nagulesapillai, V., Piano, A., Auger, J., Girard, S.-A., Christman, M., et al. (2020). Microbiota stability and gastrointestinal tolerance in response to a high-protein diet with and without a prebiotic, probiotic, and synbiotic: a randomized, double-blind, placebo-controlled trial in older women. *J. Acad. Nutr. Diet.* 120, 500–516. doi: 10.1016/j.jand.2019.12.009

Francis, P., Lyons, M., Piasecki, M., Mc Phee, J., Hind, K., and Jakeman, P. (2017). Measurement of muscle health in aging. *Biogerontology* 18, 901–911. doi: 10.1007/s10522-017-9697-5

Giallauria, F., Cittadini, A., Smart, N. A., and Vigorito, C. (2016). Resistance training and sarcopenia. *Monaldi Arch. Chest. Dis.* 84:738.

Grosicki, G. J., Fielding, R. A., and Lustgarten, M. S. (2018). Gut microbiota contribute to age-related changes in skeletal muscle size, composition, and function: biological basis for a gut-muscle axis. *Calc. Tissue Int.* 102, 433–442. doi: 10.1007/s00223-017-0345-5

Guy LeBlanc, J., Milani, C., Savoy De Giori, G., Sesma, F., Van Sinderen, D., and Ventura, M. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr. Opin. Biotechnol.* 24, 160–168. doi: 10.1016/j.copbio.2012.08.005

Ibebunjo, C., Chick, J. M., Kendall, T., Eash, J. K., Li, C., Zhang, Y., et al. (2013). Genomic and proteomic profiling reveals reduced mitochondrial function and disruption of the neuromuscular junction driving rat sarcopenia. *Mol. Cell. Biol.* 33, 194–212. doi: 10.1128/mcb.01036-12

Inglis, J. E., and Ilich, J. Z. (2015). The microbiome and osteosarcopenic obesity in older individuals in long-term care facilities. *Curr. Osteoporosis Rep.* 13, 358–362. doi: 10.1007/s11914-015-0287-7

Jäger, R., Mohr, A. E., Carpenter, K. C., Kerksick, C. M., Purpura, M., Moussa, A., et al. (2019). International society of sports nutrition position stand: probiotics. *J. Int. Soc. Sports Nutr.* 16:62.

Karlund, A., Gomez-Gallego, C., Turpeinen, A. M., Palo-Oja, O.-M., El-Nezami, H., and Kolehmainen, M. (2019). Protein supplements and their relation with nutrition, microbiota composition and health: is more protein always better for sportspeople?. *Nutrients* 11:829. doi: 10.3390/nu11040829

Kelley, D. E., Goodpaster, B., Wing, R. R., and Simoneau, J.-A. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am. J. Physiol. Endocrinol. Metab.* 277, E1130–E1141.

Khuituan, P., Sakena, K. D., Bannob, K., Hayeeawaema, F., Peerakietkhajorn, S., Tipbunjong, C., et al. (2019). Prebiotic oligosaccharides from dragon fruits alter gut motility in mice. *Biomed. Pharmacother.* 114:108821. doi: 10.1016/j.biopha.2019.108821

Kim, S., and Jazwinski, S. M. (2018). The gut microbiota and healthy aging: a mini-review. *Gerontology* 64, 513–520. doi: 10.1159/000490615

Kimura, I., Inoue, D., Hirano, K., and Tsujimoto, G. (2014). The SCFA receptor GPR43 and energy metabolism. *Front. Endocrinol.* 5:85. doi: 10.3389/fendo.2014.00085

Kobayashi, H. (2018). Amino acid nutrition in the prevention and treatment of sarcopenia. *Yakugaku Zasshi J. Pharm. Soc. Jpn.* 138, 1277–1283. doi: 10.1248/yakushi.18-00091-4

Kontis, V., Bennett, J. E., Mathers, C. D., Li, G., Foreman, K., and Ezzati, M. (2017). Future life expectancy in 35 industrialised countries: projections with a Bayesian model ensemble. *Lancet* 389, 1323–1335. doi: 10.1016/s0140-6736(16)32381-9

Korsholm, A. S., Kjaer, T. N., Ornstrup, M. J., and Pedersen, S. B. (2017). Comprehensive metabolomic analysis in blood, urine, fat, and muscle in men with metabolic syndrome: a randomized, placebo-controlled clinical trial on the effects of resveratrol after four months' treatment. *Int. J. Mol. Sci.* 18:554. doi: 10.3390/ijms18030554

Kundu, P., Blacher, E., Elinav, E., and Pettersson, S. (2017). Our gut microbiome: the evolving inner self. *Cell* 171, 1481–1493. doi: 10.1016/j.cell.2017.11.024

Kuo, H.-K., Liao, K.-C., Leveille, S. G., Bean, J. F., Yen, C.-J., Chen, J.-H., et al. (2007). Relationship of homocysteine levels to quadriceps strength, gait speed, and late-life disability in older adults. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 62, 434–439. doi: 10.1093/gerona/62.4.434

Lahiri, S., Kim, H., Garcia-Perez, I., Reza, M. M., Martin, K. A., Kundu, P., et al. (2019). The gut microbiota influences skeletal muscle mass and function in mice. *Sci. Transl. Med.* 11:eaan5662. doi: 10.1126/scitranslmed.aan5662

Larsson, L., Degens, H., Li, M., Salviati, L., Il Lee, Y., Thompson, W., et al. (2019). Sarcopenia: aging-related loss of muscle mass and function. *Physiol. Rev.* 99, 427–511. doi: 10.1152/physrev.00061.2017

Lau, K., Srivatsav, V., Rizwan, A., Nashed, A., Liu, R., Shen, R., et al. (2017). Bridging the gap between gut microbial dysbiosis and cardiovascular diseases. *Nutrients* 9:859. doi: 10.3390/nu9080859

Leonel, A. J., and Alvarez-Leite, J. I. (2012). Butyrate: implications for intestinal function. *Curr. Opin. Clin. Nutr. Metab. Care* 15, 474–479. doi: 10.1097/mco.0b013e32835665fa

Li, J., Zhao, F., Wang, Y., Chen, J., Tao, J., Tian, G., et al. (2017). Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome* 5:14.

Liguori, I., Russo, G., Aran, L., Bulli, G., Curcio, F., Della-Morte, D., et al. (2018). Sarcopenia: assessment of disease burden and strategies to improve outcomes. *Clin. Intervent. Aging* 13, 913–927. doi: 10.2147/cia.s149232

Lin, L., and Zhang, J. (2017). Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunol.* 18:2. doi: 10.1186/s12865-016-0187-3

Lin, R., Liu, W., Piao, M., and Zhu, H. (2017). A review of the relationship between the gut microbiota and amino acid metabolism. *Amino Acids* 49, 2083–2090. doi: 10.1007/s00726-017-2493-3

Lloyd-Price, J., Abu-Ali, G., and Huttenhower, C. (2016). The healthy human microbiome. *Genome Med.* 8:51.

Lu, W., Pikhart, H., and Sacker, A. (2019). Domains and measurements of healthy aging in epidemiological studies: a review. *Gerontologist* 59, e294–e310.

Lustgarten, M. S. (2019). The role of the gut microbiome on skeletal muscle mass and physical function: 2019 update. *Front. Physiol.* 10:1435. doi: 10.3389/fphys.2019.01435

Lustgarten, M. S. (2020). The kidney-gut-muscle axis in end-stage renal disease is similarly represented in older adults. *Nutrients* 12:106. doi: 10.3390/nu12010106

Maffei, V. J., Kim, S., Blanchard, E., Luo, M., Jazwinski, S. M., Taylor, C. M., et al. (2017). Biological aging and the human gut microbiota. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 72, 1474–1482.

Mammucari, C., Milan, G., Romanello, V., Masiero, E., Rudolf, R., Del Piccolo, P., et al. (2007). FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab.* 6, 458–471. doi: 10.1016/j.cmet.2007.11.001

Mangiola, F., Nicoletti, A., Gasbarrini, A., and Ponziani, F. R. (2018). Gut microbiota and aging. *Eur. Rev. Med. Pharmacol. Sci.* 22, 7404–7413.

Manickam, R., Oh, H. Y. P., Tan, C. K., Paramalingam, E., and Wahli, W. (2018). Metronidazole causes skeletal muscle atrophy and modulates muscle chronometabolism. *Int. J. Mol. Sci.* 19:2418. doi: 10.3390/ijms19082418

Manuel Marti, J., Martinez-Martinez, D., Rubio, T., Gracia, C., Pena, M., Latorre, A., et al. (2017). Health and disease imprinted in the time variability of the human microbiome. *mSystems* 2:e00144-16.

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M., Hold, G., et al. (2016). The gut microbiota and host health: a new clinical frontier. *Gut* 65, 330–339. doi: 10.1136/gutjnl-2015-309990

Marzetti, E., Calvani, R., Cesari, M., Buford, T. W., Lorenzi, M., Behnke, B. J., et al. (2013). Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials. *Int. J. Biochem. Cell Biol.* 45, 2288–2301. doi: 10.1016/j.biocel.2013.06.024

Marzetti, E., Calvani, R., Lorenzi, M., Tanganelli, F., Picca, A., Bossola, M., et al. (2016). Association between myocyte quality control signaling and sarcopenia in old hip-fractured patients: results from the Sarcopenia in HIp FracTure (SHIFT) exploratory study. *Exp. Gerontol.* 80, 1–5. doi: 10.1016/j.exger.2016.04.003

Mesinovic, J., Zengin, A., De Courten, B., Ebeling, P. R., and Scott, D. (2019). Sarcopenia and type 2 diabetes mellitus: a bidirectional relationship. *Diabetes Metab. Syndr. Obes. Targets Ther.* 12, 1057–1071. doi: 10.2147/dmso.s186600

Mitchell, C. J., Milan, A. M., Mitchell, S. M., Zeng, N., Ramzan, F., Sharma, P., et al. (2017). The effects of dietary protein intake on appendicular lean mass and muscle function in elderly men: a 10-wk randomized controlled trial. *Am. J. Clin. Nutr.* 106, 1375–1383. doi: 10.3945/ajcn.117.160325

Mitsuoka, T. (2014). Establishment of intestinal bacteriology. *Biosci. Microb. Food Health* 33, 99–116. doi: 10.12938/bmfh.33.99

Moore, D. R., Churchward-Venne, T. A., Witard, O., Breen, L., Burd, N. A., Tipton, K. D., et al. (2015). Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 70, 57–62. doi: 10.1093/gerona/glu103

Mottawea, W., Chiang, C.-K., Muelbauer, M., Starr, A. E., Butcher, J., Abujamel, T., et al. (2016). Altered intestinal microbiota-host mitochondria crosstalk in new onset Crohn's disease. *Nat. Commun.* 7:13419.

Munukka, E., Rintala, A., Toivonen, R., Nylund, M., Yang, B., Takanen, A., et al. (2017). Faecalibacterium prausnitzii treatment improves hepatic health and reduces adipose tissue inflammation in high-fat fed mice. *ISME J.* 11, 1667–1679. doi: 10.1038/ismej.2017.24

Nair, K. S. (2005). Aging muscle. *Am. J. Clin. Nutr.* 81, 953–963.

Neis, E. P. J. G., Dejong, C. H. C., and Rensen, S. S. (2015). The role of microbial amino acid metabolism in host metabolism. *Nutrients* 7, 2930–2946. doi: 10.3390/nu7042930

Ni Lochlainn, M., Bowyer, R. C. E., and Steves, C. J. (2018). Dietary protein and muscle in aging people: the potential role of the gut microbiome. *Nutrients* 10:929. doi: 10.3390/nu10070929

Nicoletti, C. (2015). Age-associated changes of the intestinal epithelial barrier: local and systemic implications. *Expert Rev. Gastroenterol. Hepatol.* 9, 1467–1469. doi: 10.1586/17474124.2015.1092872

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J.-Z., et al. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol.* 16:90. doi: 10.1186/s12866-016-0708-5

Ogawa, S., Yakabe, M., and Akishita, M. (2016). Age-related sarcopenia and its pathophysiological bases. *Inflamm. Regener.* 36, 17–17.

Partridge, L., Deelen, J., and Slagboom, P. E. (2018). Facing up to the global challenges of ageing. *Nature* 561, 45–56. doi: 10.1038/s41586-018-0457-8

Peng, L., Li, Z.-R., Green, R. S., Holzman, I. R., and Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of amp-activated protein kinase in Caco-2 cell monolayers. *J. Nutr.* 139, 1619–1625. doi: 10.3945/jn.109.104638

Petroni, M. L., Caletti, M. T., Dalle Grave, R., Bazzocchi, A., Gomez, M. P. A., and Marchesini, G. (2019). Prevention and treatment of sarcopenic obesity in women. *Nutrients* 11:1302. doi: 10.3390/nu11061302

Picca, A., Fanelli, F., Calvani, R., Mule, G., Pesce, V., Sisto, A., et al. (2018a). Gut dysbiosis and muscle aging: searching for novel targets against sarcopenia. *Mediat. Inflamm.* 2018:7026198.

Picca, A., Lezza, A. M. S., Leeuwenburgh, C., Pesce, V., Calvani, R., Bossola, M., et al. (2018b). Circulating mitochondrial DNA at the crossroads of mitochondrial dysfunction and inflammation during aging and muscle wasting disorders. *Rejuven. Res.* 21, 350–359. doi: 10.1089/rej.2017.1989

Picca, A., Ponziani, F. R., Calvani, R., Marini, F., Biancolillo, A., Coelho-Junior, H. J., et al. (2020). Gut microbial, inflammatory and metabolic signatures in older people with physical frailty and sarcopenia: results from the BIOSPHERE Study. *Nutrients* 12:65. doi: 10.3390/nu12010065

Quigley, E. M. M. (2013). Commentary: synbiotics and gut microbiota in older people - a microbial guide to healthy ageing. *Aliment. Pharmacol. Ther.* 38, 1141–1142. doi: 10.1111/apt.12495

Rajagopala, S. V., Vashee, S., Oldfield, L. M., Suzuki, Y., Venter, J. C., Telenti, A., et al. (2017). The human microbiome and cancer. *Cancer Prevent. Res.* 10, 226–234.

Rampelli, S., Candela, M., Turroni, S., Biagi, E., Collino, S., Franceschi, C., et al. (2013). Functional metagenomic profiling of intestinal microbiome in extreme ageing. *Aging Us* 5, 902–912. doi: 10.18632/aging.100623

Rodriguez-Bies, E., Bui Thanh, T., Navas, P., and Lopez-Lluch, G. (2016). Resveratrol primes the effects of physical activity in old mice. *Br. J. Nutr.* 116, 979–988. doi: 10.1017/s0007114516002920

Salarkia, N., Ghadamli, L., Zaeri, F., and Sabaghian Rad, L. (2013). Effects of probiotic yogurt on performance, respiratory and digestive systems of young adult female endurance swimmers: a randomized controlled trial. *Med. J. Islamic Republic Iran* 27, 141–146.

Salazar, N., Valdes-Varela, L., Gonzalez, S., Gueimonde, M., and De Los Reyes-Gavilan, C. G. (2017). Nutrition and the gut microbiome in the elderly. *Gut Microbes* 8, 82–97. doi: 10.1080/19490976.2016.1256525

Sato, E., Mori, T., Mishima, E., Suzuki, A., Sugawara, S., Kurasawa, N., et al. (2016). Metabolic alterations by indoxyl sulfate in skeletal muscle induce uremic sarcopenia in chronic kidney disease. *Sci. Rep.* 6:36618.

Shafiee, G., Keshtkar, A., Soltani, A., Ahadi, Z., Larijani, B., and Heshmat, R. (2017). Prevalence of sarcopenia in the world: a systematic review and meta- analysis of general population studies. *J. Diabet. Metab. Disord.* 16:21.

Shaw, S. C., Dennison, E. M., and Cooper, C. (2017). Epidemiology of sarcopenia: determinants throughout the lifecourse. *Calcified Tissue Int.* 101, 229–247. doi: 10.1007/s00223-017-0277-0

Shing, C. M., Peake, J. M., Lim, C. L., Briskey, D., Walsh, N. P., Fortes, M. B., et al. (2014). Effects of probiotics supplementation on gastrointestinal permeability, inflammation and exercise performance in the heat. *Eur. J. Appl. Physiol.* 114, 93–103. doi: 10.1007/s00421-013-2748-y

Siddharth, J., Chakrabarti, A., Pannerec, A., Karaz, S., Morin-Rivron, D., Masoodi, M., et al. (2017). Aging and sarcopenia associate with specific interactions between gut microbes, serum biomarkers and host physiology in rats. *Aging Us* 9, 1698–1720. doi: 10.18632/aging.101262

Singh, R. K., Chang, H.-W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., et al. (2017). Influence of diet on the gut microbiome and implications for human health. *J. Transl. Med.* 15:73.

Sonnenburg, J. L., and Backhed, F. (2016). Diet-microbiota interactions as moderators of human metabolism. *Nature* 535, 56–64. doi: 10.1038/nature18846

Stacchiotti, A., Favero, G., and Rodella, L. F. (2020). Impact of melatonin on skeletal muscle and exercise. *Cells* 9:288. doi: 10.3390/cells9020288

Steves, C. J., Bird, S., Williams, F. M. K., and Spector, T. D. (2016). The microbiome and musculoskeletal conditions of aging: a review of evidence for impact and potential therapeutics. *J. Bone Miner. Res.* 31, 261–269. doi: 10.1002/jbmr.2765

St-Jean-Pelletier, F., Pion, C. H., Leduc-Gaudet, J.-P., Sgarioto, N., Zovile, I., Barbat-Artigas, S., et al. (2017). The impact of ageing, physical activity, and pre-frailty on skeletal muscle phenotype, mitochondrial content, and intramyocellular lipids in men. *J. Cachexia Sarcopenia Muscle* 8, 213–228. doi: 10.1002/jcsm.12139

Takiishi, T., Fenero, C. I. M., and Camara, N. O. S. (2017). Intestinal barrier and gut microbiota: shaping our immune responses throughout life. *Tissue Barriers* 5:e1373208–e1373208.

Tang, G., Du, Y., Jia, J. S., and Yuan, W. J. (2020). The influence of gut microbiota on skeletal muscle metabolism in patients with chronic kidney disease and intervention strategies. *Zhonghua Nei Ke Za Zhi* 59, 326–328.

Tang, W. H. W., Kitai, T., and Hazen, S. L. (2017). Gut microbiota in cardiovascular health and disease. *Circ. Res.* 120, 1183–1196.

Tezze, C., Romanello, V., Desbats, M. A., Fadini, G. P., Albiero, M., Favaro, G., et al. (2017). Age-associated loss of OPA1 in muscle impacts muscle mass, metabolic homeostasis, systemic inflammation, and epithelial senescence. *Cell Metab.* 25, 1374–1389. doi: 10.1016/j.cmet.2017.04.021

Thevaranjan, N., Puchta, A., Schulz, C., Naidoo, A., Szamosi, J. C., Verschoor, C. P., et al. (2018). Age-associated microbial dysbiosis promotes intestinal permeability, systemic inflammation, and macrophage dysfunction (vol 21, pg 455, 2017). *Cell Host Microbe* 23, 570–570. doi: 10.1016/j.chom.2018.03.006

Ticinesi, A., Lauretani, F., Milani, C., Nouvenne, A., Tana, C., Del Rio, D., et al. (2017). Aging gut microbiota at the cross-road between nutrition, physical frailty, and sarcopenia: is there a gut-muscle axis?. *Nutrients* 9:1303. doi: 10.3390/nu9121303

Ticinesi, A., Lauretani, F., Tana, C., Nouvenne, A., Ridolo, E., and Nleschi, T. (2019a). Exercise and immune system as modulators of intestinal microbiome: implications for the gut-muscle axis hypothesis. *Exerc. Immunol. Rev.* 25, 8–19.

Ticinesi, A., Nouvenne, A., Cerundolo, N., Catania, P., Prati, B., Tana, C., et al. (2019b). Gut microbiota, muscle mass and function in aging: a focus on physical frailty and sarcopenia. *Nutrients* 11:1633. doi: 10.3390/nu11071633

Tieland, M., Trouwborst, I., and Clark, B. C. (2018). Skeletal muscle performance and ageing. *J. Cachexia Sarcopenia Muscle* 9, 3–19. doi: 10.1002/jcsm.12238

Vaiserman, A. M., Koliada, A. K., and Marotta, F. (2017). Gut microbiota: a player in aging and a target for anti-aging intervention. *Age. Res. Rev.* 35, 36–45. doi: 10.1016/j.arr.2017.01.001

van Nood, E., Vrieze, A., Nieuwdorp, M., Fuentes, S., Zoetendal, E. G., De Vos, W. M., et al. (2013). Duodenal infusion of donor feces for recurrent clostridium difficile. *N. Engl. J. Med.* 368, 407–415. doi: 10.1056/nejmoa1205037

Vandeputte, D., Falony, G., Vieira-Silva, S., Wang, J., Sailer, M., Theis, S., et al. (2017). Prebiotic inulin-type fructans induce specific changes in the human gut microbiota. *Gut* 66, 1968–1974. doi: 10.1136/gutjnl-2016-313271

Villa, I., Senesi, P., Montesano, A., Ferraretto, A., Vacante, F., Spinello, A., et al. (2017). Betaine promotes cell differentiation of human osteoblasts in primary culture. *J. Transl. Med.* 15:132.

Vulevic, J., Drakoularakou, A., Yaqoob, P., Tzortzis, G., and Gibson, G. R. (2008). Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. *Am. J. Clin. Nutr.* 88, 1438–1446.

Walsh, M. E., Bhattacharya, A., Sataranatarajan, K., Qaisar, R., Sloane, L., Rahman, M. M., et al. (2015). The histone deacetylase inhibitor butyrate improves metabolism and reduces muscle atrophy during aging. *Aging Cell* 14, 957–970. doi: 10.1111/acel.12387

Watanabe, M., Houten, S. M., Mataki, C., Christoffolete, M. A., Kim, B. W., Sato, H., et al. (2006). Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 439, 484–489. doi: 10.1038/nature04330

Weiss, G. A., and Hennet, T. (2017). Mechanisms and consequences of intestinal dysbiosis. *Cell. Mol. Life Sci.* 74, 2959–2977. doi: 10.1007/s00018-017-2509-x

Welch, A. A. (2014). Nutritional influences on age-related skeletal muscle loss. *Proc. Nutr. Soc.* 73, 16–33. doi: 10.1017/s0029665113003698

Wiese, M., Bashmakov, Y., Chalyk, N., Nielsen, D. S., Krych, L., Kot, W., et al. (2019). Prebiotic effect of lycopene and dark chocolate on gut microbiome with systemic changes in liver metabolism, skeletal muscles and skin in moderately obese persons. *Biomed. Res. Int.* 2019:4625279.

Wikoff, W. R., Anfora, A. T., Liu, J., Schultz, P. G., Lesley, S. A., Peters, E. C., et al. (2009). Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3698–3703. doi: 10.1073/pnas.0812874106

Wilkinson, D. J., Piasecki, M., and Atherton, P. J. (2018). The age-related loss of skeletal muscle mass and function: measurement and physiology of muscle

fibre atrophy and muscle fibre loss in humans. *Age. Res. Rev.* 47, 123–132. doi: 10.1016/j.arr.2018.07.005

Yan, H., Diao, H., Xiao, Y., Li, W., Yu, B., He, J., et al. (2016). Gut microbiota can transfer fiber characteristics and lipid metabolic profiles of skeletal muscle from pigs to germ-free mice. *Sci. Rep.* 6:31786.

Zhang, J., Guo, Z., Xue, Z., Sun, Z., Zhang, M., Wang, L., et al. (2015). A phylo-functional core of gut microbiota in healthy young Chinese cohorts across lifestyles, geography and ethnicities. *ISME J.* 9, 1979–1990. doi: 10.1038/ismej.2015.11

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 Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 10 December 2020
doi: 10.3389/fbioe.2020.614419

Anti-glioblastoma Activity of Kaempferol via Programmed Cell Death Induction: Involvement of Autophagy and Pyroptosis

Suqin Chen[1], *Jing Ma*[1], *Liu Yang*[1], *Muzhou Teng*[2], *Zheng-Quan Lai*[3], *Xiaoyu Chen*[4] *and Jingjin He*[1]*

[1] *The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, China,* [2] *School of Basic Medical Sciences, Southern Medical University, Guangzhou, China,* [3] *Department of Pharmacy, Shenzhen University General Hospital, Shenzhen University, Shenzhen, China,* [4] *Department of Neurology, The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, China*

Glioblastoma is one of the most common and lethal intracranial malignant, and is still lack of ideal treatments. Kaempferol is a major nutrient found in various edible plants, which has exhibited the potential for the treatment of glioblastoma. However, the specific anti-glioma mechanism of kaempferol is yet to be studied. Herein, we aim to explore the mechanisms underlying the anti-glioma activity of kaempferol. Our results demonstrated that kaempferol suppresses glioma cell proliferation *in vitro* and inhibits tumor growth *in vivo*. Moreover, kaempferol raises ROS and decreases mitochondrial membrane potential in glioma cells. The high levels of ROS induce autophagy then ultimately trigger the pyroptosis of glioma cells. Interestingly, when we used 3-MA to inhibit autophagy, we found that the cleaved form of GSDME was also decreased, suggesting that kaempferol induces pyroptosis through regulating autophagy in glioma cells. In conclusion, this study revealed kaempferol possesses good anti-glioma activity by inducing ROS, and subsequently leads to autophagy and pyroptosis, highlighting its clinical potentials as a natural nutrient against glioblastoma.

Keywords: kaempferol, glioblastoma, ROS, autophagy, pyroptosis

OPEN ACCESS

Edited by:
Yi Cao,
Xiangtan University, China

Reviewed by:
Yongsheng Chen,
Jinan University, China
Feifei An,
Xi'an Jiaotong University, China

***Correspondence:**
Jingjin He
he_jingjin@hotmail.com

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *06 October 2020*
Accepted: *17 November 2020*
Published: *10 December 2020*

Citation:
Chen S, Ma J, Yang L, Teng M, Lai Z-Q, Chen X and He J (2020) Anti-glioblastoma Activity of Kaempferol via Programmed Cell Death Induction: Involvement of Autophagy and Pyroptosis. Front. Bioeng. Biotechnol. 8:614419. doi: 10.3389/fbioe.2020.614419

INTRODUCTION

Glioblastoma (GBM) is one of the most aggressive type of cancers in central nervous system with poor prognosis. Patients diagnosed with GBM have the median survival time range from 5 to 15 months while the 5-year survival rates range from 0 to 5% (Wen and Kesari, 2008; Tsitlakidis et al., 2010; Ostrom et al., 2017). Despite tremendous effort has been devoted to develop novel cancer therapies, the treatment of GBM remained relatively unchanged for decades and consists of surgical resection followed by adjuvant chemoradiotherapy. Currently, Temozolomide (TMZ), a first-line drug in the treatment of GBM, is the most effective regimen that increases the median overall survival of GBM patients from 12 to 14.6 months, and increases the 2-year survival rate from 10.4 to 26.5%(Stupp et al., 2005). However, tumors will inevitably recur in most of cases and seem

to be resistant to even higher dose of chemotherapy regimens (Stupp et al., 2009; Gilbert et al., 2013). Besides, traditional chemotherapy drugs usually cause certain side effects. Therefore, it is urgently needed to develop natural anti-tumor drugs with unique curative and low toxicity side effects.

A large number of natural products derived from plants have shown great promise in treating or preventing cancer in recent years. Flavonoids is a type of natural compounds found abundantly in many herbal medicines which possesses multiple pharmacological activities. Kaempferol is one of the most common aglycone flavonoids in the form of glycoside. It can be found in many edible plants, such as tea (**Figure 1A**), most vegetables and fruits. Kaempferol has a variety of beneficial biological properties (Harborne and Williams, 2000; Rajendran et al., 2014; Imran and Salehi, 2019), including anti-oxidant, anti-carcinogenic, and anti-inflammatory. According to epidemiologic studies, high intake of kaempferol is associated with decreased risk of different types of cancers (Garcia-Closas et al., 1999; Nöthlings et al., 2007; Cui et al., 2008). Although kaempferol has shown anti-glioma effects *in vitro* (Sharma et al., 2007; Siegelin et al., 2008; Jeong et al., 2009), its *in vivo* anti-glioma activity and the specific mechanism have not been fully elucidated yet.

As we all know, there are several cell death types in cancer treatment, including necrosis, apoptosis, necroptosis, autophagy and pyroptosis. Pyroptosis is an inflammatory form of programmed cell death activated by some inflammasomes. Gasdermin D and Gasdermin E, as the essential mediators of pyroptosis, can be separately cleaved by caspase-1/4/5/11 and caspase-3 into GSDMD-NT and GSDME-NT, which further connects to the cell membranes and triggers oligomerization, forming the pores and leading the leakage of inflammatory substances into intercellular space (Schroder and Tschopp, 2010; Kayagaki et al., 2015; Fang et al., 2020). The activation of gasdermin E in chemotherapy can change the morphology of the dying tumor cells from apoptosis into a pyroptosis-like death (Wang et al., 2017). Autophagy is a process that cells use to recycle intracellular components to sustain metabolism and survival by which cellular material is delivered to lysosomes for degradation. Although autophagy normally promotes cell survival and prevents cancer development (Galluzzi et al., 2015), in advanced cancers, both enhancing and inhibiting autophagy can kill cancer cells. Moreover, inhibiting different stages of autophagy may have the opposite effect in a same cancer (Amaravadi et al., 2016). Autophagy can either promote or inhibit apoptosis under different cellular contexts, the mechanisms underlying these opposing effects are related to the degradation of different pro-apoptotic or anti-apoptotic regulators by autophagy.

It has been reported that increasing intracellular oxidative stress and inducing apoptosis were considered to be one of the anti-glioma mechanisms of kaempferol (Sharma et al., 2007; Jeong et al., 2009). Since ROS could induce a variety of complex cascades, including autophagy and pyroptosis. Therefore, in this study, we aimed to explore the anti-glioma effect of kaempferol *in vitro* and *in vivo*, and further investigate the mechanism of inducing glioma cell death, especially the involvement of autophagy and pyroptosis.

MATERIALS AND METHODS

Reagents

Kaempferol (≥97%, HPLC grade), Thiazolyl Blue Tetrazolium Bromide (MTT), and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). 2′, 7′-Dichlorodihydrofluorescein diacetate (H2DCFDA), Hoechst 33342, N-acetyl-L-cysteine (NAC), Lyso-Tracker Green, and MitoTracker Red CMXRos were obtained from Beyotime Biotechnology (Shanghai, China). Annexin V-FITC Apoptosis Detection kit was provided by BD Biosciences (Franklin Lake, New Jersey, USA). Necrosulfonamide (NSA) was obtained from MCE (New Jersey, USA).

Animals and CDX Model

The anti-glioma effect of kaempferol *in vivo* was conducted on 6-week old male immune-deficient BALB/c Nude Mice (purchased from the laboratory of Southern medical University). Mice were kept in SPF level animal room. All the investigations were approved by the Animal Care and Institutional Ethics Committee of Sun-Yat Sen University and conformed to the US National Institutes of Health (Bethesda, MD, USA) Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines.

Six BALB/c Nude Mice were randomly divided into two groups (with Two biological repetitions), and U87 MG cells (10^7/100 μL PBS/mouse) were injected subcutaneously to establish cell line derived xenografts (CDX) models. Mice were checked every other day to monitor the tumor growth and the weight. Then the kaempferol treatment group were received kaempferol (40 mg/kg, dissolved in plant oil) by gavage daily for 3 weeks, while the control group only received the plant oil instead. When the experiment was terminated, mice were euthanized by overdose anesthesia. The tumor tissues and the viscera were removed for further analysis.

Cell Culture

U87 MG and U251 Glioblastoma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured with DMEM (Gibco BRL, Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Gibco BRL, Invitrogen, Australia, USA) at 37°C with a 5% CO_2–humidified atmosphere.

Measurement of Cell Viability

Cell viability was evaluated using MTT assay as previous described (Liu et al., 1997). U87 MG and U251 cells were seeded on 96-well-plates at a density of 5.0×10^3 cells/well. After adhesion, cells were treated with different concentrations (0, 20, 40, 80, and 120 μM) of kaempferol for 24 h. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. Then the cells were incubated for 4 h at 37°C, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 100 μL of dimethyl sulfoxide and the absorbance of each well was measured at 570 nm with microplate Reader (Varioskan Lux, Thermo Scientific, USA). Values were expressed as a percentage relative to those obtained in controls. The ratio of viability of control cells in the absence of kaempferol was taken as 100%.

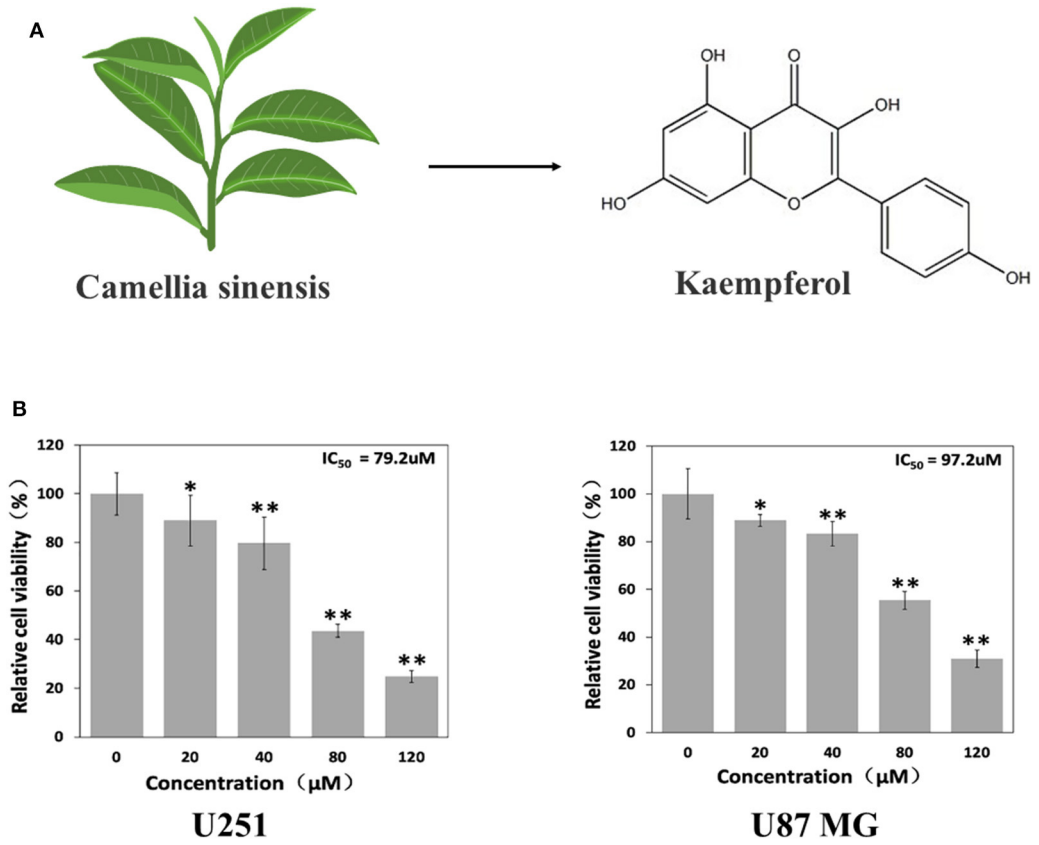


FIGURE 1 | Kaempferol suppressed glioma cells. **(A)** Structure of kaempferol. **(B)** U251 and U87 MG cells were treated with different concentrations of kaempferol for 24 h, and cell viability was assessed by MTT assay, $n = 6$. Data was presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using JC-1 probe. For flow cytometric analysis of JC-1 Staining, U87 MG and U251 cells were cultured in 6-well-plates and treated with different concentrations (0, 40, and 80 μM) of kaempferol for 24 h. Then, the cells were collected and stained with 500 μL 1×JC-1 staining solution and 500 μL of DMEM for 30 min at room temperature, and the stained cells were tested using a BD LSRFortessa flow cytometer with emission at 590 and 529nm. JC-1 accumulates as J-aggregates (590 nm; red) only in metabolically active mitochondria and depolarization of mitochondrial membranes leads to JC-1 monomer formation (527 nm; green). For fluorescence image assay, U87 MG and U251 cells were cultured in cover glass bottom dishes and treated with 80 μM of kaempferol for 24 h. After stimulation, the cells were stained with JC-1 staining solution (1×) for 30 min and then observed and photographed using a Confocal microscope (LSM 880 with fast Airyscan, Zeiss, Germany).

Measurement of ROS

Intracellular ROS generation in cells treated with kaempferol was assessed using H2DCFDA probe. After treating with 80 μM of kaempferol for 24 h, Cells cultured in cover glass bottom dishes were incubated with 10 μM H2DCFHDA for 30 min at 37°C and washed twice with PBS. Then cells were observed and photographed using a Confocal microscope (LSM 880 with fast Airyscan, Zeiss, Germany).

Assays for Mitochondrial Membrane Potential and Autophagy

U87 MG and U251 cells were cultured in cover glass bottom dishes overnight before treating with or without kaempferol (80 μM) for 24 h. Then, the cells were stained with MitoTracker Red CMXRos working solutions (100 nM) and LysoTracker Green working solutions (100 nM) for 1 h. After that, the cells were stained with Hoechst working solutions (1×) or 4′,6′-diamidino-2-phenyl-indole (DAPI) working solutions (1×) for another 20 min. Then, the cells were imaged with a confocal microscope (LSM 880 with fast Airyscan, Zeiss, Germany).

Western Blotting

Western blotting was used to determine the protein expression level. U87 MG and U251 cells cultured in 6-well-culture plates were treated with different concentrations (0, 40, and 80 μM) of kaempferol for 48 h. The samples of whole-cell protein were prepared with radioimmunoprecipitation assay (RIPA)

buffer containing protease/phosphatase inhibitor cocktail (CST), separated by 10–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Millipore). Antibodies including PARP (46D11, CST), LC3 (3868s, CST), γH2AX (9718s, CST), β-actin (4967s, CST), p62/SQSTM1 (18420-1-AP, Proteintech), α-tubulin (5886s, CST), and gasdermin E (GSDME) (ab215191, Abcam) were used in this study. After addition of chemiluminescence reagent (Thermo), the blots were exposed to ChemiDoc Touch imaging System (Singapore) and the images were analyzed using Image J version 1.52 (National Institutes of Health).

Real-Time-Polymerase Chain Reaction

The mRNA expressions were detected using quantitative polymerase chain reaction (qPCR) assay. Briefly, total RNA was isolated using RNeasy Mini Kit (Qiagen) and reversely transcribed to cDNA via PrimerScript RT reagent Kit with gDNA Eraser (Takara). The primer sequences used in qPCR are as follows, IL-1β: 5′-AGCTACGAATCTCCGACCAC-3′ and 5′-CGTTATCCCATGTGTCGAAGAA-3′; ASC: 5′-TGGATGCTCTGTACGGGAAG-3′ and 5′-CCAGGCTGGTGT-GAAACTGAA-3′. We used TB Green Premix (Takara) to performed qPCR according to the directions.

Statistical Analysis

Analyses were managed using SPSS version 17.0 (USA). Data in this study were presented as mean ± standard deviation (SD) and analyzed using the *T*-test analysis or Repeated Measures ANOVA. $^{*}P < 0.05$ was considered to be statistically significant. All experiments were performed in triplicate at a minimum.

RESULT

Kaempferol Suppressed Human GBM Cells *in vitro*

To evaluate whether kaempferol affects viability of GBM cells, MTT assay was done on U87MG and U251 cells. As shown in **Figure 1B**, kaempferol showed a dose-dependent manner in inhibiting GBM proliferation. The half maximal inhibitory concentration (IC50) of kaempferol against U87MG and U251 cells was 97.2 and 79.2 μM, respectively.

Kaempferol Suppressed GBM Growth *in vivo*

The nude-mouse CDX model was used to investigate the anti-glioma effect of kaempferol *in vivo*. The result showed that tumor size was significantly suppressed in mice treated with kaempferol when compared with the control group (**Figures 2A,B**). The weight of mice was measured and found that kaempferol has no detectable impact on the weight of nude-mice at the dose of 40 mg/kg (**Figure 2C**).

Kaempferol Induced Reactive Oxygen Species Generation

Reactive Oxygen Species (ROS) is one of the causes for apoptosis. Many chemotherapeutic drugs kill cancer cells by inducing ROS generation. To explore whether kaempferol increases ROS levels in GBM cells, we used fluorescent probe H2DCFDA to measure the levels of ROS in GBM cells. Our results showed that kaempferol induced ROS generation in both U87MG and U251 cells (**Figure 3A**). Apoptosis assay and WB result of γH2AX indicated that kaempferol could induce apoptosis via triggering DNA damage (**Supplementary Figures 1A–C**), which was consistent with previous reports (Sharma et al., 2007).

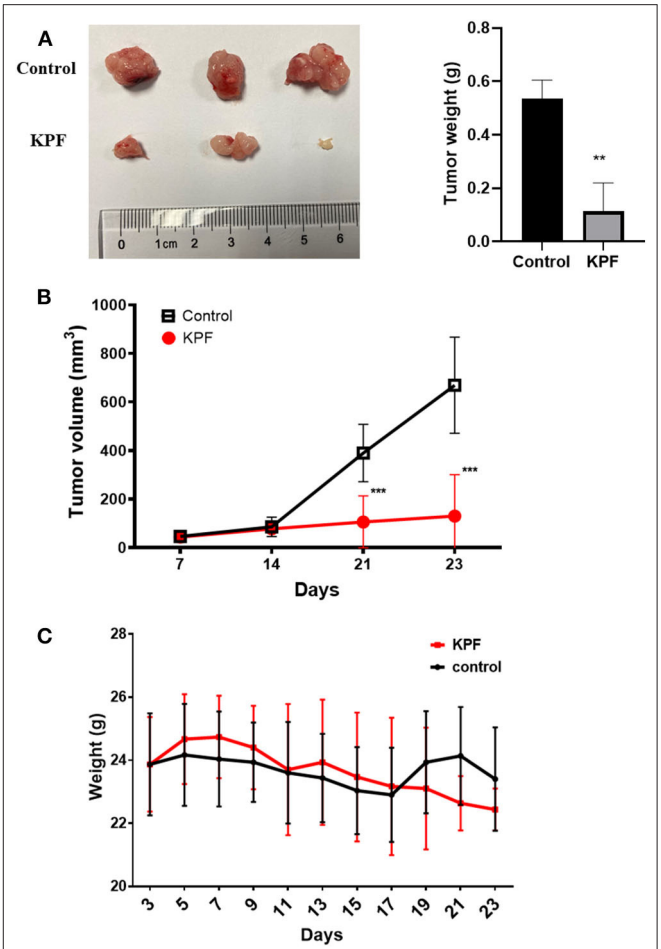


FIGURE 2 | Kaempferol inhibited glioma growth *in vivo* ($n = 3$, with two biological duplication). **(A)** Tumor weight compared between control and kaempferol treatment group. **(B)** Comparison of the tumor volume between control and kaempferol treatment group. **(C)** The body weight changes of mice in control group and kaempferol treatment group were compared. Data was presented as mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control.

Kaempferol Decreased Mitochondrial Membrane Potential in Human GBM Cells

Since ROS generation is inversely correlated with mitochondrial membrane potential by disrupting the outer mitochondrial potential to release the death-promoting proteins, we investigated mitochondrial changes by JC-1 staining. In intact mitochondria, JC-1 aggregates in the matrix of mitochondria

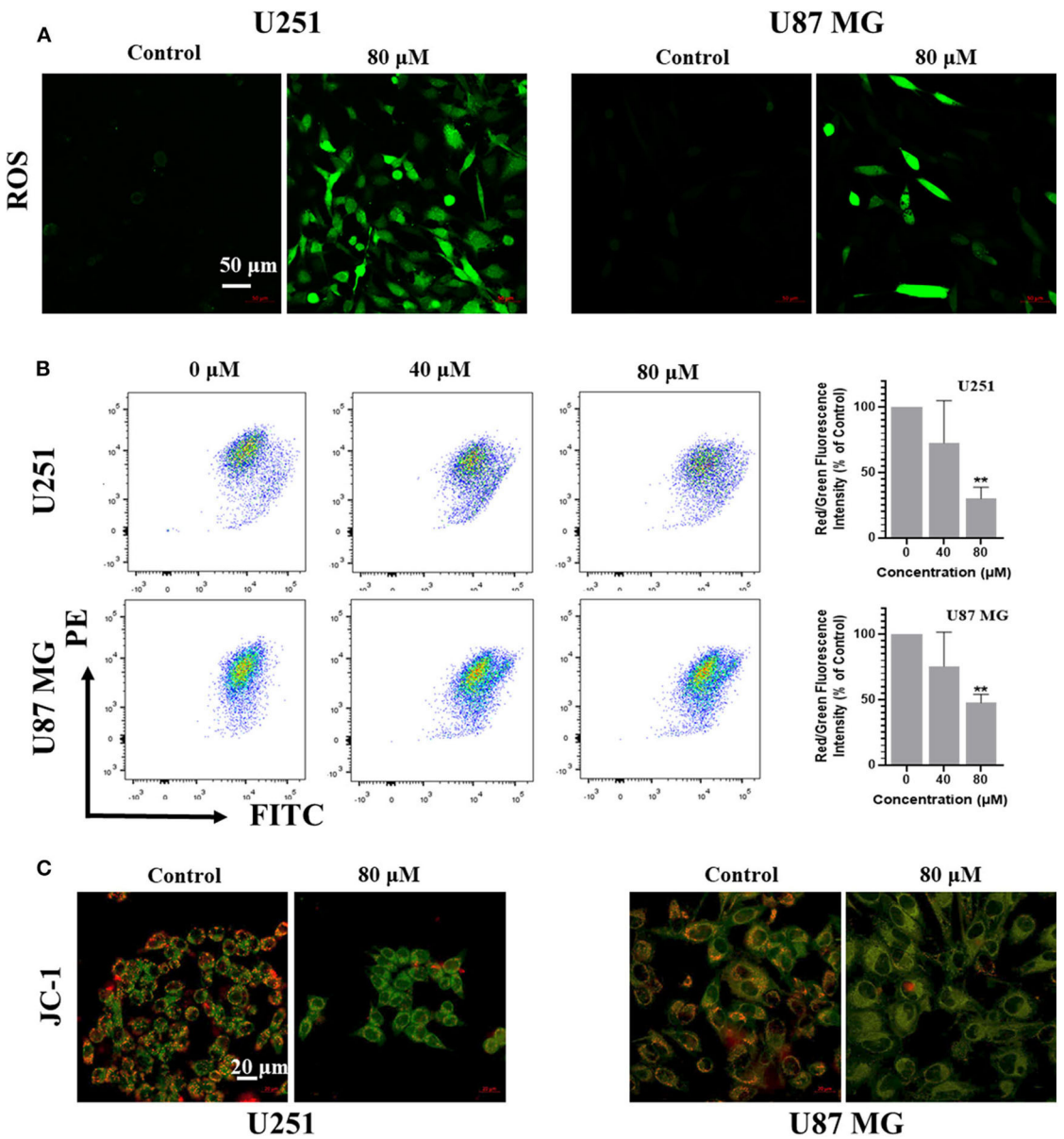


FIGURE 3 | Kaempferol inhibited IL-6 secretion, induced ROS generation, and decreased mitochondrial membrane potential in glioma cells. **(A)** ROS was detected by DCFDA-H2 staining. **(B)** Mitochondrial membrane potential was measured by JC-1 probe. Red/green fluorescence intensity was measured by flow cytometry. **(C)** Fluorescence photographs of mitochondrial membrane potential in glioma cells. Data was presented as mean ± SD. $^{**}P < 0.01$ compared with the control.

to form polymers, which can produce red fluorescence; while the membrane potential of mitochondria is low, JC-1 is not able to gather in the matrix of mitochondria, results in the form of monomer and produce green fluorescence. A significant reduction of mitochondrial membrane potential in a dose-dependent manner was observed in kaempferol-treated U87MG and U251 cells (**Figures 3B,C**), indicating that kaempferol induces ROS generation and decreases mitochondrial membrane potential in GBM cells.

Kaempferol Induced Autophagy in Human GBM Cells

The role of autophagy in tumor development is complicated, it can be tumor-suppressing or tumor-promoting at different tumor stage or tumor microenvironment condition (Amaravadi et al., 2016). Autophagy can also be triggered by ROS and cross-link with apoptosis or pyroptosis via regulating the degradation of the proteins involving in the different pathways (Su et al., 2015). To investigate whether kaempferol could induce autophagy in GBM cells, we detected the level of several

autophagy markers, ubiquitin-like molecule Light chain 3 (LC3) and Sequestosome 1 (SQSTM1, also known as p62). Cleaved LC3 will further lead to form phagophore and autophagosome. The LC3-binding protein p62 is a specific substrate for autophagy. Extensive accumulation of p62 as a scaffold protein is associated with several signal pathways, including apoptosis. Immunofluorescence images showed that kaempferol treatment could increase LC3 expression in GBM cells (**Figure 4A**). Further quantitative analysis by western blotting showed that kaempferol treatment led to a conversion from LC3-I to LC3-II and increased the expression of p62 in a dose-dependent manner (**Figure 4B**), which supported that kaempferol induces autophagy in GBM cells.

Mitophagy is a form of autophagy that selectively degrades damaged or aged mitochondria, which occurred especially when the mitochondrial membrane potential decreased. Since our previous studies has confirmed that kaempferol could decrease mitochondrial membrane potential and induce autophagy, we wondered if kaempferol could induce mitophagy in GBM cells. MitoTracker Red is a red fluorescent probe, which is specifically used to stain mitochondria in living cell. LysoTracker Green is a Green fluorescent labeled lysosome probe, which can selectively

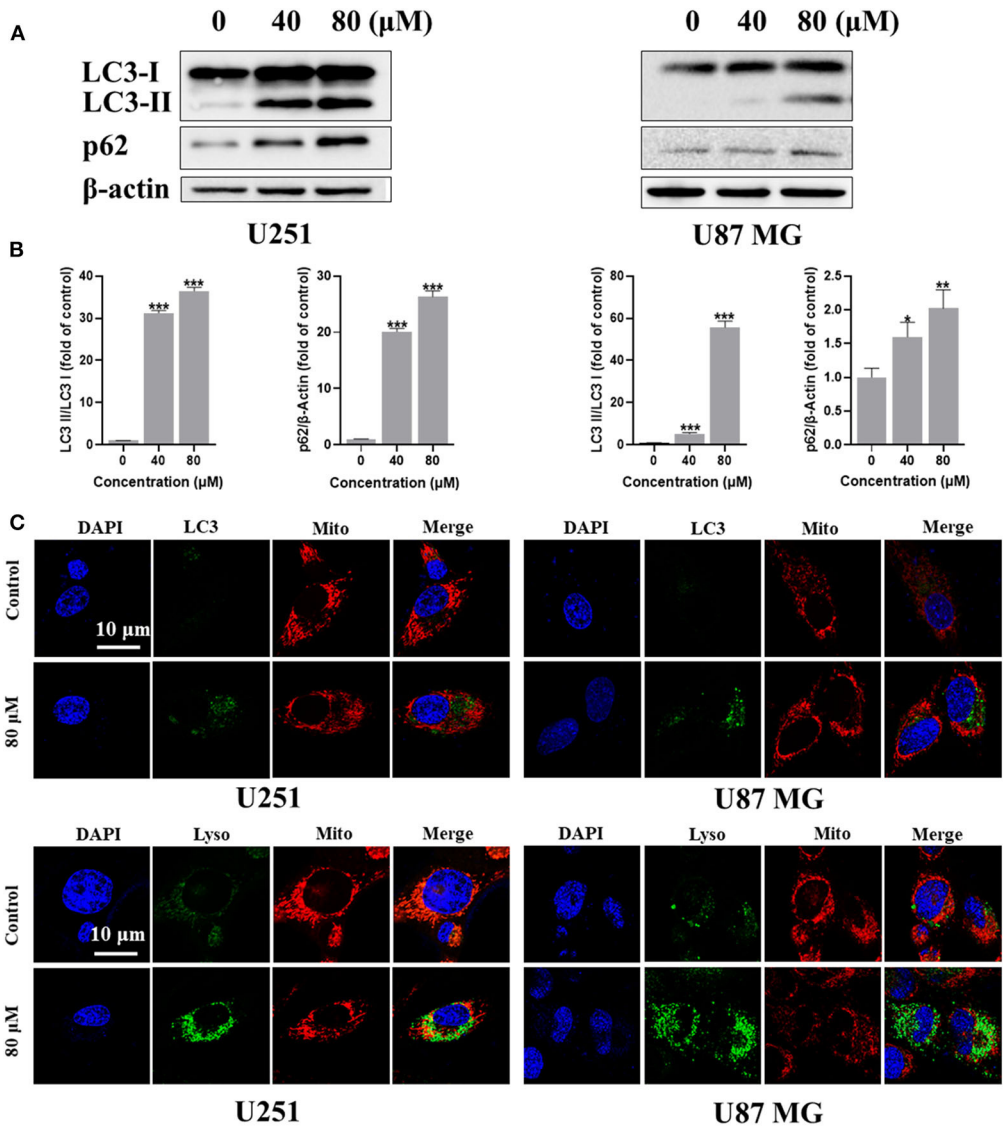


FIGURE 4 | Kaempferol induced autophagy in U251 and U87 MG cells. **(A)** The protein expression of LC3-I, LC3-II, and p62 detected by Western blotting. **(B)** Immunofluorescence images of the LC3 and mitochondria. **(C)** U251 and U87 MG cells stained with MitoTracker Red and LysoTracker Green. Lyso: lysosome; and Mito: mitochondria. Data was presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control.

stain the acid regions in living cells. LysoTracker Green and MitoTracker Red co-staining showed that kaempferol increased the expression and colocalization of lysosomes and mitochondria (**Figure 4C**), indicating kaempferol could induce mitophagy in GBM cells.

Kaempferol Induced Pyroptosis in Human GBM Cells

ROS serves as an important inflammasome activation signal, while inflammasome activation could further lead to pyroptosis, a process of programmed cell death distinct from apoptosis through activation of caspase and further leading to activation of inflammatory cascade. Based on our findings that kaempferol induces ROS generation, we were curious to know whether kaempferol could induce pyroptosis in GBM cells. There are three kinds of activation pathways in pyroptosis, canonical inflammasome pathway, noncanonical inflammasome pathway and a new-found GSDME-mediated pathway. GSDME-mediated pyroptosis shares some early stage activation molecular with apoptosis, such as caspase 3. We wondered if kaempferol can induced apoptosis and trigger pyroptosis while activating the same pro-caspase3, so we detected the cleaved form of GSDME. As shown in **Figure 5A**, kaempferol could increase the cleavage levels of GSDME, which suggested kaempferol could induce pyroptosis in GBM cells. We also measured the mRNA expression of the proinflammatory factor IL-1β and ASC, which presented that the mRNA levels of IL-1β and ASC increased after a 24 h treatment of kaempferol (**Figure 5B**).

Kaempferol Induced Pyroptosis Through Regulating Autophagy in Human GBM Cells

In order to clarify the relationship between autophagy and pyroptosis, we blocked ROS generation and autophagy pathway by using antioxidant reagent N-acetyl-L-cysteine (NAC) and PI3K inhibitor 3-Methyladenine (3-MA), respectively. The result showed that NAC reduced the levels of kaempferol-inducing ROS in both U87MG and U251 cells (**Figure 6A**). Furthermore, we found that NAC reversed autophagy by decreasing the cleavage

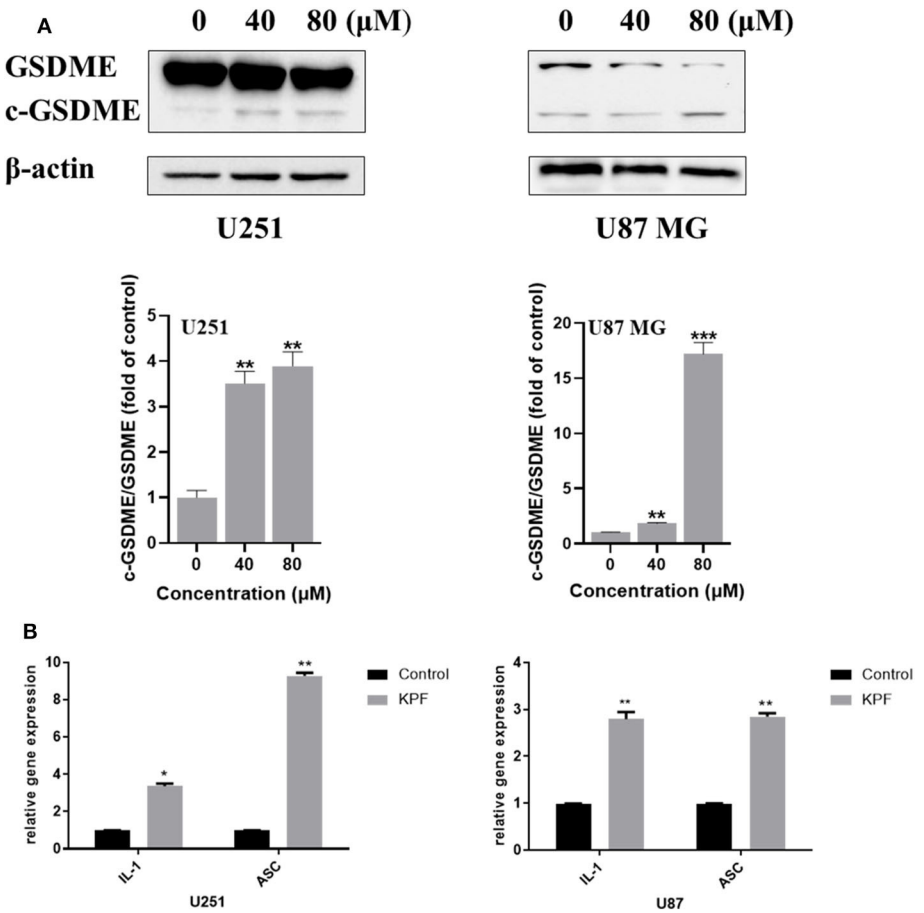


FIGURE 5 | Kaempferol induced pyroptosis in glioma cells. **(A)** Western blotting showed the protein expression of GSDME, and c-GSDME in U251 and U87 MG cells. **(B)** qPCR showed the mRNA expression of IL-1β and ASC in glioma cells. Data was presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the control.

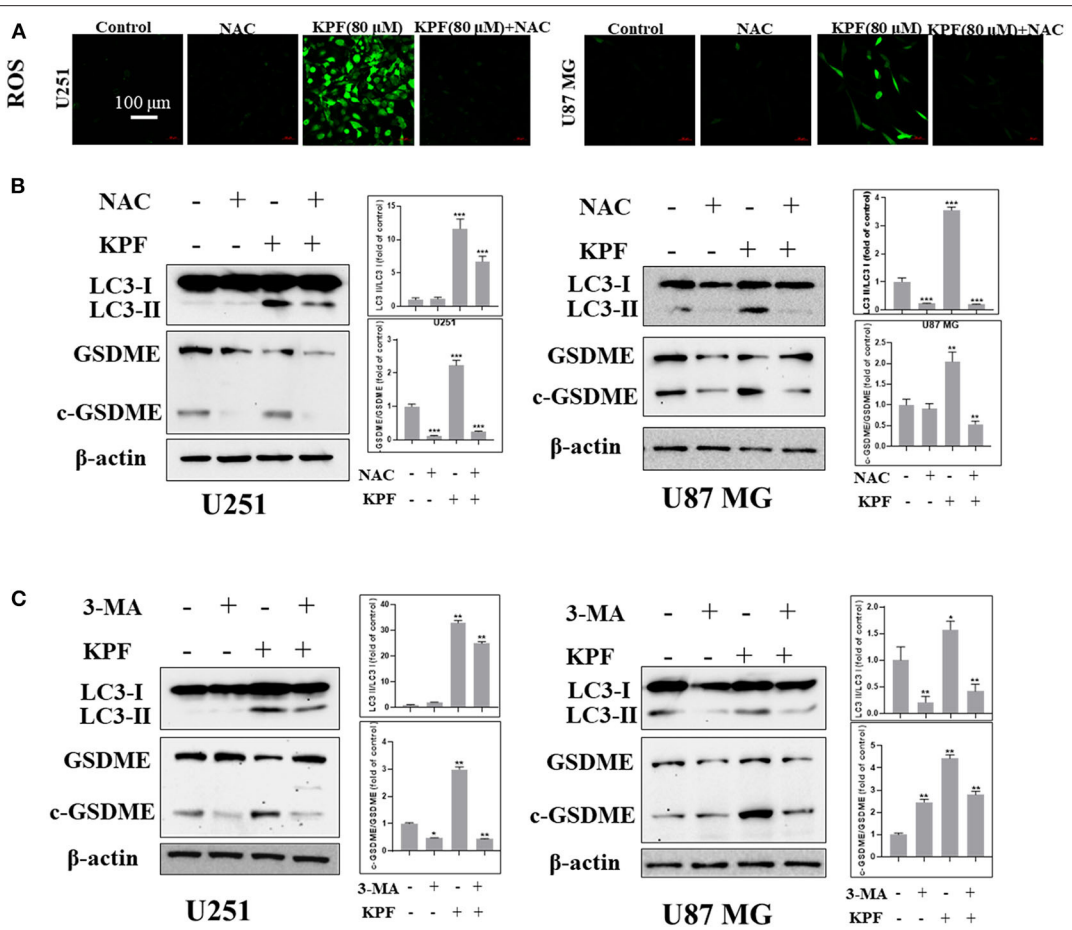


FIGURE 6 | Kaempferol induced pyroptosis through regulating autophagy in glioma cells. **(A,B)** U251 and U87 MG cells were treated with or without NAC (5 mM) for 2 h before a 12 h treatment of kaempferol (80 μM). **(A)** ROS was detected by DCFDA-H2 staining. **(B)** Western blotting showed the protein expression of LC3, GSDME, and c-GSDME in glioma cells. **(C)** Glioma cells were treated with or without 3-MA (5 mM) for 2 h before a 12 h treatment of kaempferol (80 μM), the expression of LC3, GSDME, and c-GSDME was detected. Data was presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the control.

levels of LC3 and reversed the pyroptosis by decreasing the cleavage levels of GSDME (**Figure 6B**), which indicates that ROS generation induced by kaempferol contributed to autophagy and pyroptosis in GBM cells. We further used 3-MA to inhibit the pathway of autophagy, and found that the levels of cleaved form of LC3 was decreased as expected, while the levels of cleaved form of GSDME also decreased (**Figure 6C**), suggesting autophagy induced by kaempferol may lead to pyroptosis in GBM cells.

DISCUSSION

Cancer is one of the biggest challenges to human health and the second major cause of death in the world (Bray et al., 2018). Most of the current chemotherapy drugs have systemic toxicity, which limits their application in treating certain type of cancers. Increasing evidence showed that diverse compounds derived from natural products exhibited anti-cancer activities (Cragg and Pezzuto, 2016). Most of these agents are abundantly present in different plant-based food items, with low toxicity and multiple biological activities, including antioxidant, antimetastatic and anti-inflammatory activities, making them good candidates as new antitumor drugs.

Flavonoids are widely present in plants in response to microbial infection, with the structure of 2-phenylchromone and a keto carbonyl group in their molecules, flavonoids have a variety of biological activities, including antioxidant, anti-inflammatory, anti-tumor, antibacterial and antiviral activities. As one of the most common flavonoids, with broad range of activity, and low toxicity compared with other compounds, kaempferol is widely reported (Imran et al., 2019). Glioblastoma is one of the most invasive and aggressive brain tumors, most of them have high resistance against current therapies (Stupp et al., 2005). It has been reported that kaempferol inhibited both growth and migration of GBM cells, and triggered ROS generation and apoptosis (Sharma et al., 2007; Jeong et al., 2009). However, the underlying mechanisms of kaempferol in inducing glioma cell death are still elusive. Here, we verified that kaempferol could significantly inhibit glioma *in vitro* and *in vivo* via triggering ROS and further inducing autophagy and pyroptosis besides the commonly known apoptosis.

Increased generation of intracellular ROS may induce programmed cell death, such as apoptosis, autophagy and pyroptosis, via execution by lysosomal proteases or caspases (Li et al., 2015). Autophagy is a highly sensitive cellular process which can be induced in response to a wide range of stresses. A number of studies have suggested that ROS induces autophagy as upstream modulators (Filomeni et al., 2010; Szumiel, 2011). In this study, we found that inhibition of ROS can reverse the autophagy induced by kaempferol in human glioblastoma cells, indicating that ROS generation induced by kaempferol contributed to autophagy.

Pyroptosis is a type of programmed cell death with the feature of pro-inflammatory and can be triggered by increasing ROS and the subsequent activation of inflammasome-caspase-1-IL-1β signaling. GSDME belongs to the gasdermin superfamily and cleavage form of GSDME is an important marker of pyroptosis (Wang et al., 2018). In this study, we found that blocking ROS generation could reverse pyroptosis, suggesting that kaempferol induced ROS and further contributing to GSDME-mediated pyroptosis in glioma cells. Based on our current findings, kaempferol could induce ROS and further lead to both autophagy and pyroptosis. We were curious that whether pyroptosis was functionally associated with autophagy. The further results showed that inhibition of autophagy using 3-MA could reverse pyroptosis, demonstrating that pyroptosis induced by kaempferol could be regulated by autophagy in glioma cells.

In conclusion, kaempferol significantly inhibits the proliferation of GBM cells both *in vitro* and *vivo* without obvious toxicity and side effects. In addition to the common apoptosis, kaempferol cause ROS and autophagy, which further leads to pyroptosis in GBM cells. Taken together, kaempferol represents a promising new anti-glioma candidate by inducing apoptosis and triggering ROS-mediated pyroptosis through the regulation of autophagy in GBM cells.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

JH and SC conceived of the original research idea. SC and JH performed the majority of experiments with the help of JM, LY, Z-QL and XC. MT helped with the animal experiments. SC and JH interpreted the data. SC and JH were responsible for the initial draft of the manuscript, whereas other authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (81900238), the China Postdoctoral Science Foundation funded project (2018M643300), and the Shenzhen Scientific Research Project of Health and Family Planning System (SZBC2018002).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.614419/full#supplementary-material

REFERENCES

Amaravadi, R., Kimmelman, A. C., and White, E. (2016). Recent insights into the function of autophagy in cancer. *Genes Dev* 30, 1913–1930. doi: 10.1101/gad.287524.116

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Cragg, G. M., and Pezzuto, J. M. (2016). Natural products as a vital source for the discovery of cancer chemotherapeutic and chemopreventive agents. *Med Princ Pract.* 25(Suppl 2), 41–59. doi: 10.1159/000443404

Cui, Y., Morgenstern, H., Greenland, S., Tashkin, D. P., Mao, J. T., Cai, L., et al. (2008). Dietary flavonoid intake and lung cancer–a population-based case-control study. *Cancer* 112, 2241–2248. doi: 10.1002/cncr.23398

Fang, Y., Tian, S., Pan, Y., Li, W., Wang, Q., Tang, Y., et al. (2020). Pyroptosis: a new frontier in cancer. *Biomed. Pharmacother.* 121, 109595. doi: 10.1016/j.biopha.2019.109595

Filomeni, G., Desideri, E., Cardaci, S., Rotilio, G., and Ciriolo, M. R. (2010). Under the ROS…thiol network is the principal suspect for autophagy commitment. *Autophagy* 6, 999–1005. doi: 10.4161/auto.6.7.12754

Galluzzi, L., Pietrocola, F., Bravo-San Pedro, J. M., Amaravadi, R. K., Baehrecke, E. H., Cecconi, F., et al. (2015). Autophagy in malignant transformation and cancer progression. *EMBO J.* 34, 856–880. doi: 10.15252/embj.201490784

Garcia-Closas, R., Gonzalez, C. A., Agudo, A., and Riboli, E. (1999). Intake of specific carotenoids and flavonoids and the risk of gastric cancer in Spain. *Cancer Causes Control* 10, 71–75. doi: 10.1023/A:1008867108960

Gilbert, M. R., Wang, M., Aldape, K. D., Stupp, R., Hegi, M. E., Jaeckle, K. A., et al. (2013). Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial. *J. Clin. Oncol.* 31, 4085–4091. doi: 10.1200/JCO.2013.49.6968

Harborne, J. B., and Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry* 55, 481–504. doi: 10.1016/S0031-9422(00)00235-1

Imran, M., Rauf, A., Shah, Z. A., Saeed, F., Imran, A., Arshad, M. U., et al. (2019). Chemo-preventive and therapeutic effect of the dietary flavonoid kaempferol: a comprehensive review. *Phytother. Res.* 33, 263–275. doi: 10.1002/ptr.6227

Imran, M., and Salehi, B. (2019). Kaempferol: a key emphasis to its anticancer *Potential* 24:2277. doi: 10.3390/molecules24122277

Jeong, J. C., Kim, M. S., Kim, T. H., and Kim, Y. K. (2009). Kaempferol induces cell death through ERK and Akt-dependent down-regulation of XIAP and survivin in human glioma cells. *Neurochem. Res.* 34, 991–1001. doi: 10.1007/s11064-008-9868-5

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., et al. (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 526, 666–671. doi: 10.1038/nature15541

Li, L., Tan, J., Miao, Y., Lei, P., and Zhang, Q. (2015). ROS and autophagy: interactions and molecular regulatory mechanisms. *Cell. Mol. Neurobiol.* 35, 615–621. doi: 10.1007/s10571-015-0166-x

Liu, Y., Peterson, D. A., Kimura, H., and Schubert, D. (1997). Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem.* 69, 581–593. doi: 10.1046/j.1471-4159.1997.69020581.x

Nöthlings, U., Murphy, S. P., Wilkens, L. R., Henderson, B. E., and Kolonel, L. N. (2007). Flavonols and pancreatic cancer risk: the multiethnic cohort study. *Am. J. Epidemiol.* 166, 924–931. doi: 10.1093/aje/kwm172

Ostrom, Q. T., Gittleman, H., Liao, P., Vecchione-Koval, T., Wolinsky, Y., Kruchko, C., et al. (2017). CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014. *Neuro Oncol.* 19, v1–v88. doi: 10.1093/neuonc/nox158

Rajendran, P., Rengarajan, T., Nandakumar, N., Palaniswami, R., Nishigaki, Y., and Nishigaki, I. (2014). Kaempferol, a potential cytostatic and cure for inflammatory disorders. *Eur. J. Med. Chem.* 86, 103–112. doi: 10.1016/j.ejmech.2014.08.011

Schroder, K., and Tschopp, J. (2010). The inflammasomes. *Cell* 140, 821–832. doi: 10.1016/j.cell.2010.01.040

Sharma, V., Joseph, C., Ghosh, S., Agarwal, A., Mishra, M. K., and Sen, E. (2007). Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. *Mol. Cancer Ther.* 6, 2544–2553. doi: 10.1158/1535-7163.MCT-06-0788

Siegelin, M. D., Reuss, D. E., Habel, A., Herold-Mende, C., and von Deimling, A. (2008). The flavonoid kaempferol sensitizes human glioma cells to TRAIL-mediated apoptosis by proteasomal degradation of survivin. *Mol. Cancer Ther.* 7, 3566–3574. doi: 10.1158/1535-7163.MCT-08-0236

Stupp, R., Hegi, M. E., Mason, W. P., van den Bent, M. J., Taphoorn, M. J., Janzer, R. C., et al. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 10, 459–466. doi: 10.1016/S1470-2045(09)70025-7

Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* 352, 987–996. doi: 10.1056/NEJMoa043330

Su, Z., Yang, Z., Xu, Y., Chen, Y., and Yu, Q. (2015). Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol. Cancer* 14, 48. doi: 10.1186/s12943-015-0321-5

Szumiel, I. (2011). Autophagy, reactive oxygen species and the fate of mammalian cells. *Free Radic. Res.* 45, 253–265. doi: 10.3109/10715762.2010.525233

Tsitlakidis, A., Foroglou, N., Venetis, C. A., Patsalas, I., Hatzisotiriou, A., and Selviaridis, P. (2010). Biopsy versus resection in the management of malignant gliomas: a systematic review and meta-analysis. *J. Neurosurg.* 112, 1020–1032. doi: 10.3171/2009.7.JNS09758

Wang, Y., Gao, W., Shi, X., Ding, J., Liu, W., He, H., et al. (2017). Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. *Nature* 547, 99–103. doi: 10.1038/nature22393

Wang, Y., Yin, B., Li, D., Wang, G., Han, X., and Sun, X. (2018). GSDME mediates caspase-3-dependent pyroptosis in gastric cancer. *Biochem. Biophys. Res. Commun.* 495, 1418–1425. doi: 10.1016/j.bbrc.2017.11.156

Wen, P. Y., and Kesari, S. (2008). Malignant gliomas in adults. *N. Engl. J. Med.* 359, 492–507. doi: 10.1056/NEJMra0708126

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 Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 25 January 2021
doi: 10.3389/fbioe.2020.623866

Chrysophanol Exerts Anti-inflammatory Activity by Targeting Histone Deacetylase 3 Through the High Mobility Group Protein 1-Nuclear Transcription Factor-Kappa B Signaling Pathway *in vivo* and *in vitro*

Quan Wen[1,2†], Ngaikeung Lau[3†], Huandi Weng[1], Peng Ye[2], Shaohui Du[4], Chun Li[5], Jianping Lv[6] and Hui Li[2*]*

[1] Guangdong-HongKong-Macau Institute of CNS Regeneration, Jinan University, Guangzhou, China, [2] School of Basic Medical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China, [3] Guangdong Provincial Hospital of Chinese Medicine, Guangzhou, China, [4] Shenzhen Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, China, [5] School of Nursing Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China, [6] Department of Neurosurgery, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, Guangzhou, China

Chrysophanol (Chr) is the main monomer isolated from *Rheum rhabarbarum*. This study aimed to identify the potential *in vitro* and *in vivo* cytoprotective effects of Chr on lipopolysaccharide (LPS)-triggered acute lung injury (ALI). We used an ALI-murine model and constructed an inflammatory macrophage *in vitro* cell model to determine the cellular mechanisms involved in Chr-mediated activity. To observe the vital role of histone deacetylase 3 (HDAC3) in abolishing inflammation action, HDAC3 was downregulated using small interfering RNA. Analysis of the expression of nuclear transcription factor-kappa B p65 (NF-κB p65) and molecules of its downstream signaling pathway were assessed *in vitro* and in lung tissue samples using the mouse model. Concentrations of tumor necrosis factor-α, interleukin-1β, high mobility group protein 1 (HMGB1), and interleukin-16 in supernatants and the bronchoalveolar lavage fluid were measured using enzyme-linked immunosorbent assay. A reporter gene assay measured HMGB1 activity, and NF-κB p65 and HMGB1 intracellular localization was determined by immunofluorescence detection on histological lung samples from Chr-treated mice. The protein interactions between HMGB1, HDAC3, and NF-κB p65 were tested by co-immunoprecipitation. Chr treatment relieved LPS-induced lung lesions. Chr also enhanced superoxide dismutase levels in ALI mice. Chr reduced the LPS-induced protein expression of NF-κB and its related pathway molecules in both *in vivo* and *in vitro* models. Moreover, Chr downregulated LPS-enhanced HMGB1 expression, acetylation, and nuclear nucleocytoplasmic translocation. However, HDAC3 knockdown substantially reduced Chr-mediated HDAC3/NF-κB expression.

OPEN ACCESS

Edited by:
Miaomiao Yuan,
Sun Yat-sen University, China

Reviewed by:
Heqing Huang,
Sun Yat-sen University, China
Zhiren Zhang,
Army Medical University, China

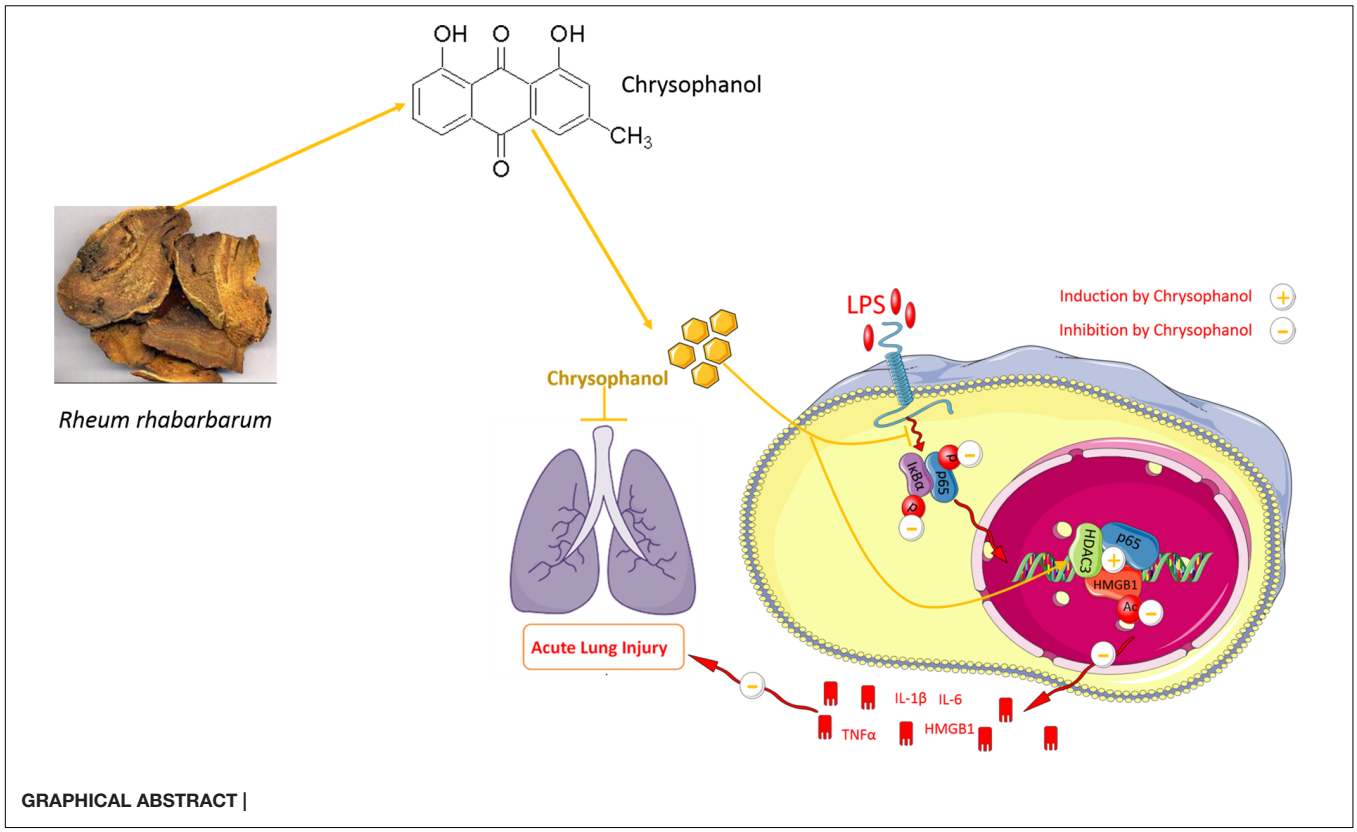
***Correspondence:**
Hui Li
lihui@gzucm.edu.cn;
lihuigzucm163@163.com
Jianping Lv
ljpwhx@163.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *30 October 2020*
Accepted: *17 December 2020*
Published: *25 January 2021*

Citation:
Wen Q, Lau N, Weng H, Ye P, Du S, Li C, Lv J and Li H (2021) Chrysophanol Exerts Anti-inflammatory Activity by Targeting Histone Deacetylase 3 Through the High Mobility Group Protein 1-Nuclear Transcription Factor-Kappa B Signaling Pathway in vivo and in vitro. Front. Bioeng. Biotechnol. 8:623866. doi: 10.3389/fbioe.2020.623866



GRAPHICAL ABSTRACT |

Furthermore, Chr enhanced HMGB1/HDAC3/NF-κB p65 complex interaction, whereas HDAC3 knockdown reduced Chr-mediated HMGB1/HDAC3/NF-κB p65 formation. This study showed that the protective effects induced by Chr were associated with the regulation of the HMGB1/NF-κB pathway via HDAC3.

Keywords: chrysophanol, sepsis, HMGB1, HDAC3, NF-κB

INTRODUCTION

Sepsis, a generalized inflammatory state induced by lipopolysaccharide (LPS) infection, usually leads to acute lung injury (ALI). Sepsis can induce multi-organ dysfunction syndrome or septic shock (Drewry et al., 2017). Septic ALI is induced by a lung inflammatory response syndrome, which leads to high mortality rates, high patient management costs, and accelerated morbidity (Ishii, 2011). LPS is the main component of the outer membrane of Gram-negative bacteria and induces lung leukocyte activation and promotes the secretion of inflammatory cytokines. Effective treatment for sepsis shock for ALI remains limited, and it is necessary to find novel agents.

As a late pro-inflammatory factor, the extracellular high mobility group box 1 (HMGB1) protein triggers responses that cause damage to tissues leading to the activation of the inflammatory cascade in several conditions, including lung injury and septic shock (Czura et al., 2004; El Gazzar, 2007). LPS diffuses into the lung *via* blood circulation, activates macrophages, and promotes HMGB1 release. Extracellular HMGB1 recognizes and binds to its receptors, for example, Toll-like receptor 4, and may activate NF-κB by its translocation to the nucleus where it triggers the transcription and release of inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 (Valdes-Ferrer et al., 2015). Therefore, HMGB1/NF-κB activation and nucleocytoplasmic transport might show promise as a crucial pathway in the development of ALI.

A crucial epigenetic factor present in macrophages is a histone deacetylase (HDAC). Its activation is triggered by transient inflammatory stimuli and results in the increased synthesis and release of pro-inflammatory cytokines in numerous diseases, including septic shock and ALI. In the latter, attenuation of HDAC activity has been reported to decrease the expression of inflammatory cytokines (Leoni et al., 2002; Pooladanda et al., 2019). Moreover, histone deacetylase 3 (HDAC3) has been reported to promote hyperacetylation and translocation of

HMGB1, suggesting a pivotal role for HDACs in regulating the HMGB1 translocation (Zou and Crews, 2014; Banerjee et al., 2015; Pooladanda et al., 2019). Conversely, inhibition of HDAC3 decreased TNF-α levels and was accompanied by a co-dependent increase in acetylated p65, a subunit of NF-κB, which has been reported to play a vital role in abolishing IκBα-mediated NF-κB transcriptional activity (Kiernan et al., 2003; Zhu et al., 2010).

Rhubarb (*Rheum rhabarbarum*) is a well-known traditional medicinal herb that has been widely used in clinical and pharmacological studies. Chrysophanol (Chr), a bioactive compound isolated from Rhubarb, has been reported to protect the human body from LPS-induced toxicity (Kim et al., 2010; Menghini et al., 2016). In the treatment of lung diseases, Chr can reverse lung injury via its anti-inflammatory and immunosuppressive effects (Qian et al., 2011; Lian et al., 2017). Our previous studies have reported that Chr inhibited the LPS-triggered release of pro-inflammatory factors, downregulating NF-κB gene expression and activation *via* regulation of the PPARγ pathway (Wen et al., 2018); however, the detailed molecular mechanisms involved in Chr-mediated effects on the NF-κB pathway are still elusive.

Hence, we propose that Chr attenuates macrophage activation, reduces levels of intrapulmonary inflammation-associated cytokines, and improves LPS-induced sepsis shock through the HMGB1/NF-κB axis by regulating HDAC3.

MATERIALS AND METHODS

Reagents

Chr (purity ≥ 99%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LPS (055:B5), the HMGB1 antagonist sodium butyrate (SB), and the HDAC3 antagonist RGFP966 were obtained from MedChemExpress (New Jersey, United States). Dexamethasone (DEX) was purchased from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). TNF-α, IL-6, IL-1β, and HMGB1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Multi Sciences Biotech Co. Ltd. (Hangzhou, China). Malondialdehyde (MDA), myeloperoxidase (MPO), and superoxide dismutase (SOD) kits were purchased from the Jiancheng Bioengineering Institute (Nanjing, China). Dual-Luciferase® reporter assay kits were purchased from Promega (Madison, WI, United States). Antibodies against HMGB1 (#6893), HDAC3 (#3949), TNF-α (#11948), IL-1β (#12703), IL-6 (#12912), inhibitor of nuclear factor kappa B (IκBα) (#4814), acetylated-lysine (#9441), NF-κB p65 (#8242), β-actin (#4970), lamin B (#13435), phospho-p65 (#3031), and phospho-IκBα (#2859) were purchased from Cell Signaling Technology (Danvers, MA, United States). All reagents used for cell culture were obtained from Gibco (Grand Island, NY, United States).

Experimental Animals and Protocols

Male BALB/c mice (18–22 g) were supplied and housed in the laboratory animal services center (Guangzhou University of Chinese Medicine, Guangzhou, China). The animals were housed in specific pathogen-free surroundings, provided with food and sterilized water *ad libitum*, and exposed to 12 h light/dark cycles and appropriate temperature and humidity. All experimental methods were followed in accordance with the Institutional and National Institutes of Health guidelines for humane animal experimentation. Animal handling followed the dictates of the National Animal Welfare Law of China.

Measurement of Blood Pressure and Mortality

Sodium pentobarbital (30 mg/kg) was used to anesthetize mice by intraperitoneal injection. To monitor blood pressure and drug administration, the neck skin was dissected to expose and cannulate the right carotid artery and connected to a blood pressure transducer (BL-420 Apparatus). The mice were grouped into the control group (isotonic saline, n = 12) and the LPS-treated group (15 mg/kg, n = 72). After LPS treatment, mice went into shock (30 min later, blood pressure reduced by ∼30%); we divided the 72 shock-induced mice into six groups: six groups received increasing does of Chr (7.5, 15, and 30 mg/kg) (Kim et al., 2010; Lian et al., 2017), the DEX treatment group (2 mg/kg) and the LPS group (LPS only). Every 30 min, the blood pressure [mean arterial pressure (MAP)] was recorded for 5 h. The survival rate was tested at the 24 h time point to assess the efficacy of Chr treatment.

Tissue Extraction

Mice (n = 12 per group) were killed after 24 h, the right lung tissues were rapidly collected under aseptic conditions, and after washing, approximately 50% of the total lung tissue was transferred to −80°C for subsequent quantitative real-time polymerase chain reaction or Western blotting (WB), and another portion of the tissue was fixed in 10% formalin for histological studies.

Histological Analysis

The fixed left lungs were rendered transparent for 20 h, embedded in paraffin wax, and sliced into 4 μm sections. After staining, the morphological structure and pathological index of the lung tissues were determined under a light microscope. The lung inflammation was scored by its histological severity as follows: grade 0: no inflammatory cells, grade (1) few cells, grade (2) a ring of cells surrounding the vessels with infiltration 1 cell layer deep, (3) a ring of cells 2–4 cell layers deep; and (4) a ring of cells with more than 4 cell layers deep.

Evaluation of Lung Wet-to-Dry Ratio

After blunt dissection, the right lung trachea and esophagus were exposed from the right middle lobe, and the level of pulmonary edema was determined. Next, samples were placed in an oven at 60°C for 48 h and allowed to dry by eliminating moisture, then wet-to-dry (W/D) ratios were calculated.

Collection of Bronchoalveolar Lavage Fluid

After treatment with Chr, lung tissue samples were collected and infused three times in phosphate-buffered saline. Bronchoalveolar lavage fluid (BALF) fluid was collected, centrifuged, and then stored at −80°C for subsequent analyses.

Biochemical Tests

The activities of MPO, SOD, and MDA in the BALF samples from the mouse model were detected following the manufacturer's instructions using a commercial detection product (Jiancheng Bioengineering).

Cell Culture and Treatment

RAW264.7 cells (China Center for Type Culture Collection, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin and incubated at 37°C in 5% carbon dioxide. There were six groups: control, LPS (0.2 μg/ml), LPS-SB (10 mM SB plus 0.2 μg/ml LPS), and LPS-Chr groups treated with increasing doses of Chr (5, 10, and 5 μM plus 0.2 μg/ml LPS) (Wen et al., 2018). We stimulated cells by LPS for 0.5 h before Chr was added.

MTT Assay

The MTT assay detected cell activity. First, RAW264.7 cells (5×10^3 cells/ml) were seeded in a 96-well plate overnight. The cells were treated with increasing concentrations of Chr (5, 10, 15, and 20 μM) for an additional 24 h. Next, MTT was added for the final 4 h of culture at 37°C away from light, and the absorbance values were detected at 480 nm.

Transient Transfection and Luciferase Reporter Assays

RAW264.7 cells were seeded in 24-well plates for 12 h. HMGB1 promoter–reporter plasmids and corresponding negative control (NC) vectors were transiently transfected into cells using Lipofectamine 2000 (Invitrogen, United States). Transfected cells were treated with LPS and/or Chr for another 24 h. Luminescence was determined by the Dual-Luciferase Reporter Assay System (Promega). The ratio of Firefly luciferase/Renilla luciferase was used to normalize the luminescence intensity.

Total RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

RAW264.7 cells or lung tissue samples were lysed by TRIzol reagent (Invitrogen). For qRT-PCR, the reaction steps were performed according to the protocol indicated by the manufacturer (Fermentas, United States). Thermal cycling conditions were 9 s at 95°C, 5 s at 95°C, followed by 40 cycles at 95°C for 13 s, 60°C for 1 min, and 95°C for 17 s in a StepOnePlus thermocycler (Applied Biosystems). The primer sequences are listed in **Table 1**.

Immunofluorescence

After fixation in 3% paraformaldehyde for 40 min, cells were permeabilized by 0.1% Triton X-100 for 30 min and then incubated using the following antibodies overnight at 4°C: rabbit anti-HMGB1 Abs (1:500, CST) and rabbit anti-NF-κB p65 antibody (1:500, CST). Next, cells were exposed to fluorescein isothiocyanate-conjugated secondary antibody (1:1,000, Abcam) for another 1 h, followed by observation by confocal microscopy (Zeiss LSM 710 Meta; Carl Zeiss).

Cytokine Assay by Enzyme-Linked Immunosorbent Assay

ELISA kits were used to measure the levels of inflammatory factors (TNF-α, IL-6, IL-1β, and HMGB1) in supernatant or tissues in accordance with the manufacturer's protocols. The emission absorbance of 480 nm was detected using a Thermomax microplate reader.

Preparation of Protein Extracts and Western Blotting

Briefly, cells were washed, lysed, and centrifuged. Next, cytoplasmic protein extracts were obtained, and the pellets containing nuclei were resuspended, placed on ice for 20 min, and the nuclear debris was centrifuged at 15,000×*g* for 15 min. Cells were lysed in mammalian protein extraction reagent (Thermo Fisher Scientific, Pierce). Total proteins and nuclear and cytoplasmic fractions were collected. The protein concentrations were analyzed using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Pierce) and were stored at −80°C until use. The lung tissues were quickly lysed using ice–cold radioimmunoprecipitation assay lysis buffer.

The concentration of total protein of lung tissue lysates was determined for WB. After electrophoresis, proteins were transferred into polyvinylidene fluoride membranes (Roche Ltd, Basel, Switzerland). Membranes were incubated with 5% bovine serum albumin at 37°C for 1 h to block membranes, which were subsequently exposed to the following primary antibodies at 4°C overnight: anti-acetylated lysine (1:1,000), anti-β-actin (1:1,000), anti-glyceraldehyde 3-phosphate dehydrogenase (1:1,000), anti-NF-kB p65 (1:1,000), anti-IkBa (1:1,000), anti-TNF-a (1:1,000), anti-IL-1β (1:1,000), anti-lamin B (1:1,000), anti-HMGB1 (1:1,000), and anti-HDAC3 (1:1,000). Next, an anti-rabbit secondary antibody (1:2,000) was used to incubate the membranes at 37°C for 1 h. The Bio-Rad imaging system imaged the immunoreactive bands.

Small Interfering RNA Transfection

HDAC3 small interfering RNA (siRNA) and the non-specific NC were constructed by GenePharma (Shanghai, China). The transient siRNA transfection was performed by the protocol indicated by the manufacturer. Cells were transfected for 24 h and then treated with 0.2 μg/ml LPS for 30 min; after that, cells were treated with increasing concentrations of Chr (5, 10, 15, and 20 μM) for 24 h. At the end of the treatment period, we collected cells for WB and immunofluorescence analysis.

Co-immunoprecipitation

Protein immunoprecipitation (IP) was carried out with antibodies against HMGB1, HDAC3, and NF-κB (CST).

TABLE 1 | Primers for real time-PCR.

Gene	Forward primer	Reverse primer	Species
GAPDH	CGTGTTCCTACCCCCAATGT	TGTCATCATACTTGGCAGGTTTCT	Mouse
HMGB1	GTTCAAGGACCCCAATGCAC	TGGATAAGCCAGGATGCTCG	Mouse
HDAC3	CAGAACTCACGCCAGTATCTGG	TCTGCCGGGACATCATGAAT	Mouse
NF-kB	CGCAAGCCCTTCAGTGACATC	GGTACTGGCTGTCAGGGTGGTT	Mouse
IL-1β	TCGCAGCAGCACATCAACAAGAG	TGCTCATGTCCTCATCCTGGAAGG	Mouse
TNF-α	ATGTCTCAGCCTCTTCTCATTC	GCTTGTCACTCGAATTTTGAGA	Mouse

Immunoglobulin G was used as a parallel-group. First, samples were incubated with immunoglobulin G reagent to be precleared. Samples were then treated with anti-HMGB1 (1:500), HDAC3 (1:500), and NF-κB (1:500) antibodies; 24 h later, lysates were incubated with protein A/G-agarose for another 2 h. Finally, samples were washed three times in phosphate-buffered saline and subjected to WB analysis.

Statistical Analysis

GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, United States) was used to evaluate the data. Multiple comparisons between the groups were performed using one-way analysis of variance, followed by variance with Tukey's test. A value of $P < 0.05$ was considered statistically significant; data are expressed as the means ± *SD*.

RESULTS

Chrysophanol Treatment Improved the Pathological Changes in Lung Tissues, Survival Rates, and Mean Arterial Pressure of Mice With Lipopolysaccharide-Induced Shock

We used hematoxylin–eosin staining to reveal the pathological changes on pulmonary tissue. Compared with the control group, the LPS group presented distinct neutrophil sequestration and infiltration in the lung tissue. The Chr- and DEX-treated groups showed varying degrees of protective effects in the LPS-induced mouse model (**Figures 1A,B**).

As an essential indicator of the therapeutic outcome of Chr, the survival rate of mice was measured (**Figure 1C**). Administration of 15 mg/kg LPS led to 70% mice mortality ($P < 0.001$). Chr treatment (Chr 30 mg/kg) protected mice from LPS-induced lethality, as shown by the higher survival rates; in addition, survival rates were better than those of the DEX group. Next, we evaluated the impact of Chr on MAP in our mouse model. LPS administration lowered the MAP by > 30% ($P < 0.01$). In contrast, Chr significantly restored the reduction in MAP in ALI mice, more strongly than with DEX treatment (**Figure 1D**).

Effects of Chrysophanol on Wet-to-Dry, Myeloperoxidase in Lung Tissues, Malondialdehyde, and Superoxide Dismutase Activities in Bronchoalveolar Lavage Fluid

To estimate the degree of pulmonary edema, the W/D weight ratio, MDA, and SOD levels in BALF were calculated.

Chr notably decreased the LPS-induced lung W/D weight ratio (**Figure 2A**). LPS sharply increased the production of MPO and MDA, whereas Chr treatment (7.5, 10, and 20 mg/kg) remarkably decreased MPO and MDA levels in the BALF (**Figures 2B,D**). The SOD index was downregulated in the LPS-induced sepsis model group, and Chr (7.5, 10, and 20 mg/kg) treatment led to a significant increase in SOD activity in BALF (**Figure 2C**).

Chrysophanol Inhibited High Mobility Group Protein 1/Nuclear Transcription Factor-Kappa B p65 Signaling Pathway Activity in the Acute Lung Injury Model

As shown in **Figures 3A,B**, we explored the inflammatory response in murine lung tissue. We detected NF-κB pathway-related proteins using WB analysis. The expression of NF-κBp65, p-NF-κB p65, IκBα, p-IκBα, HMGB1, TNF-α, and IL-1β increased in the LPS-treated group. As anticipated, Chr (7.5 and 15 mg/kg) or DEX treatment significantly inhibited the expression of these proteins. The results indicated that Chr possessed the anti-inflammatory activity in treating LPS-induced ALI through the HMGB1/NF-κB pathway. Moreover, we also found that Chr significantly downregulated LPS-induced HDAC3 protein levels. The ELISA result confirmed that the LPS group presented significantly enhanced TNF-α, IL-6, IL-1β, and HMGB1 release in BALF; however, their concentrations were decreased after Chr treatment (**Figures 3C–F**). These data indicated that Chr could sharply inhibit the levels of inflammatory factors in the ALI mouse model.

Effects of Chrysophanol on the Viability of RAW264.7 Cells

We used the MTT assay to investigate the effects of Chr at different concentrations (5, 10, 15, or 20 μM) on RAW264.7 cells viability in the LPS-induced *in vitro* cell model. There was no obvious cytolethality induced by Chr on cells (**Figure 4A**), except for the 20 μM Chr-treatment group. Instead, the viability

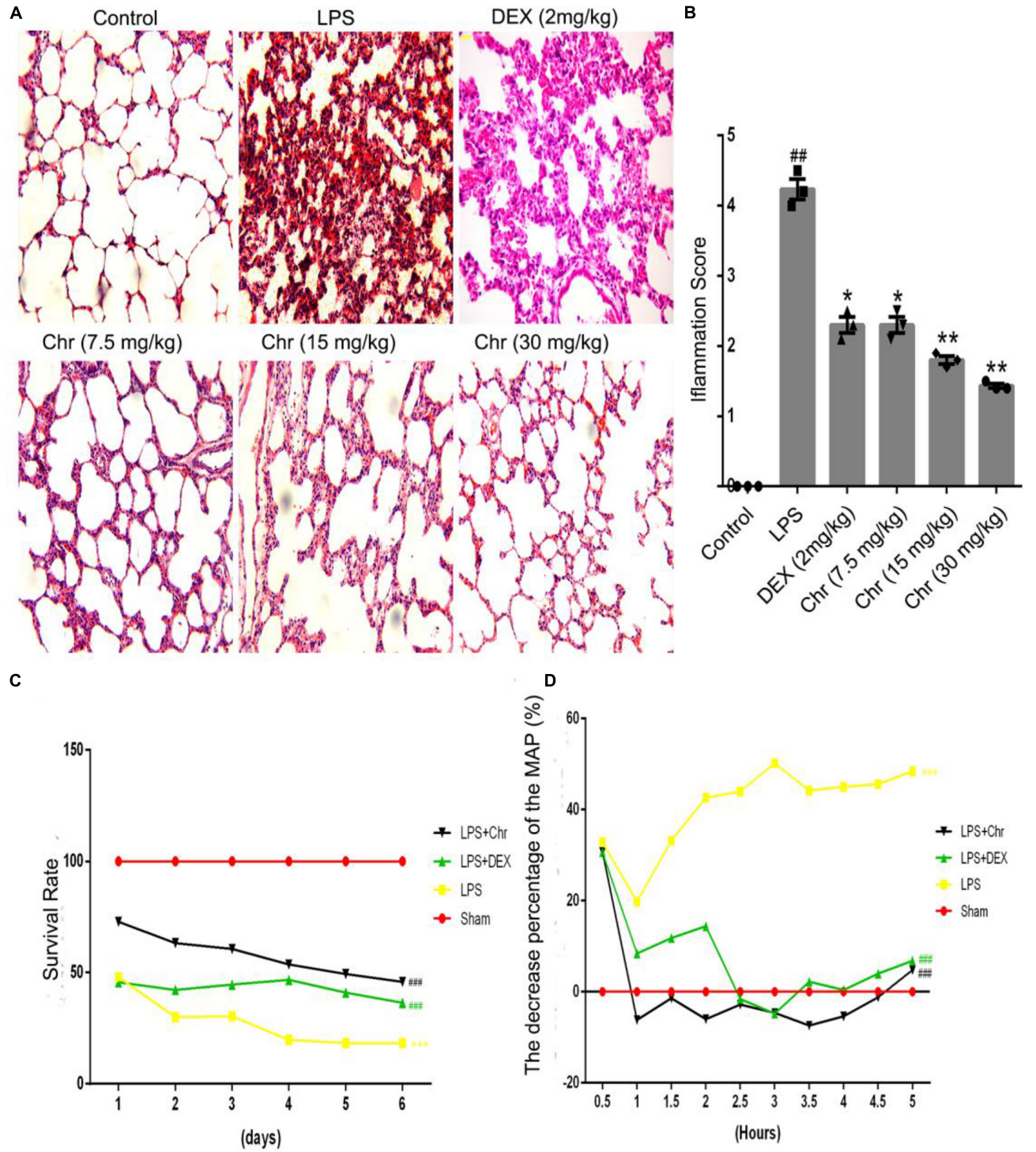


FIGURE 1 | Effects of Chr on the pathological changes, survival rate, and MAP of ALI mice (n = 12). **(A,B)** Pathological effects of Chr (7.5, 15, 30 mg/kg) in lung tissues (×200). **(C)** Survival rate of different groups. **(D)** MAP was recorded over 5 h at 30 min intervals. $^{\#}P < 0.01$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. LPS group.

of cells stimulated by LPS was 80.0 ± 1.0% of the control group, and treatment with Chr (5, 10, and 15 μM) protected cells against LPS-induced cellular injury, increasing cell activity up to 90.67 ± 1.15%, 96.0 ± 1.73%, and 98.0 ± 1.0%, respectively (**Figure 4B**). Therefore, Chr (5, 10, and 15 μM) were decided to use for the next experiment.

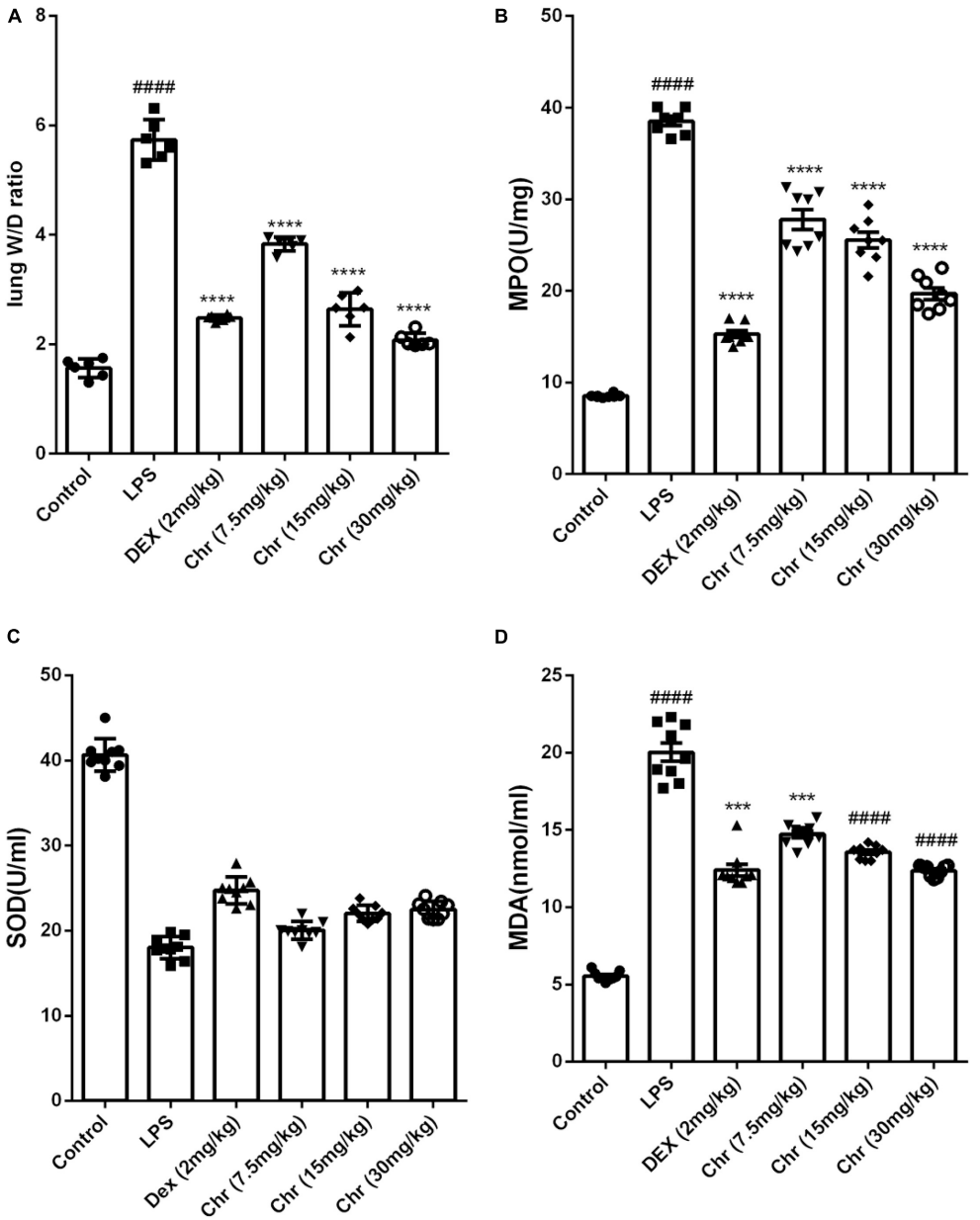


FIGURE 2 | Effects of Chr on **(A)** wet-to-dry weight ratio, **(B)** MPO, **(C)** SOD, and **(D)** MDA activities. Data are expressed as mean ± *SD*. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with LPS.

Chrysophanol Reduced Lipopolysaccharide-Induced High Mobility Group Protein 1 Expression, Nucleocytoplasmic Translocation, and Acetylation in Macrophages

Our previous study found Chr could attenuate inflammatory factor levels in LPS-induced macrophages. To investigate the potential role of Chr, we examined the effects of Chr on HMGB1 *in vitro*. We evaluated HMGB1 promoter activity and mRNA levels. Treatment with Chr (5, 10, and 15 μM) and the HMGB1 inhibitor SB (10 μM) significantly inhibited LPS-induced HMGB1 promoter activity (**Figure 5A**) and mRNA expression (**Figure 5B**), compared with the LPS-treatment group. We also evaluated the influence of Chr on total, cytoplasmic, and nuclear HMGB1 protein levels by WB. As shown in **Figures 5C–E**, total and cytoplasmic HMGB1 levels increased, whereas the nuclear levels decreased after LPS stimulation. However, all these effects were reversed by exposure to Chr.

We then examined the levels of HMGB1 in the supernatant by ELISA assay. The levels of HMGB sharply increased after LPS exposure; however, this increase was inhibited in

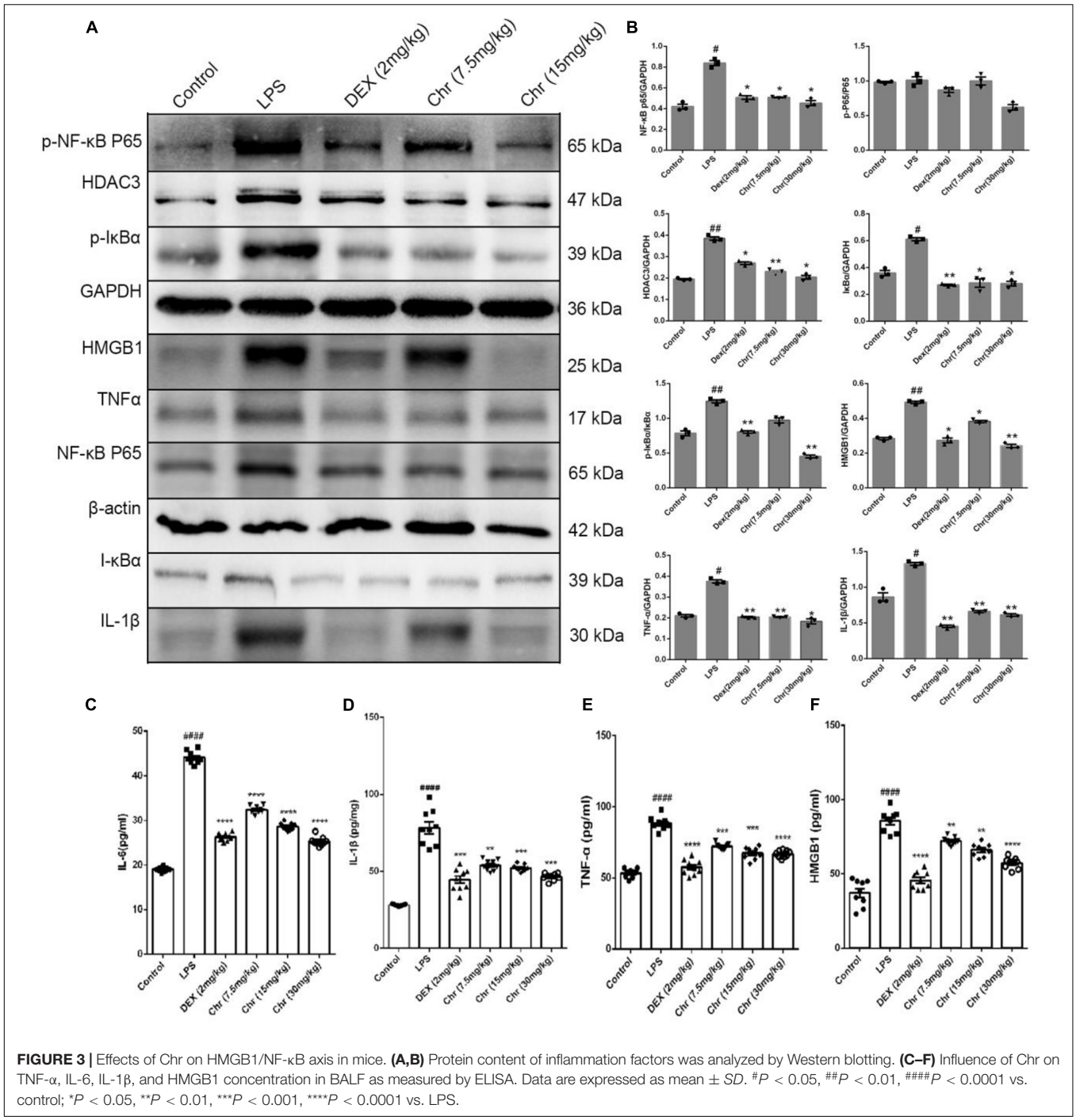


FIGURE 3 | Effects of Chr on HMGB1/NF-κB axis in mice. **(A,B)** Protein content of inflammation factors was analyzed by Western blotting. **(C–F)** Influence of Chr on TNF-α, IL-6, IL-1β, and HMGB1 concentration in BALF as measured by ELISA. Data are expressed as mean ± *SD*. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. LPS.

the presence of 10 μM SB (**Figure 5F**). Furthermore, Chr nearly completely abolished LPS-triggered intracellular HMGB1 production, similar to that in the SB-treated group (**Figure 5F**). Our results indicated the important inhibitory activity by Chr (5, 10, and 15 μM) on LPS-induced HMGB1 release in a dose-dependent fashion.

Immunofluorescent assay results also supported our conclusion. Nuclear–cytoplasmic translocation of HMGB1 was induced by LPS (**Figure 5G**, middle). Simultaneous treatment with Chr reduced cytoplasmic HMGB1 and enhanced nuclear localization of HMGB1 (**Figure 5G**, right), similar to unstimulated cells (**Figure 5G**, left), which provided morphological evidence to support our results.

To explore whether the HMGB1 translocation is related to its deacetylation, we analyzed the content of acetylated HMGB1 by IP (**Figure 5H**). When LPS-induced RAW264.7 cells to release HMGB1, we found acetylated HMGB1 levels were enhanced. Conversely, HMGB1 acetylation was markedly decreased after

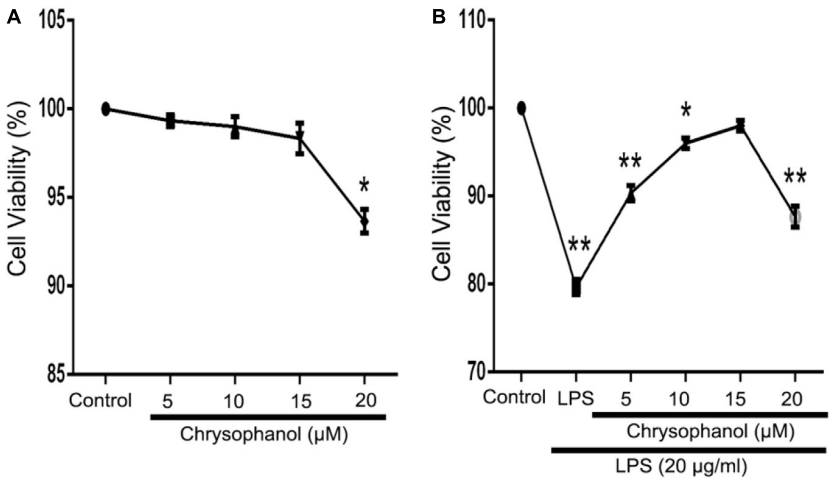


FIGURE 4 | Effects of Chr on the viability of RAW264.7 cells. MTT assay was used to evaluate effects of **(A)** Chr in RAW264.7 cells and in **(B)** LPS-stimulated model pretreated with Chr with increasing concentration of Chr. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

Chr treatment, indicating that Chr interfered with the acetylation of HMGB1 (**Figure 5H**).

Effects of Chrysophanol on Nuclear Transcription Factor-Kappa B Pathway After Histone Deacetylase 3 Knockdown

Our previous study demonstrated that Chr significantly reduced LPS-activated NF-κB promoter and the expression of mRNA, protein, and phosphorylation of components of the NF-κB pathway (Wen et al., 2018). Because Chr significantly downregulated HDAC3 protein expression and inhibited NF-κB pathway activation in the sepsis shock mice model, we then performed *in vitro* model experiments to clarify the potential mechanisms involved. To specifically knockdown HDAC3 levels, we used targeted siRNA inhibition. After LPS induction, Chr (15 μM) and HDAC3 antagonist RGFP966 (10 μM) were added to cells for 24 h. Then, qPCR and WB were performed. As shown in **Figures 6A–E**, mRNA and protein expression (NF-kB p65, IκBα, IL-1β, and TNFα) in the control group and siNC group were unchanged. Instead, after siHDAC3 transfection, the mRNA and protein levels of NF-kB p65, IκBα, IL-1β, and TNFα were more remarkably downregulated than the control group ($P < 0.01$). After transfected HDAC3 siRNA, in contrast with the siHDAC3-treated group, the NF-kB p65 pathway activity was enhanced by LPS ($P < 0.01$), exposure to RGFP966 significantly inhibited LPS-enhanced NF-kB p65 pathway activation ($P < 0.05$). In contrast, we observed that pathway inhibition by Chr treatment was reversed after HDAC3 gene knockdown (**Figure 6**).

Next, immunofluorescence analysis was performed. NF-κB p65 nuclear localization signals were increased in the siHDAC3-LPS group, consistent with the expression of inflammation genes, HDAC3 knockdown almost entirely abrogated the inhibitory capacity of Chr-modulated NF-κB p65 nuclear translocation (**Figure 6F**).

In brief, the Chr inhibitory effect on NF-κB p65 expression and on downstream proinflammatory gene expression was notably reversed in cells transfected with siRNA targeting HDAC3.

Chrysophanol Reduced Lipopolysaccharide-Induced High Mobility Group Protein 1 Acetylation and Translocation Were Reversed After Knockdown of Histone Deacetylase 3 Expression by Small Interfering RNA

As a part of the HDAC protein family, HDAC3 has been reported to play a key role in blocking HMGB1 secretion (Bonaldi et al., 2003). To further investigate the influence of Chr on HDAC3 activity and levels of acetylated HMGB1 and cytoplasmic to nuclear translocation, we transfected HDAC3 siRNA and NC-siRNA into RAW264.7; in addition, the HMGB1 inhibitor SB was used as a positive control. The distribution of HMGB1 was analyzed to determine the nucleocytoplasmic localization using fluorescence signals, HMGB1production was analyzed through ELISA, and IP was used to detect the HMGB1 acetylation level. As shown in **Figures 7A,B**, LPS induced translocation of HMGB1 protein from the nucleus to the cytoplasm; meanwhile, the inhibitory effects on HMGB1 translocation by Chr treatment were reversed in HDAC3 siRNA-transfected cells. In addition, ELISA results showed that HDAC3 siRNA eliminated the inhibitory effects of Chr on HMGB1 production in the cell culture supernatants (**Figure 7C**).

Immunofluorescent staining for HMGB1 recapitulated the results of WB and ELISA. In the HDAC3 siRNA control group and siRNA NC group, HMGB1 staining was only positive in the nucleus, whereas HMGB1 fluorescence signals were present in both the nuclear and cytoplasmic compartments in both the LPS- and the Chr-treated groups (**Figure 7D**). As shown in **Figure 7E**, after siRNA transfection, Chr treatment did not reduce LPS-induced HMGB1 acetylation.

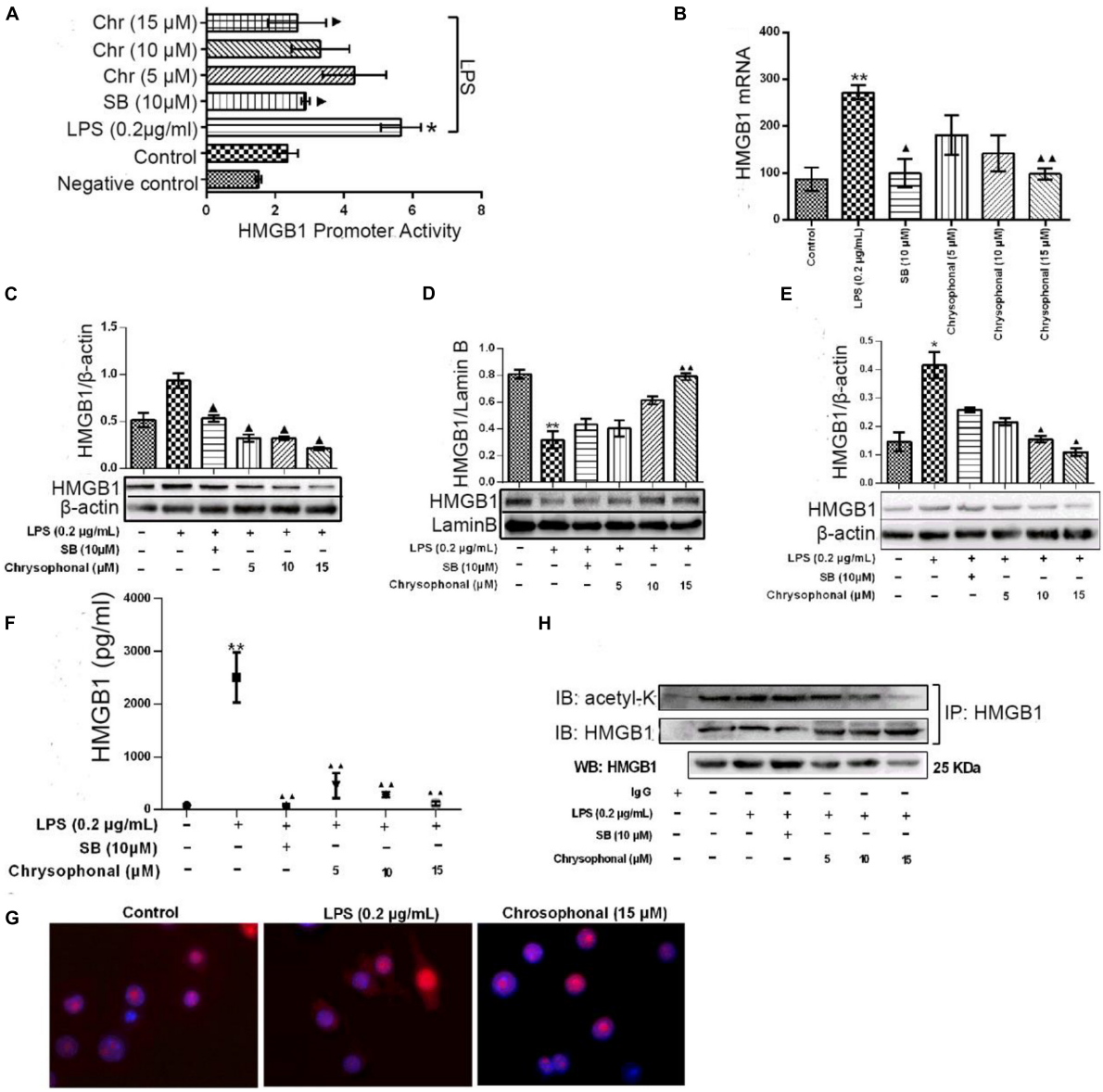


FIGURE 5 | Chr reversed LPS-induced HMGB1 release and inhibited HMGB1 acetylation. Cells were treated with LPS (0.2 μg/ml) for 30 min before being exposed to Chr (5, 10, and 15 μM) for 24 h. **(A)** HMGB1 promoter activity was determined. Mean value of the relative luciferase activity is shown. **(B)** Inhibition of HMGB1 mRNA expression by Chr in RAW264.7 cells. **(C)** Protein expression of HMGB1. **(D)** Protein expression of the nuclear HMGB1. **(E)** Protein expression of cytosolic HMGB1. **(F)** HMGB1 concentration as determined by ELISA. **(G)** Localization of HMGB1 was visualized in RAW264.7 cells by confocal microscopy. **(H)** Western blotting was performed to determine acetylated HMGB1 levels. Data are presented as means ± *SD* for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{▲}P < 0.05$, $^{▲▲}P < 0.01$ vs. LPS.

Collectively, these results demonstrated that Chr reversed LPS-triggered HMGB1 acetylation and nucleocytoplasmic translocation by enhancing HDAC3 expression.

Chrysophanol Enhances Formation of the HDAC3:HMGB1:NF-κB p65 Complex

Protein–protein interactions have an important role in multiple diseases (Gao et al., 2006). We speculated that HDAC3 deacetylates HMGB1 and regulates the NF-κB p65-mediated inflammatory signaling pathway by enhancing the interaction between HDAC3:HMGB1:NF-κB p65. As shown in **Figure 8**, LPS-induced RAW264.7 cell extracts were immunoprecipitated with HMGB1, HDAC3, and NF-κB p65 antibodies, respectively, and were subjected to WB using HDAC3, NF-κB p65, and HMGB1 antibodies. The results indicated a positive interaction between these proteins. After stimulation with LPS, the amount of HDAC3 immunoprecipitated with anti-HMGB1, NF-κB p65 antibodies were decreased. By comparison, the interaction of HMGB1:NF-κBp65 and HMGB1:HDAC3 were dramatically

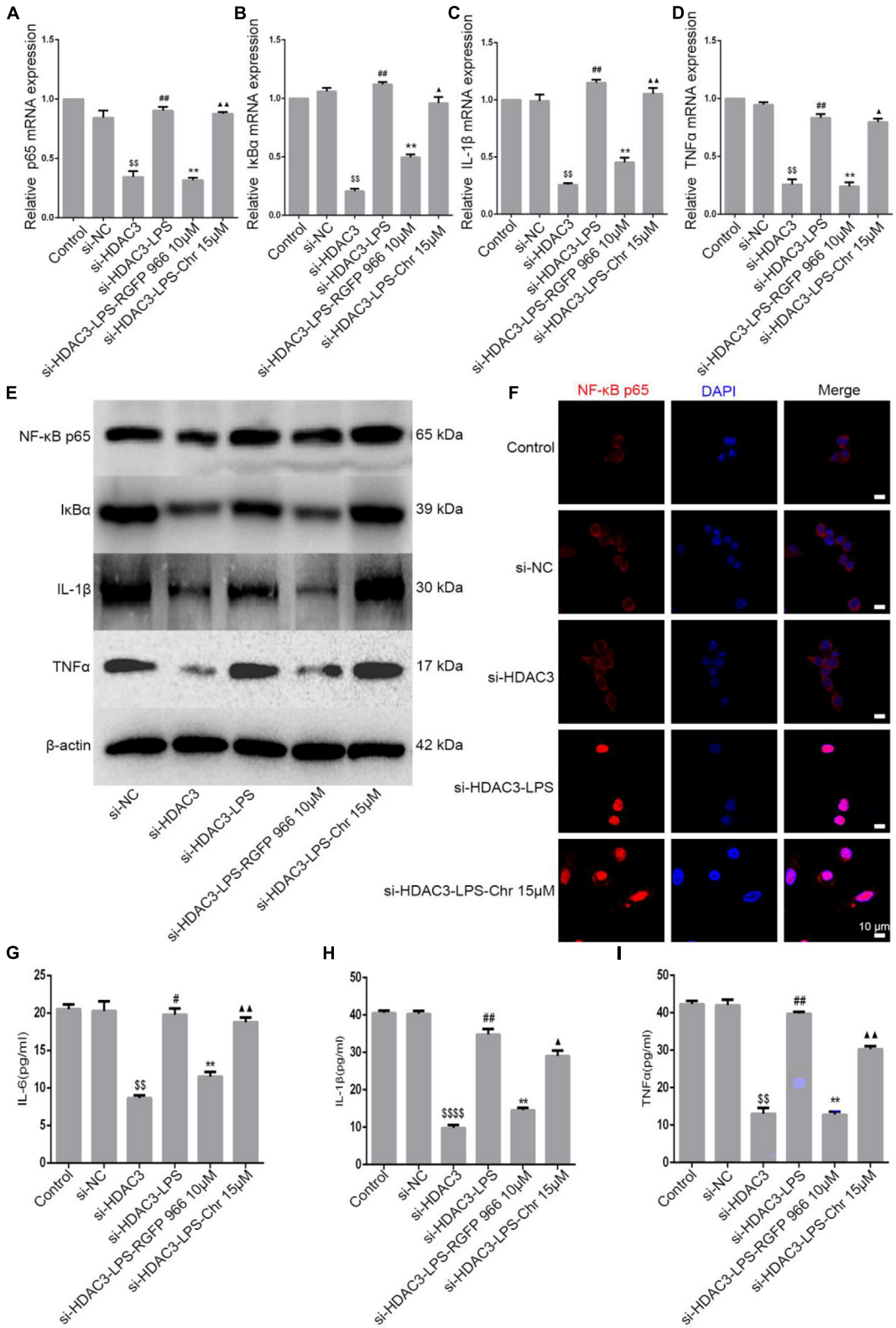


FIGURE 6 | Effect of Chr on NF-κB pathway after HDAC3 knockdown. siHDAC3 was transfected into RAW264.7 cells. **(A–D)** Expression of mRNA levels of NF-κB signaling pathway. **(E)** Protein expression of components of NF-κB signal pathway. **(F)** Immunofluorescence staining results. **(G–I)** Concentrations of NF-κB downstream factors as determined by ELISA. Data shown are mean ± *SD*. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$\$}P < 0.0001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. siHDAC3 group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. siHDAC3-LPS group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, vs. RGFP966 group.

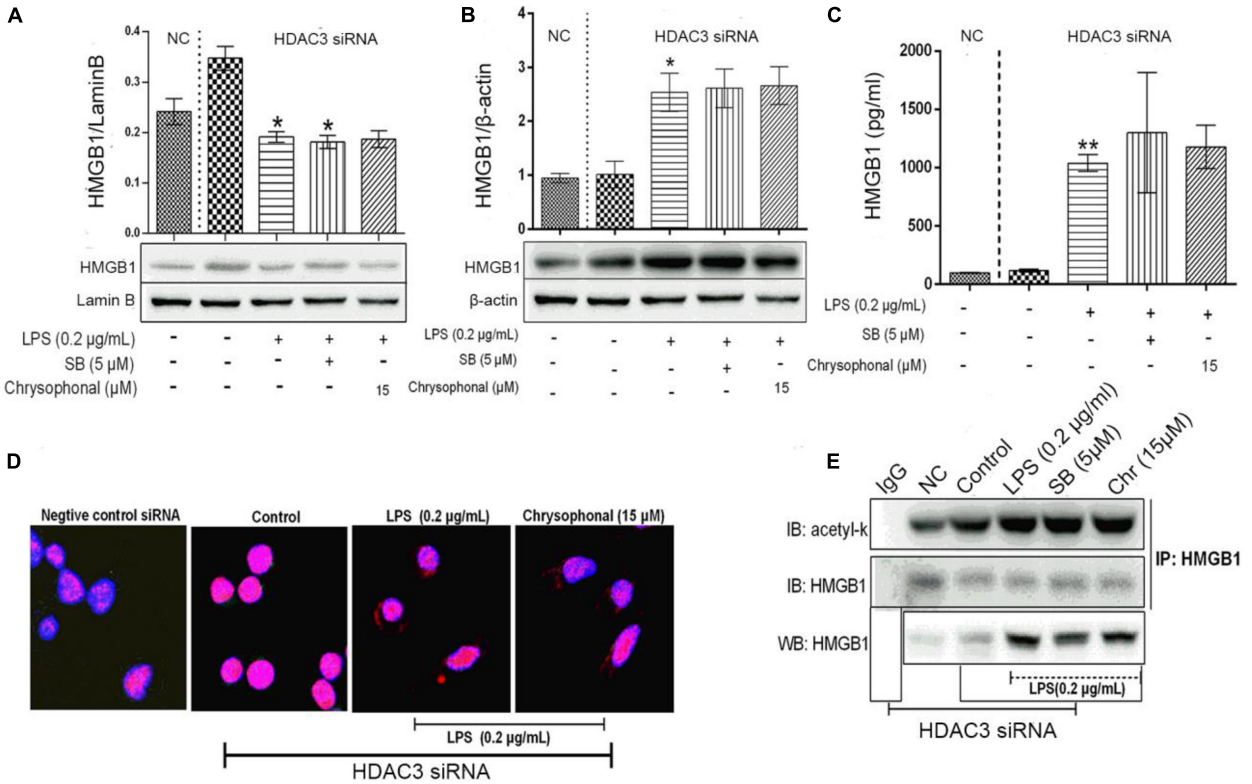


FIGURE 7 | Knockdown of HDAC3 expression promotes nuclear HMGB1 translocation to the cytoplasm, enhances HMGB1 release, and reverses Chr effects. **(A)** Protein expression of nuclear HMGB1. **(B)** Protein expression of cytosol HMGB1. **(C)** HMGB1 concentration. **(D)** Localization of HMGB1was visualized in RAW264.7 cells by confocal microscopy. Red, HMGB1; blue, nuclei. **(E)** Western blotting was performed to determine acetylated HMGB1 levels. Data are analyzed as means ± *SD* for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control group.

enhanced through treatment with 15 μM Chr (**Figure 7A**). Moreover, similar results were observed when the cell extract was immunoprecipitated with the HDAC3 or NF-κB p65 antibody and followed by immunoblotting with HMGB1, NF-κB p65, or HDAC3, HMGB1 antibodies (**Figures 8B,C**).

Results verifying the relationship of the three proteins indicated that LPS induced HMGB1, NF-κB p65, and HDAC3 complex dissociation, whereas Chr enhanced the interaction between HMGB1, NF-κB p65, and HDAC3, which changes reflected our intended meaning.

Histone Deacetylase 3 Knockdown Suppressed Chrysophanol-Mediated Enhancement of HDAC3:HMGB1:NF-κB p65 Complex Formation

Then, we used siRNA to knockdown HDAC3 expression *in vitro*. HDAC3 was decreased after transfection into RAW264.7 cells with siRNA for 48 h. Co-immunoprecipitation assays were carried out to test the interaction between the HDAC3:HMGB1:NF-κB p65 protein complex after HDAC3 knockdown by HMGB1 and NF-κB p65 antibodies, respectively. We found that under the NC or HDAC3-siRNA conditions, HMGB1 protein was detected in the IP product using the p65 antibody, and conversely, p65 protein was detected in the IP product using the HMGB1 antibody (**Figures 9A,B**). After LPS stimulation, the coexisting protein–protein interactions between HMGB1 and p65 were inhibited, which was observed in the siHDAC3-LPS group. Moreover, we observed from the IP product that HDAC3 knockdown potently abolished the Chr-augmented p65:HMGB1 proteins complex formation, indicating the regulation role of HDAC3 in Chr-mediated enhancement of HDAC3:HMGB1:NF-κB p65 complex formation.

DISCUSSION

Sepsis is a life-threatening pathological condition characterized by a dysregulated inflammatory response, a disordered blood coagulation cascade, and multiple organ dysfunctions (Falagas and Kopterides, 2006; Jean-Baptiste, 2007; Prescott et al., 2016). The effective treatment of sepsis involves the inhibition of the expression of proinflammatory mediators. In our study, we examined the underlying mechanisms and protective effects of Chr in LPS-induced ALI in *in vitro* cell lines and in an *in vitro* LPS-induced murine model of ALI. Our findings explicitly demonstrated that Chr could significantly downregulate the HMGB1/NF-κB axis and inhibit the inflammatory response via modulation of HDAC3 expression. Finally, Chr treatment

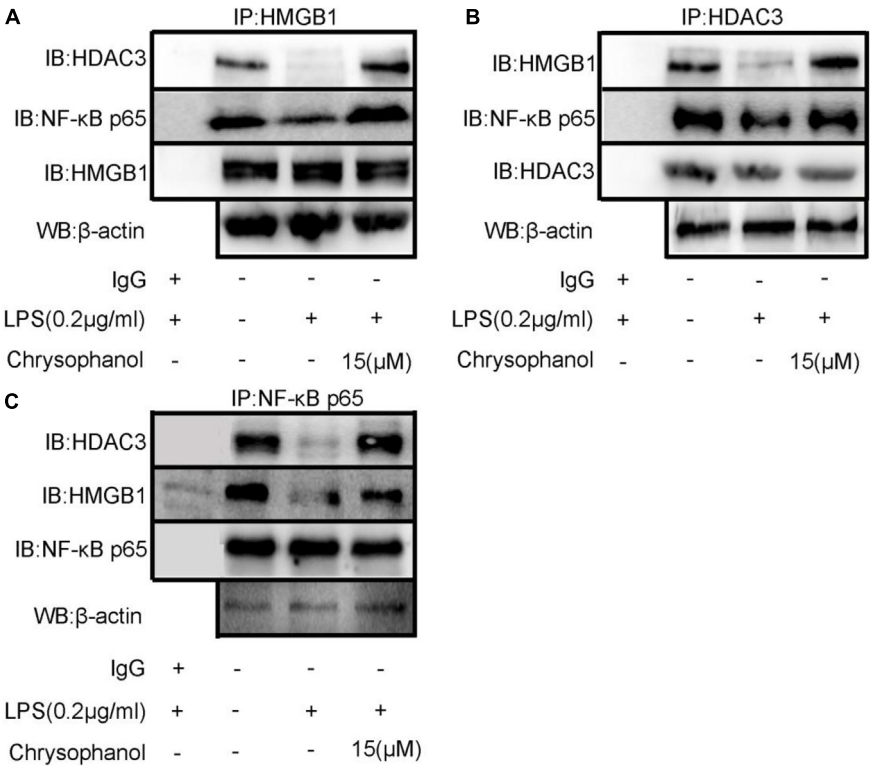


FIGURE 8 | Chr enhances interaction between HDAC3, HMGB1, and NF-κB p65. Immunoprecipitation of total and **(A)** anti- HMGB1-, **(B)** anti- HDAC3-, or **(C)** anti-NF-κB p65-antibody-treated lysates, respectively, subjected to WB.

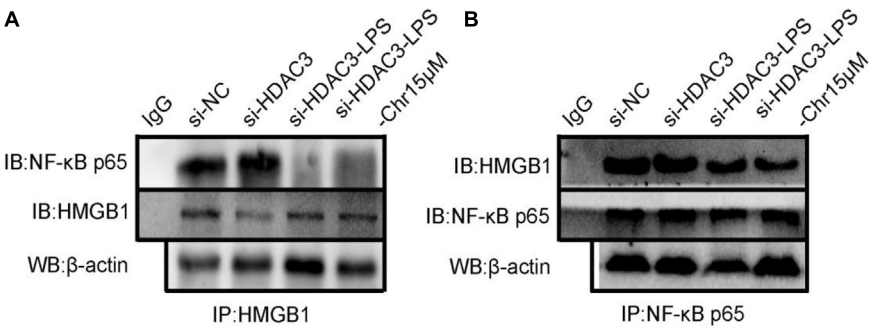


FIGURE 9 | (A) Co-immunoprecipitation was performed using protein lysate from cells to observe relationship between HMGB1/NF-κB p65 complex formation in HDAC3 knockdown condition with specific siRNA and after LPS activation and Chr treatment. **(B)** Co-immunoprecipitation was performed to determine the interactions between p65-HMGB1.

markedly enhanced protein–protein interactions and relieving symptoms of ALI.

The overproduction of inflammatory mediators has been associated with the pathogenesis of ALI; thus, the identification of effective measures to control lung injury is important. The therapeutic activity of DEX is attributed to its anti-inflammatory (Tolaj et al., 2017), immunosuppressive (Wang et al., 2013), anti-endotoxic (Zhang et al., 2017), and anti-shock (Yang et al., 2010) activity. Recent studies have determined that DEX possesses the capacity to activate the remission of organ injury and to overcome the infection, although the limitation of DEX treatment is that it is not recommended for long-term use, as it causes numerous adverse effects (Wang et al., 2013; Sun et al., 2017). Thus, there is an urgent need to identify novel effective treatments for ALI having fewer secondary adverse effects.

LPS induces the same disease characteristics as ALI and thus has been applied as the best model for ALI. We studied the protective effects of Chr in the ALI mouse model. The results showed that Chr sharply reduced MAP, the lung W/D weight ratio, lung MPO activity, MDA content in LPS-stimulated mice,

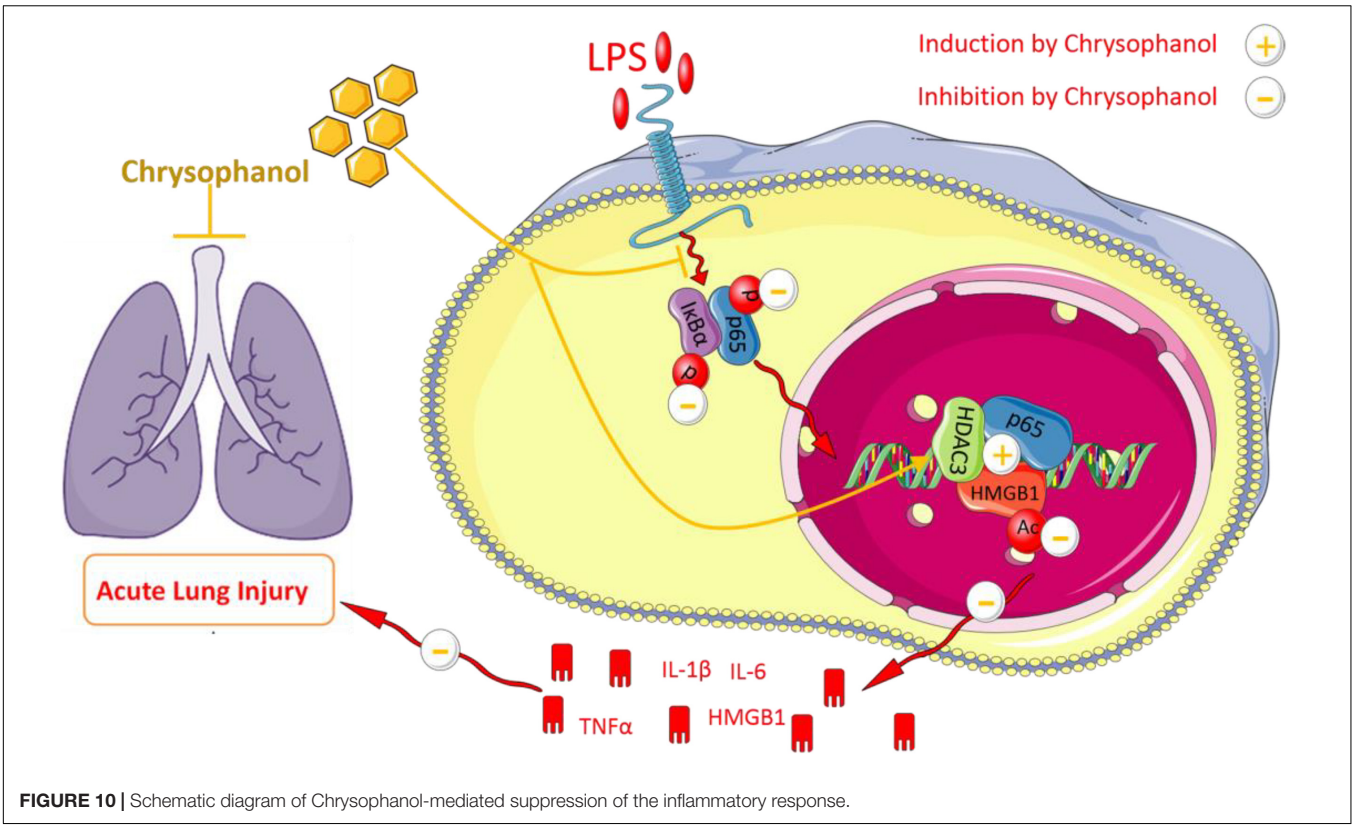


FIGURE 10 | Schematic diagram of Chrysophanol-mediated suppression of the inflammatory response.

and the levels of several inflammatory mediators in the BALF. In addition, Chr also increased SOD levels and the survival rate and, thus, effectively relieved ALI.

The level of lysosomal-specific MPO is an indicator of the active condition of macrophages (Yang et al., 2016), which contribute to inducing the oxidative stress reaction and causing organ or tissue damage (Jiang et al., 2016). Researchers have determined that the lipid peroxidation reaction induced by MDA (Diao et al., 2016) can lead to substantial cytotoxicity. SOD has been reported to be the major enzymatic antioxidative enzyme able to detoxify and eliminate free radicals (Wang et al., 2016). Our study suggested that Chr treatment could enhance SOD levels in contrast to the changes observed in the LPS group.

We then performed WB and ELISA to identify mechanisms involved in Chr protection against LPS-induced ALI. ALI is well known to develop from the increased exposure to cytokines released during the inflammation process. Our results indicated that the activation of LPS led to a significant increase in the activation of the NF-κB p65 pathway, as detected in the BALF, whereas Chr treatment disrupted the LPS-induced pro-inflammatory effects. Bacterial LPS triggering activation of the NF-κB signaling pathway has been considered to be a major component of ALI. LPS interacts with TLRs. In addition, as a late-onset risk factor, released HMGB1 induces the inflammatory cascade and accelerates the synthesis of inflammatory cytokines by binding to NF-κB receptors, which triggers NF-κB kinase phosphorylation, and signals that converge on the IkB/NF-kB, allowing the disruption of the complex, and shifting of NF-κB to the nucleus, leading to the release of large quantities of lethal factors (Jiang et al., 2005). Moreover, the results suggested that Chr treatment decreased LPS-enhanced expression of HDAC3, which is considered a transcriptional co-repressor of NF-κB p65. The HDAC3 inhibitor effectively inhibited NF-κB p65 transcriptional activity, likely by binding to the co-repressor transcription complex PPARγ (Jennewein et al., 2008).

In line with our *in vivo* study, we assumed that Chr could also inhibit activated cells and intrapulmonary lethal cytokines and relieve ALI through the HMGB1/NF-κB pathway via the HDAC3 signaling. The *in vitro* study was designed to define better the underlying molecular mechanisms involving Chr activity on the HMGB1/NF-κB signaling pathway. First, we found that Chr suppressed LPS-induced HMGB1 acetylation, which was in line with the reduction in HMGB1 nuclear translocation and extracellular HMGB1 production, suggesting a mechanism in which HDAC3 deacetylates HMGB1 and leads to the inhibition of HMGB1 release. Our previous study confirmed that Chr treatment inhibited the NF-κB p65 signaling pathway via inhibition of sIκB phosphorylation and NF-κB p65 relocation triggered by LPS-activated RAW264.7 cells (Wen et al., 2018). To provide a more in-depth exploration of how and whether Chr-regulated HMGB1 suppression influenced the HDAC3-mediated inactivation of NF-kB p65, we used synthetic siRNA targeting HDAC3. We found that the inhibitory effect of Chr was essentially lost in cells where the expression of HDAC3 was silenced, suggesting an important role for HDAC3 in the effects induced by Chr.

Next, we explored the mechanisms involved in Chr-regulation of the p65:HDAC3:HMGB1 complex. Co-immunoprecipitation findings indicated that the members of the p65:HDAC3:HMGB1 complex were tightly bound to each other in the quiescent state. Then, we confirmed that after LPS activation, the binding between the components of the complex was disrupted, which might be a consequence of protein degradation. Furthermore, we showed that Chr treatment before LPS stimulation led to the stronger interaction between the complex components. When HDAC3 was knocked down, the enhancement effect of Chr was sharply abolished, suggesting an essential role of HDAC3 in the effects of Chr (**Figure 10**). In brief, our research showed that Chr possesses an intense anti-inflammatory effect by regulating HMGB1/NF-κB signaling through HDAC3.

Our study has some limitations. In our experiments, the potential mechanism of Chr via macrophages *in vivo* was not investigated. Thus, to better understand and characterize LPS-induced ALI, influence functions of other cell types (e.g., pulmonary macrophages and epithelial cells) are required to assess the clinical benefits of Chr further.

CONCLUSION

In conclusion, the present study showed that Chr exerts an intense anti-inflammatory effect by regulating HMGB1/NF-κB signaling through HDAC3 expression.

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 in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

QW and NL conceived and designed the experiments. QW performed the experiments, analyzed the data, prepared manuscript, and wrote the manuscript. NL contributed to the reagents, materials, and analysis tools. All authors read and approved the manuscript.

FUNDING

This work was supported by grants from the Chinese National Natural Science Foundation (Nos. 81072908, 81173377, and 81273962) and the Guangdong Provincial Science and Technology Project (grant no. 2009B030801283).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.623866/full#supplementary-material

REFERENCES

Banerjee, S., Rakshit, T., Sett, S., and Mukhopadhyay, R. (2015). Interactions of histone acetyltransferase p300 with the nuclear proteins histone and HMGB1, as revealed by single molecule atomic force spectroscopy. *J. Phys. Chem. B* 119, 13278–13287. doi: 10.1021/acs.jpcb.5b07795

Bonaldi, T., Talamo, F., Scaffidi, P., Ferrera, D., Porto, A., Bachi, A., et al. (2003). Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J.* 22, 5551–5560. doi: 10.1093/emboj/cdg516

Czura, C. J., Yang, H., Amella, C. A., and Tracey, K. J. (2004). HMGB1 in the immunology of sepsis (not septic shock) and arthritis. *Adv. Immunol.* 84, 181–200. doi: 10.1016/s0065-2776(04)84005-7

Diao, M., Zhang, S., Wu, L., Huan, L., Huang, F., Cui, Y., et al. (2016). Hydrogen gas inhalation attenuates seawater instillation-induced acute lung injury via the Nrf2 pathway in rabbits. *Inflammation* 39, 2029–2039. doi: 10.1007/s10753-016-0440-1

Drewry, A. M., Ablordeppey, E. A., Murray, E. T., Stoll, C. R., Izadi, S. R., Dalton, C. M., et al. (2017). Antipyretic therapy in critically Ill septic patients: a systematic review and meta-analysis. *Crit. Care Med.* 45, 806–813. doi: 10.1097/ccm.0000000000002285

El Gazzar, M. (2007). HMGB1 modulates inflammatory responses in LPS-activated macrophages. *Inflamm. Res.* 56, 162–167. doi: 10.1007/s00011-006-6112-0

Falagas, M. E., and Kopterides, P. (2006). Risk factors for the isolation of multi-drug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: a systematic review of the literature. *J. Hosp. Infect.* 64, 7–15. doi: 10.1016/j.jhin.2006.04.015

Gao, Z., He, Q., Peng, B., Chiao, P. J., and Ye, J. (2006). Regulation of nuclear translocation of HDAC3 by IkappaBalpha is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor gamma function. *J. Biol. Chem.* 281, 4540–4547. doi: 10.1074/jbc.m507784200

Ishii, Y. (2011). [Acute lung injury/acute respiratory distress syndrome: progress in diagnosis and treatment topics. I: pathogenesis and pathophysiology; 2. Etiology and epidemiology]. *Nihon Naika Gakkai Zasshi* 100, 1522–1528.

Jean-Baptiste, E. (2007). Cellular mechanisms in sepsis. *J. Intensive Care Med.* 22, 63–72. doi: 10.1177/0885066606297123

Jennewein, C., Kuhn, A. M., Schmidt, M. V., Meilladec-Jullig, V., von Knethen, A., Gonzalez, F. J., et al. (2008). Sumoylation of peroxisome proliferator-activated receptor gamma by apoptotic cells prevents lipopolysaccharide-induced NCoR removal from kappaB binding sites mediating transrepression of proinflammatory cytokines. *J. Immunol.* 181, 5646–5652. doi: 10.4049/jimmunol.181.8.5646

Jiang, W., Li, M., He, F., Yao, W., Bian, Z., Wang, X., et al. (2016). Protective effects of asiatic acid against spinal cord injury-induced acute lung injury in rats. *Inflammation* 39, 1853–1861. doi: 10.1007/s10753-016-0414-3

Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., et al. (2005). CD14 is required for MyD88-independent LPS signaling. *Nat. Immunol.* 6, 565–570. doi: 10.1038/ni1207

Kiernan, R., Bres, V., Ng, R. W., Coudart, M. P., El Messaoudi, S., Sardet, C., et al. (2003). Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. *J. Biol. Chem.* 278, 2758–2766. doi: 10.1074/jbc.m209572200

Kim, S. J., Kim, M. C., Lee, B. J., Park, D. H., Hong, S. H., and Um, J. Y. (2010). Anti-Inflammatory activity of chrysophanol through the suppression of NF-kappaB/caspase-1 activation in vitro and in vivo. *Molecules* 15, 6436–6451. doi: 10.3390/molecules15096436

Leoni, F., Zaliani, A., Bertolini, G., Porro, G., Pagani, P., Pozzi, P., et al. (2002). The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proc. Natl. Acad. Sci. U.S.A.* 99, 2995–3000. doi: 10.1073/pnas.052702999

Lian, Y., Xia, X., Zhao, H., and Zhu, Y. (2017). The potential of chrysophanol in protecting against high fat-induced cardiac injury through Nrf2-regulated anti-inflammation, anti-oxidant and anti-fibrosis in Nrf2 knockout mice. *Biomed. Pharmacother.* 93, 1175–1189. doi: 10.1016/j.biopha.2017.05.148

Menghini, L., Ferrante, C., Leporini, L., Recinella, L., Chiavaroli, A., Leone, S., et al. (2016). An hydroalcoholic chamomile extract modulates inflammatory and immune response in HT29 cells and isolated rat colon. *Phytother. Res.* 30, 1513–1518. doi: 10.1002/ptr.5655

Pooladanda, V., Thatikonda, S., Bale, S., Pattnaik, B., Sigalapalli, D. K., Bathini, N. B., et al. (2019). Nimbolide protects against endotoxin-induced acute respiratory distress syndrome by inhibiting TNF-alpha mediated NF-kappaB and HDAC-3 nuclear translocation. *Cell Death Dis.* 10:81.

Prescott, H. C., Osterholzer, J. J., Langa, K. M., Angus, D. C., and Iwashyna, T. J. (2016). Late mortality after sepsis: propensity matched cohort study. *BMJ* 353:i2375. doi: 10.1136/bmj.i2375

Qian, Z. J., Zhang, C., Li, Y. X., Je, J. Y., Kim, S. K., and Jung, W. K. (2011). Protective effects of emodin and chrysophanol isolated from marine fungus *Aspergillus* sp. on ethanol-induced toxicity in HepG2/CYP2E1 cells. *Evid. Based Complement. Alternat. Med.* 2011:452621.

Sun, G., Yang, W., Zhang, Y., and Zhao, M. (2017). Esculentoside A ameliorates cecal ligation and puncture-induced acute kidney injury in rats. *Exp. Anim.* 66, 303–312. doi: 10.1538/expanim.16-0102

Tolaj, I., Ramadani, H., Mehmeti, M., Gashi, H., Kasumi, A., Gashi, V., et al. (2017). Does dexamethasone helps in meningococcal sepsis? *Med. Arch.* 71, 173–177. doi: 10.5455/medarh.2017.71.173-177

Valdes-Ferrer, S. I., Papoin, J., Dancho, M. E., Olofsson, P., Li, J., Lipton, J. M., et al. (2015). HMGB1 mediates anemia of inflammation in murine sepsis survivors. *Mol. Med.* 21, 951–958. doi: 10.2119/molmed.2015.00243

Wang, G., Song, Y., Feng, W., Liu, L., Zhu, Y., Xie, X., et al. (2016). Activation of AMPK attenuates LPS-induced acute lung injury by upregulation of PGC1alpha and SOD1. *Exp. Ther. Med.* 12, 1551–1555. doi: 10.3892/etm.2016.3465

Wang, Z. H., Liang, Y. B., Tang, H., Chen, Z. B., Li, Z. Y., Hu, X. C., et al. (2013). Dexamethasone down-regulates the expression of microRNA-155 in the livers of septic mice. *PLoS One* 8:e80547. doi: 10.1371/journal.pone.0080547

Wen, Q., Mei, L., Ye, S., Liu, X., Xu, Q., Miao, J., et al. (2018). Chrysophanol demonstrates anti-inflammatory properties in LPS-primed RAW 264.7 macrophages through activating PPAR-gamma. *Int. Immunopharmacol.* 56, 90–97. doi: 10.1016/j.intimp.2018.01.023

Yang, S., Yu, Z., Yuan, T., Wang, L., Wang, X., Yang, H., et al. (2016). Therapeutic effect of methyl salicylate 2-O-beta-d-lactoside on LPS-induced acute lung injury by inhibiting TAK1/NF-kappaB phosphorylation and NLRP3 expression. *Int. Immunopharmacol.* 40, 219–228. doi: 10.1016/j.intimp.2016.08.041

Yang, T. H., Shih, M. F., Wen, Y. S., Ho, W. Y., Leu, K. L., Wang, M. Y., et al. (2010). Attenuation of circulatory shock and cerebral ischemia injury in heat stroke by combination treatment with dexamethasone and hydroxyethyl starch. *Exp. Transl. Stroke Med.* 2:19. doi: 10.1186/2040-7378-2-19

Zhang, Y., Ran, K., Zhang, S. B., Jiang, L., Wang, D., and Li, Z. J. (2017). Dexmedetomidine may upregulate the expression of caveolin1 in lung tissues of rats with sepsis and improve the shortterm outcome. *Mol. Med. Rep.* 15, 635–642. doi: 10.3892/mmr.2016.6050

Zhu, H., Shan, L., Schiller, P. W., Mai, A., and Peng, T. (2010). Histone deacetylase-3 activation promotes tumor necrosis factor-alpha (TNF-alpha) expression in cardiomyocytes during lipopolysaccharide stimulation. *J. Biol. Chem.* 285, 9429–9436. doi: 10.1074/jbc.m109.071274

Zou, J. Y., and Crews, F. T. (2014). Release of neuronal HMGB1 by ethanol through decreased HDAC activity activates brain neuroimmune signaling. *PLoS One* 9:e87915. doi: 10.1371/journal.pone.0087915

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 Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 27 January 2021
doi: 10.3389/fbioe.2021.575724

Effects of Banana Resistant Starch on the Biochemical Indexes and Intestinal Flora of Obese Rats Induced by a High-Fat Diet and Their Correlation Analysis

*Jinfeng Fu†, Yuting Wang†, Simin Tan and Juan Wang**

School of Food Science and Engineering, South China University of Technology, Guangzhou, China

The effects of banana resistant starch (BRS) on obesity-related metabolic and intestinal flora were investigated in a high-fat diet-induced obesity model. After 6 weeks of intervention, the glucolipid metabolism index [blood glucose (GLU), total cholesterol (TC), triacylglycerol (TG), low density lipoprotein-cholesterol (LDL-C), and high density lipoprotein-cholesterol (HDL-C)], hormone index [leptin (LEP), insulin (INS), ghrelin, adiponectin (ADP), and thyroxine (T4)], and 16S rRNA sequencing analyses were performed for each group to explore the regulating effect of intestinal flora and the mechanism of weight loss in obese rats. The results showed that (1) BRS intervention significantly reduced the levels of GLU, TG, TC, LDL-C, LEP, and INS ($p < 0.01$) and increased the contents of ghrelin ($p < 0.05$) and ADP ($p < 0.01$). (2) BRS could improve the diversity of intestinal flora and regulate the overall structure of intestinal microorganisms, mainly by upregulating the *Bacteroides*/*Firmicutes* ratio and the relative abundance of *Cyanobacteria* and downregulating the relative abundances of *Deferribacteres* and *Tenericutes* (at the phylum level). BRS could inhibit the proliferation of *Turicibacter*, *Romboutsia*, and *Oligella* and increase the abundances of *Bacteroides*, *Ruminococcaceae*, and *Lachnospiraceae* (at the genus level). (3) Some significant correlations were observed between the gut microbiota and biomarkers. *Turicibacter*, *Romboutsia*, and *Oligella* were positively correlated with GLU, TG, TC, LEP, and INS and negatively correlated with ghrelin and ADP. *Bacteroides*, *Parabacteroides*, and *Akkermansia* were negatively correlated with GLU, TG, and TC. Conclusion: BRS had promising effects on weight loss, which could be associated with the improvement in host metabolism by regulating intestinal flora.

Keywords: banana resistant starch, biochemical index, gut microbiota, correlation analysis, obesity

OPEN ACCESS

Edited by:
Wenzhen Liao,
Southern Medical University, China

Reviewed by:
Sastia Prama Putri,
Osaka University, Japan
Yingbin Shen,
Guangzhou University, China

***Correspondence:**
Juan Wang
wangjuan@scut.edu.cn

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *24 June 2020*
Accepted: *05 January 2021*
Published: *27 January 2021*

Citation:
Fu J, Wang Y, Tan S and Wang J (2021) Effects of Banana Resistant Starch on the Biochemical Indexes and Intestinal Flora of Obese Rats Induced by a High-Fat Diet and Their Correlation Analysis. Front. Bioeng. Biotechnol. 9:575724. doi: 10.3389/fbioe.2021.575724

INTRODUCTION

Obesity (BMI $\geq$ 30 kg/m^2) is a metabolic disease that results in weight gain due to the accumulation of a large amount of adipose tissue in the body (Prospective Studies Collaboration, 2009). The prevalence of obesity likely results from the interaction of multiple factors: heredity, environment, dietary intervention, physical activity, lifestyle, and so on. Sedentary lifestyle and a high-sugar

high-fat diet appear to be the most important factors causing obesity (Chooi et al., 2019). With the development of society, the prevalence of obesity has risen dramatically year after year. In 2015, approximately 603 million adults and 107 million children were obese (Collaborators et al., 2017), which means that obesity has become a worldwide epidemic. Moreover, obesity is a prevalent manifestation of metabolic disorders, and accumulating evidence has demonstrated that TC, TG, LDL-C, INS, and LEP levels were significantly higher in obese than in normal weight people, while the concentrations of ADP and ghrelin decreased (Yildiz et al., 2004; Addante et al., 2011). Vekic's work (Vekic et al., 2019), which focused on metabolic disorders in obesity, indicated that high concentrations of TG and LDL-C accompanied by decreased HDL-C concentrations are the main characteristics of dyslipidaemia. LEP and ADP are associated with inflammation since LEP stimulates adipose tissue to secrete inflammatory cytokines, while ADP acts as an anti-inflammatory adipokine. At the same time, INS has a higher concentration in plasma and can lead to insulin resistance, hyperglycaemia, and hyperinsulinaemia. In summary, obesity will increase the risk of a variety of diseases, such as cardiovascular diseases (hypertension, atherosclerosis, and hyperlipidaemia) (Lavie et al., 2014), type 2 diabetes (Malik et al., 2010) and some cancers (esophageal cancer, cholangiocarcinoma, and pancreatic cancer) (Nam, 2017), which cause great threats to global public health and have passive effects on the quality of human life and healthcare costs (Tremmel et al., 2017).

Approximately 1.5 kg of bacteria exists in our gut, and they are not only the densest but also the most diverse microbiome in the human body (Zhao, 2013). The intestinal flora is closely related to the health status of the host, and the composition of the gut microbiota varies due to the age of the host, living environment, dietary habits, and other factors (Delzenne and Cani, 2011). It plays a very important role in the physiological processes of the host, such as nutrient digestion, absorption, energy utilization and storage, and metabolism (Saad et al., 2016). Diet is considered to be a major factor affecting the structure of intestinal flora that transforms food ingredients into bioactive metabolites with different functions, which further regulate the composition of intestinal microorganisms and influence the host phenotype (Laparra and Sanz, 2010). A large number of studies have demonstrated that obesity is related to gut microbiota dysfunction and that dietary intervention has important impacts on intestinal flora to a certain extent, mainly manifesting as changes in gut microbiota structure and function, hindering the development of obesity (Portune et al., 2017).

Resistant starch (RS) is defined as the sum of the starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Anonymous, 1991). RS is subdivided into five major types: RS_1, RS_2, RS_3, RS_4, and RS_5 (Meenu and Xu, 2019). RS_1 is found mainly in grains or seeds and is composed of a matrix of proteins that makes it difficult for enzymes to get close to the starch granules. RS_2 is resistant to enzyme digestion and present in food such as raw potatoes and unripe bananas. RS_3 is retrograded starch formed during the cooling of gelatinized starch in moist-heated food. RS_4 is chemically modified starch due to crosslinking, esterification, and etherification. RS_5 is formed by amylose with lipids, and the long carbon chains are the cause of RS_5 resistance. As a new prebiotic (Sajilata et al., 2006), RS can prevent colon cancer, slow the release of glucose, and control weight gain and other physiological effects, and the fermentation of RS in the colon produces short-chain fatty acids (SCFAs), which improve the intestinal barrier environment and play a key role in the prevention and relief of metabolic syndrome.

Recent studies have pointed to the composition of intestinal flora in connection with RS. The higher levels of *Bifidobacterium*, *Akkermansia*, and *Allobaculum,* which were colonized by RS, could alleviate the development of obesity, and the proportions of *Bifidobacterium* and *Akkermansia* were positively correlated with gut weight and GLP-1 (Tachon et al., 2013). Furthermore, RS could stimulate a cluster of bacteria in the *Clostridia* class and increase the concentration of fecal butyrate to decrease the inflammatory response and improve insulin sensitivity (Sanchez-Tapia et al., 2020). In addition, *Bacteroides plebeius*, *Blautia producta*, and *Prevotella stercorea* were negatively associated with TC, while *Bacteroides ovatus*, *Bacteroides uniformis*, and *Bacteroides acidifaciens* were positively correlated with ADP, and all of them were enriched after RS intervention, although RS did not significantly enrich *Methanobrevibacter* spp. and *Eubacterium dolichum,* but they were correlated with weight and SCFA levels. *Methanobrevibacter* spp., *Ruminococcus gnavus*, and *Prevotella stercorea* were negatively correlated with LDL (Upadhyaya et al., 2016).

Banana resistant starch (BRS) belongs to the RS_2 type and is the main ingredient of green banana, which comprises approximately 50% of unripe banana pulp (dry base). Our preceding study showed that BRS had a good effect on weight loss and improved the condition of the obese rats. After obese rats were treated with a dose of 2.5 g/kg BRS for 6 weeks, the body weight of rats in BRS group was significantly decreased by 9.06% ($p < 0.05$) and the fat accumulation was reduced, which especially decreased by 34.32% in the epididymis fat and 31.48% in the ratio of adipose tissues (including epididymis fat and renal fat) and weight ($p < 0.05$), compared with that of obese rats in model group. But there was no significant difference in food intake compared with obese rats. Histology analysis revealed that BRS alleviated hepatic steatosis and fatty liver in obese rats. All of these above will be reported in detail in another article.

There are some studies about food intervention to relieve obesity. Our preceding researches (Tan and Wang, 2018; Wang, 2018) report that BRS has a good effect on weight loss and improved the condition of the obese rats. Moreover, some literatures (Alvarado-Jasso et al., 2020; Wu et al., 2020) show that banana flour is benefit for reducing body fat. But few studies focused on the mechanistic role of the BRS-induced weight loss. Therefore, the anti-obesity mechanism of BRS mediating intestinal flora was studied in this article. It is reported that RS has many physiological benefits, including the management and control of glucose-metabolism related diseases such as type 2 diabetes and obesity (Niba, 2002). However, the microstructures of RS from different food sources are not same, so the physiological functions of RS may be different. Therefore, the health benefits of BRS could not be predicted without test.

The structures and physicochemical properties of BRS differ from variety to variety (Wang et al., 2014, 2016). We found that some banana cultivar do not have good effects on weight loss (Tan and Wang, 2018). Thus, the cultivar of banana used here is proved with a positive influence on alleviating obesity by our previous studies.

The present study aimed to explore the effect of BRS on intestinal flora and discuss the correlation among intestinal flora, the glucolipid metabolism index and serum hormones in a high-fat diet-induced obesity model in order to understand the mechanism of BRS-induced weight loss. The significances of this study are as follows. Firstly, it could enrich the theoretical basic research related to RS. Secondly, it is a fundamental research about the functional ingredient of banana.

MATERIALS AND METHODS

Materials

Banana resistant starch was provided by Natural Banana Healthy Food Co., Ltd., (Guangdong, China). Banana cultivar is *Musa ABB Dajiao*. Orlistat (approval number H20123131) was purchased from Zhien Pharmaceutical Co., Ltd., (Chongqing, China).

Animals and Experimental Design

Male SD rats (100–110 g, 6 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (GDMLAC, Guangdong, China) with the laboratory animal license number SCXK (Guangdong) 2013-0002. The normal chow diet (NCD: 55% nitrogen-free extract, 18% crude protein, 10% water, 8% ash, 4% crude fat, 5% crude fiber, 1.8% calcium, and 1.2% phosphorus, with total calorific value 327.6 Kcal/100 g) was obtained from Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd., (Jiangsu, China). A high-fat diet (HFD: containing 64% normal control diet, 15.0% lard, 15.0% sucrose, 5% casein, 0.6% calcium hydrogen phosphate, and 0.4% stone powder, with total calorific value 404.84 Kcal/100 g) was obtained from Guangdong Medical Laboratory Animal Center (GDMLAC, Guangdong, China).

All animals were raised in the specific pathogen-free (SPF) experimental animal room (constant temperature 21 ± 2°C, relative humidity 50 ± 10% and a 12-h light-dark cycle) of the Experimental Animal Center of South China Agricultural University with free access to water and food. The protocol and design of the animal experiment was showed in **Figure 1**. After 6 weeks of the experiment, all rats were fasted for 12 h and sacrificed with an intraperitoneal injection of 10% chloral hydrate. Blood samples were collected from the abdominal aorta and centrifuged at 3,000 r/min for 15 min at 4°C to obtain serum, which was stored at −80°C for measurements. The intestinal tract contents were obtained and stored at −80°C until analysis.

Biochemical Assays

Total cholesterol (TC), total triacylglycerol (TG), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), and blood glucose (GLU) were determined by commercially available kits (Mindray Biomedical Electronics Co., Ltd., Shenzhen, China). Serum leptin (LEP), insulin (INS), adiponectin (ADP), thyroxine (T4), and ghrelin were measured using ELISA kits (ColorfulGene Biological Technology Co., Ltd., Wuhan, China). All measurements were carried out according to the manufacturer's protocol.

DNA Extraction and Sequencing

Total genomic DNA from large intestine samples (n = 3 per group) was extracted using the CTAB method, and the DNA concentration and purity were monitored on 1% agarose gels. Then, the V3–V4 hypervariable region of the microbiota 16S rRNA was amplified with the primers 341F (5′- CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). The PCRs consisted of the following program: initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, and finally 72°C for 5 min. PCRs were carried out in 30-μL reactions with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and approximately 10 ng of template DNA. The same volume of 1 × loading buffer (containing SYBR green) was mixed with PCR products, and electrophoresis was performed on a 2% agarose gel for detection. Then, mixed PCR products were purified with a GeneJET™ Gel Extraction Kit (Thermo Scientific, MA, United States). Purified amplicons were sequenced on an Ion S5™ XL platform (Thermo Scientific, MA, United States), and 400/600 bp single-end reads were generated following the manufacturer's recommendations by Nuohe Zhiyuan Technology Co., Ltd (Beijing, China).

Bioinformatics Analysis

Cutadapt software (version V1.9.1) was used to filter and quality control the data to obtain raw reads, and then raw reads were detected and the chimera sequences were removed by usearch software (version v7.0.1090[1]) to obtain clean reads. Operational taxonomic units (OTUs) were performed by Uparse (version v7.0.1001[2]) with $a \geq 97\%$ similarity threshold, and taxonomic analysis was conducted in comparison with the Silva database[3] using the RDP classifier Mothur algorithm to annotate the taxonomic information of representative sequences for each OTU. The alpha diversity index and PCA analysis were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). A heatmap was generated by using the vegan package in R software. Linear discriminant effect size analysis (LEfSe) was performed to characterize the differences among groups. The non-parametric factorial Kruskal-Wallis (KW) sum-rank test was used to evaluate species with significant differences in abundance between different groups, and linear discriminant analysis (LDA) was used to assess the magnitude of the impact of significantly different species. Spearman association analyses between gut microbiota and the metabolites and the r- and p-values were conducted using the MANTEL function. Moreover,

[1]http://www.drive5.com/usearch/manual/chimera_formation.html

[2]http://drive5.com/uparse/

[3]http://drive5.com/uchime/uchime_download.html

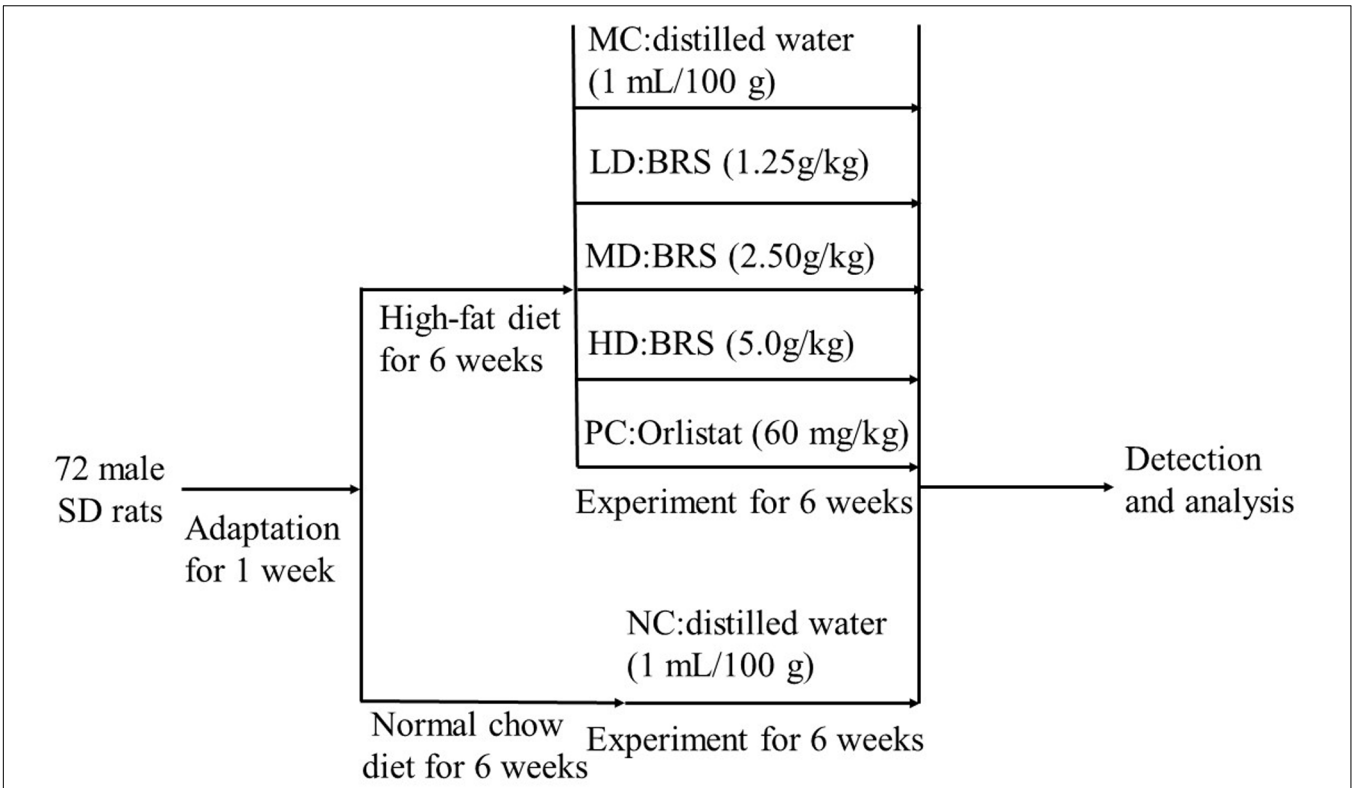


FIGURE 1 | Animal experimental protocol and design. MC-model control group, LD-low dose group of BRS, MD-middle dose group of BRS, HD-high dose group of BRS, PC-positive control group, NC-normal control group.

the visualization work was done by the PHEATMAP function in the pheatmap package.

Statistical Analysis

The data are shown as the mean ± SD. Statistical analysis was implemented using a one-way analysis of variance (ANOVA) followed by LSD *post hoc* test to determine the differences between groups. The results were considered significant when $p < 0.05$. Analyses were performed using IBM SPSS Statistics 24.0 (IBM, Chicago, IL, United States).

RESULTS

BRS Ameliorated the Levels of Glucolipid Metabolism

The results showed that, compared with the NC group, the levels of GLU, TG, and TC markedly increased in the MC group ($p < 0.01$). The BRS treatment groups displayed a significant decrease in the levels of GLU ($p < 0.01$) and TG ($p < 0.01$) in comparison with the MC group, which gradually approached the NC group. The TC level in the LD ($p < 0.01$) and HD ($p < 0.05$) groups was extremely reduced, except for the MD group. In addition, an eventful reduction in LDL-C ($p < 0.01$) in the HD group, as well as a significant enrichment of HDL-C ($p < 0.05$) in the PC group, were observed compared with the MC group, but the HDL-C ($p < 0.05$) level in the HD group was lower than that in the MC group (**Table 1**). The above results showed that BRS could improve glucose and lipid metabolism abnormalities by lowering levels of TG and GLU. Effects of BRS on serum hormone levels.

To further explain the relationship between serum hormones and obesity, the levels of LEP, INS, ghrelin, ADP, and T4 were analyzed. As shown in **Table 2**, LEP and INS levels ($p < 0.01$) in the MC group significantly increased, while ADP ($p < 0.01$), ghrelin ($p < 0.01$), and T4 ($p < 0.05$) levels significantly decreased compared with the NC group. Compared with the MC group, the LEP and INS levels were crucially lower, while the ADP levels were sharply higher in the BRS and PC groups ($p < 0.01$). Additionally, the ghrelin levels ($p < 0.05$) of the HD group were obviously increased, but the LD and MD groups lacked a notable increase. The contents of T4 ($p < 0.01$) in the MD and HD groups were substantially enhanced compared with the MC group. It was interesting that the above serum hormones have a dose-effect relationship with BRS. It was implied that BRS were effective on reducing LEP and INS levels, increasing ghrelin and ADP levels, and then inhibiting obesity.

BRS Changed the Diversity of the Gut Bacterial Communities

Alpha diversity is used to assess the diversity of the microbial community in the sample, usually expressed as the alpha

diversity index (Good's coverage, Chao1, ACE, and Shannon) (Li et al., 2020). The Good's coverage index reflects the depth and rationality of sample sequencing, which means that the closer the value is to 1, the lower is the probability of new OTUs in the sample. The Chao1 and ACE indexes were positively correlated with species richness. The Shannon index considers the uniformity of species distribution on the basis of richness; the higher the richness index and the uniformity, the stronger is the sample diversity. Therefore, the higher the Shannon index, the higher the biodiversity. The species diversity in different groups is shown in **Table 3**. The Good's coverage of each group reached more than 0.99 ($p > 0.05$), indicating that each group had sufficient samples and that almost all of the sequences were detected. Compared to the NC group, the Chao1 and ACE indexes were not significantly different in the MC group ($p > 0.05$), but the Shannon index was significantly lower ($p < 0.05$). There was no difference in species richness in the intestinal flora of rats induced by the high-fat diet, but the highly uneven colony distribution led to a decrease in the community diversity of the MC group, suggesting that the high-fat diet caused an imbalance in the proportion of intestinal flora structure in rats. In the MD group, the Shannon index was significantly increased compared with that in the MC group ($p < 0.05$), reflecting that supplementation with BRS regulates intestinal microbial community diversity to a certain extent.

Beta diversity is the analysis of microbial community structure of different samples to reveal the similarity of community composition between groups. Principal component analysis (PCA) is one of the methods used to evaluate the diversity of phylogenetic differences. In the PCA plot (**Figure 2A**), all groups exhibited an obviously distinct clustering of microbiota composition, suggesting that the samples in each group have a high degree of parallelism. The MC group was markedly separated from the NC group, with the LD, HD, and PC groups distributed in between, although the MD group overlapped with the MC group. The results implied that BRS intervention evidently altered the overall structure of the gut microbiota and improved disorders of the intestinal bacteria. It was observed that

TABLE 1 | BRS improved glucose and lipid metabolism in obese rats (mmol /L).

Groups	GLU	TG	TC	LDL-C	HDL-C
NC	$3.82 \pm 0.15^{\#\#}$	$0.44 \pm 0.07^{\#\#}$	$1.46 \pm 0.06^{\#\#}$	0.46 ± 0.09	0.70 ± 0.08
MC	$9.77 \pm 0.21^{**}$	$0.65 \pm 0.03^{**}$	$1.79 \pm 0.04^{**}$	0.48 ± 0.05	0.64 ± 0.05
LD	$4.16 \pm 0.52^{\#\#}$	$0.44 \pm 0.05^{\#\#}$	$1.45 \pm 0.02^{\#\#}$	0.49 ± 0.10	0.61 ± 0.10
MD	$7.20 \pm 0.87^{**\#\#}$	$0.45 \pm 0.03^{\#\#}$	$1.78 \pm 0.09^{**}$	0.43 ± 0.04	0.70 ± 0.06
HD	$3.78 \pm 0.3^{\#\#}$	$0.47 \pm 0.07^{\#\#}$	$1.61 \pm 0.07^{\#}$	$0.33 \pm 0.01^{**\#\#}$	$0.53 \pm 0.04^{**\#}$
PC	$6.76 \pm 0.56^{**\#\#}$	$0.57 \pm 0.03^{**}$	$1.66 \pm 0.07^{**\#}$	0.46 ± 0.04	$0.78 \pm 0.05^{\#}$

*The values were expressed as mean ± standard error (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by LSD post hoc test. *p < 0.05 and **p < 0.01 vs. NC; #p < 0.05 and ##p < 0.01 vs. MC. "*"Means the difference was significant with NC at the 0.05 level.*

TABLE 2 | BRS ameliorated the levels of serum hormones in obese rats.

Groups	ADP (ng/mL)	INS (U/L)	LEP (ng/mL)	Ghrelin (mU/L)	T4 (ng/mL)
NC	$35.17 \pm 0.59^{\#\#}$	$8.46 \pm 0.19^{\#\#}$	$1.20 \pm 0.09^{\#\#}$	$1.05 \pm 0.11^{\#\#}$	$17.52 \pm 0.41^{\#}$
MC	$23.60 \pm 0.39^{**}$	$15.15 \pm 0.40^{**}$	$2.10 \pm 0.13^{**}$	$0.76 \pm 0.03^{**}$	$14.51 \pm 0.33^{*}$
LD	$24.71 \pm 0.46^{**\#\#}$	$11.51 \pm 0.24^{**\#\#}$	$1.82 \pm 0.14^{**\#\#}$	$0.79 \pm 0.02^{**}$	$14.73 \pm 0.72^{*}$
MD	$28.16 \pm 0.41^{**\#\#}$	$11.18 \pm 1.73^{\#\#}$	$1.59 \pm 0.07^{**\#\#}$	$0.85 \pm 0.05^{**}$	$17.91 \pm 0.67^{\#\#}$
HD	$34.44 \pm 0.68^{\#\#}$	$9.29 \pm 0.54^{\#\#}$	$1.37 \pm 0.09^{**\#\#}$	$0.92 \pm 0.06^{*\#}$	$18.66 \pm 1.27^{\#\#}$
PC	$26.18 \pm 0.51^{**\#\#}$	$9.23 \pm 0.23^{\#\#}$	$1.62 \pm 0.11^{**\#\#}$	$0.86 \pm 0.04^{**}$	$16.27 \pm 1.18^{\#}$

*The values were expressed as mean ± standard error (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by LSD post hoc test. *p < 0.05 and **p < 0.01 vs. NC; #p < 0.05 and ##p < 0.01 vs. MC.*

TABLE 3 | Alpha diversity analysis of the gut microbiota in different groups.

Groups	Good's coverage	Chao1	ACE	Shannon
NC	0.9990 ± 0.0000	796.61 ± 24.63	793.79 ± 21.42	$7.65 \pm 0.02^{\#}$
MC	0.9990 ± 0.0000	807.69 ± 20.23	808.05 ± 21.63	$7.31 \pm 0.01^{*}$
LD	0.9990 ± 0.0000	$696.37 \pm 16.12^{*\#}$	$711.48 \pm 18.51^{*\#}$	$6.53 \pm 0.05^{*\#}$
MD	0.9990 ± 0.0000	781.74 ± 23.12	784.88 ± 20.20	$7.43 \pm 0.07^{*\#}$
HD	0.9990 ± 0.0000	766.78 ± 3.97	$765.30 \pm 3.22^{\#}$	$7.28 \pm 0.03^{*}$
PC	0.9987 ± 0.0005	$826.09 \pm 38.17^{*}$	821.53 ± 28.29	$7.59 \pm 0.01^{\#}$

*The values were expressed as mean ± standard error (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by LSD post hoc test. *p < 0.05 vs. NC; #p < 0.05 vs. MC.*

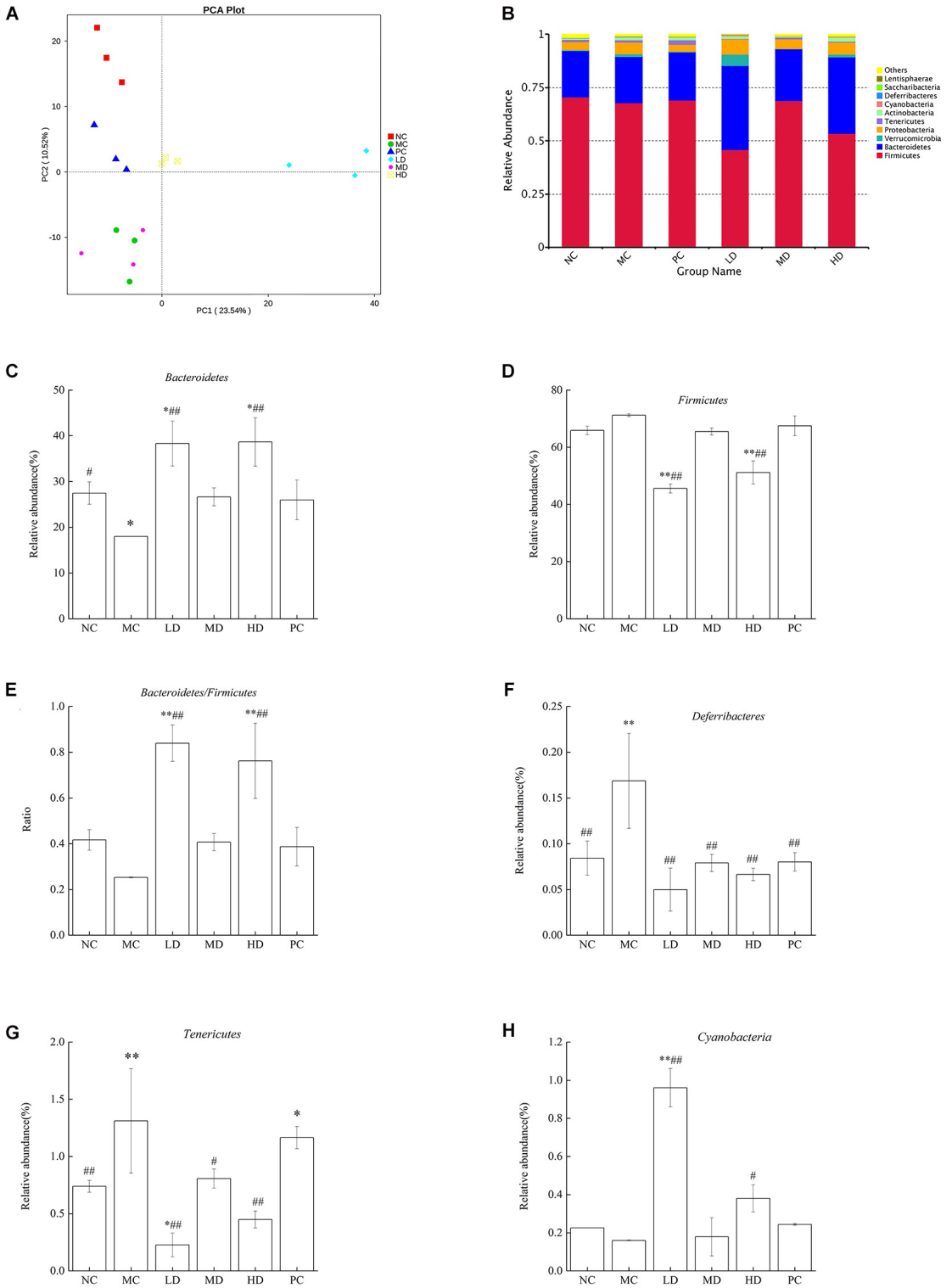


FIGURE 2 | BRS alters the composition based on OTUs and the relative abundance of gut microbiota at the phylum level. **(A)** Principal component analysis (PCA) of gut microbiota based on OTUs. The abscissa represents the first principal component, the ordinate represents the second principal component, and the percentage represents the contribution of the principal component to the sample difference. Each point in the figure represents a sample, and samples from the same group are represented in the same color. **(B)** Bacterial taxonomic profiling at the phylum level of gut microbiota. **(C)** *Bacteroidetes*, **(D)** *Firmicutes*, **(E)** *Bacteroidetes/Firmicutes* ratio, **(F)** *Deferribacteres*, **(G)** *Tenericutes*, and **(H)** *Cyanobacteria*. Differences were based on ANOVA analysis followed by LSD *post hoc* test and denoted in graph bars as follows: $*p < 0.05$ and $**p < 0.01$ compared with NC; $\#p < 0.05$ and $\#\#p < 0.01$ compared with MC.

the diversity of intestinal flora was reduced significantly in obese rats. But the diversity could be recovered by BRS.

Effects of BRS on the Composition and Structure of Intestinal Bacteria

To provide a better comprehension of the changes in the composition and structure of intestinal bacteria in response to BRS treatment, the bacterial species and abundance were determined at the phylum and genus levels among groups. In total, 12 phyla, 25 classes, 52 orders, 88 families, 192 genera, 78 species, and 1,043 OTUs were detected in this research. The study indicated that *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria*, *Tenericutes*, *Actinobacteria*, *Cyanobacteria*, *Deferribacteres*, *Saccharibacteria*, and *Lentisphaerae* were phyla found in each group, and the bacterial composition was dominated by *Firmicutes* and *Bacteroidetes* (**Figure 2B**). Meanwhile, the relative abundance of different phyla in the gut microbes of each group was discussed for the purpose of accounting for the effect of BRS in intestinal bacteria. It was clear that the MC group fed a high-fat diet induced a major reduction in the relative abundance of *Bacteroidetes* ($p < 0.05$); however, although the difference was not significant, *Firmicutes* had a slight increase combined with the *Bacteroidetes*/*Firmicutes* ratio (B/F) and had a mild decrease (**Figures 2C–E**) in comparison with the NC group ($p > 0.05$), which was consistent with the literature (Dong et al., 2016). After 6 weeks of BRS treatment in obese rats, the LD and HD groups had a higher abundance of *Bacteroidetes* (38.31 ± 4.94%, 38.65 ± 5.33% vs. 17.98 ± 0.01%), a lower abundance of *Firmicutes* (45.55 ± 1.56%, 51.11 ± 4.03% vs. 71.18 ± 0.47%) and a higher B/F ratio (0.84 ± 0.08, 0.76 ± 0.16 vs. 0.25 ± 0.02) than the MC group ($p < 0.01$). As shown in **Figures 2F–H**, compared with the NC group, *Deferribacteres* and *Tenericutes* were significantly enhanced ($p < 0.01$), and *Cyanobacteria* was decreased in the MC group ($p > 0.05$), but the result did not achieve significance. Notably, BRS treatment had a beneficial effect on intestinal flora. The LD ($p < 0.01$) and HD ($p < 0.05$) groups had a considerable improvement in *Cyanobacteria* compared with the MC group (0.96 ± 0.10%, 0.38 ± 0.07% vs. 0.16 ± 0.02%); the LD ($p < 0.01$), MD ($p < 0.05$), and HD ($p < 0.01$) groups had a substantial reduction in *Tenericutes* compared with the MC group (0.23 ± 0.10%, 0.81 ± 0.08%, 0.45 ± 0.07% vs. 1.31 ± 0.46%); the *Deferribacteres* abundance of MC, LD, MD, HD, and PC was 0.17 ± 0.05%, 0.05 ± 0.02%, 0.08 ± 0.01%, 0.07 ± 0.01%, and 0.08 ± 0.01%, respectively, which were extremely lower with BRS treatment than that of the MC group ($p < 0.01$).

The Top10 genera of relative abundance in different groups were *Bacteroides*, *Akkermansia*, *Lachnospiraceae_NK4A136_group*, *Desulfovibrio*, *[Eubacterium]_coprostanoligenes_group*, *Ruminococcaceae_NK4A214_group*, *Romboutsia*, *unidentified_Ruminococcaceae*, *Roseburia*, and *Ruminococcaceae_UCG-014* (**Figure 3A**). In addition, the heatmap analysis showed that the MC group had more *Turicibacter*, *Romboutsia*, *Oligella*, *Roseburia*, *Coprococcus_2*, and *Bifidobacterium* than the NC group. As expected, BRS could regulate intestinal flora; the relative abundances of *Turicibacter*, *Romboutsia*, and *Oligella* were lower, and *Bacteroides*, *Parabacteroides*, *Desulfovibrio*, *[Eubacterium]_coprostanoligenes_group*, *Psychrobacter*, *Akkermansia*, *Ruminococcaceae_UCG-014*,*Ruminococcaceae_UCG-005*,*Ruminococcaceae_NK4A214_group*, *Alistipes*, *Lactobacillus*, *Oscillibacter*, *Alloprevotella*, *Parasutterella*, *Christensenellaceae_R-7_group*, and *Corynebacterium_1* were greater in response to BRS intake (**Figure 3B** and **Table 4**). In particular, the relative abundance of some bacteria was reversed to close to that of normal rats by BRS and orlistat (**Figures 3C–E**). *Turicibacter* in MC (2.47 ± 1.09%) and NC (0.72 ± 0.03%) decreased in LD (0.60 ± 0.12%), MD (0.91 ± 0.11%), HD (0.67 ± 0.17%), and PC (1.02 ± 0.15%). *Romboutsia* in MC (5.06 ± 1.97%) and NC (1.89 ± 0.08%) decreased in LD (2.27 ± 0.36%), MD (2.44 ± 0.29%), HD (1.87 ± 0.44%), and PC (3.10 ± 0.31%). *Oligella* in MC (1.51 ± 0.39%) and NC (0.31 ± 0.07%) decreased in LD (0.18 ± 0.08%), MD (0.33 ± 0.21%), HD (0.68 ± 0.47%), and PC (0.52 ± 0.36%), which fell sharply with BRS and orlistat treatment ($p < 0.01$). Therefore, BRS intervention increased the abundance of beneficial bacteria, such as *Cyanobacteria*, *Alistipes*, *Parabacteroides*, *Bacteroides*, *Ruminococcaceae*, *Lachnospiraceae*, and *Akkermansia*. At the same time, it inhibited the growth of bacteria including *Deferribacteres*, *Tenericutes*, *Turicibacter*, *Romboutsia*, and *Oligella*.

BRS Modulated the Key Phylotypes of Gut Microbiota

LEfSe was able to search for biomarkers with statistically significant differences from group to group and was applied to determine characteristic bacteria in each group. The results revealed that the NC group was rich in *Clostridiales*; in contrast, the MC group was characterized by a greater increase in the abundance of *Coprococcus_2*. After gavage of BRS, the intestinal flora of the LD group was markedly enhanced in *Bacteroides* and *Ruminococcaceae_UCG-014*, while the MD group was rich in *Lachnospiraceae_bacterium_28-4*. Orlistat treatment also notably increased the abundance of *Ruminococcaceae_NK4A214_group*. However, there was no significant change in the HD group, and only the NC group was detected to have a lower abundance of *Firmicutes* at the phylum level (**Figures 4A,B**). The results showed that BRS administration modulated the key phylotypes of gut microbiota by elevating the *Bacteroides*, *Ruminococcaceae_UCG-014*, and *Lachnospiraceae_bacterium_28-4* levels.

Association Between Obesity-Related Biological Parameters and Gut Microbiota

Spearman correlation analysis was applied to assess the relationship between intestinal flora and the obesity-related biochemical indexes to identify whether there was a correlation between gut microbiota and host metabolism. The results showed that *unidentified_Ruminococcaceae*, *Romboutsia*, and *Turicibacte* were positively associated with the levels of GLU, TG, TC, LDL and HDL. *Bacteroides*, *Akkermansia*,

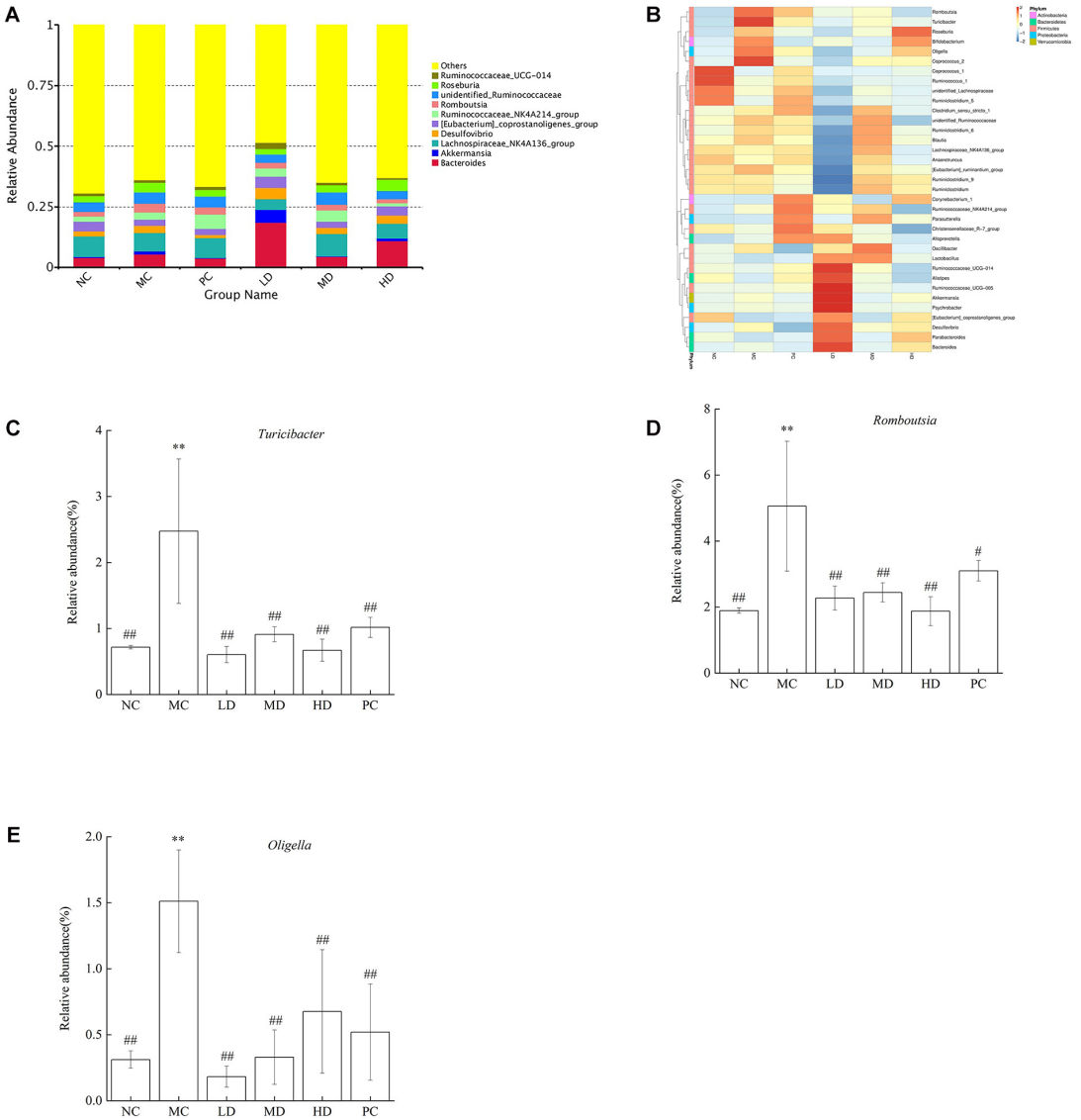


FIGURE 3 | Relative abundance of gut microbiota at the genus level. **(A)** Bacterial taxonomic profiling at the genus level of gut microbiota. **(B)** Heatmap analysis at the genus level among different test groups displayed the changes in the relative abundance of identified genus. **(C)** *Turicibacter*, **(D)** *Romboutsia*, and **(E)** *Oligella*. Differences were based on ANOVA analysis followed by LSD *post hoc* test and denoted in graph bars as follows: $*p < 0.05$ and $**p < 0.01$ compared with NC; $\#p < 0.05$ and $\#\#p < 0.01$ compared with MC.

TABLE 4 | Effects of BRS on gut bacteria at the genus level.

Genus name	NC (%)	MC (%)	LD (%)	MD (%)	HD (%)	PC (%)
Bacteroides	3.99 ± 0.94	3.88 ± 1.24	↑ 18.65 ± 9.43$^{**\#\#}$	4.35 ± 1.64	↑ 10.92 ± 2.36$^{*\#}$	3.71 ± 0.90
Ruminococcaceae_UCG-014	1.00 ± 0.21	0.87 ± 0.12	↑ 1.39 ± 0.56$^{\#}$	1.10 ± 0.17	↓ 0.49 ± 0.09*	1.28 ± 0.21
Ruminococcaceae_UCG-005	0.40 ± 0.08	0.40 ± 0.06	↑ 1.53 ± 0.68$^{**\#\#}$	0.42 ± 0.11	0.33 ± 0.07	0.48 ± 0.12
Alistipes	1.14 ± 0.18	0.95 ± 0.17	↑ 1.71 ± 0.25$^{*\#\#}$	1.04 ± 0.29	0.87 ± 0.16	1.16 ± 0.16
Akkermansia	0.69 ± 0.13	0.63 ± 0.43	↑ 1.29 ± 0.16$^{*\#}$	0.33 ± 0.38	0.64 ± 0.09	0.38 ± 0.26
Parabacteroides	0.39 ± 0.13	0.25 ± 0.07	↑ 0.88 ± 0.36$^{*\#\#}$	0.29 ± 0.13	↑ 0.69 ± 0.34$^{\#}$	0.31 ± 0.15

The values were expressed as mean ± standard error (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by LSD post hoc test. $*p < 0.05$ and $**p < 0.01$ vs. NC; $\#p < 0.05$ and $\#\#p < 0.01$ vs. MC.

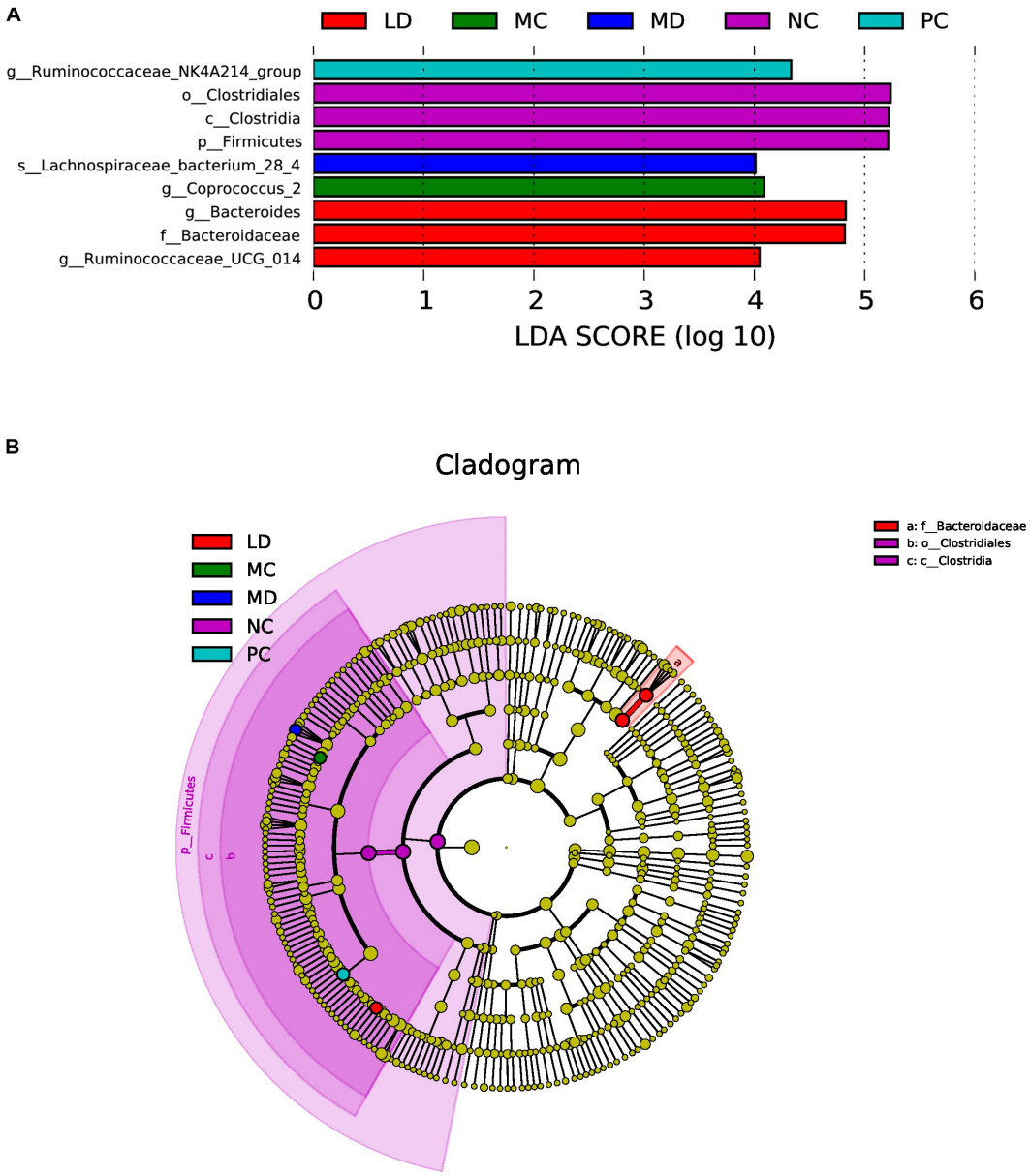


FIGURE 4 | LEfSe analysis of gut bacteria in each group. **(A)** Linear discriminant analysis (LDA) score plot. Species with an LDA Score threshold >4 were listed and the length of the bar chart represented the impact of different species. **(B)** Taxonomy cladogram. The circle of radiation from inside to outside represented the taxonomic rank from phylum to genus (or species) and the diameter of the circles was based on relative abundance.

Desulfovibrio, *X.Eubacterium._coprostanoligenes_ group*, and *Parabacteroides* were negatively associated with GLU, TG, TC, and HDL levels and positively correlated with LDL levels. In contrast, bacteria including *Blautia*, *Ruminiclostridium_5*, *Clostridium_sensu_stricto_1*, and *Ruminiclostridium_6* appeared to have a positive relationship with GLU, TG, TC, and HDL levels and had a negative correlation with LDL levels. *Oligella*, *Coprococcus_2* and *Bifidobacterium* were positively related to GLU, TG, and TC levels, while they were negatively related to HDL levels (**Figure 5A** and **Supplementary Table 1**). The relationship between serum hormones and intestinal flora was studied, as shown in **Figure 5B** and **Supplementary Table 2**. *Romboutsia*, *Turicibacter*, and *Oligella* presented a negative relationship with ghrelin, ADP, and T4 and displayed a positive relationship with LEP and INS. However, *X.Eubacterium._coprostanoligenes_group*, *Coprococcus_1*, and *Anaerotruncus* were positively correlated with ghrelin, ADP, and T4 and negatively correlated with LEP and INS. Additionally, *Roseburia* was found to have a positive relationship with T4, ADP, and INS and to have a negative relationship with ghrelin and LEP. In summary, the change in gut microbiota regulated by BRS was linked with obesity-related blood indicators, suggesting

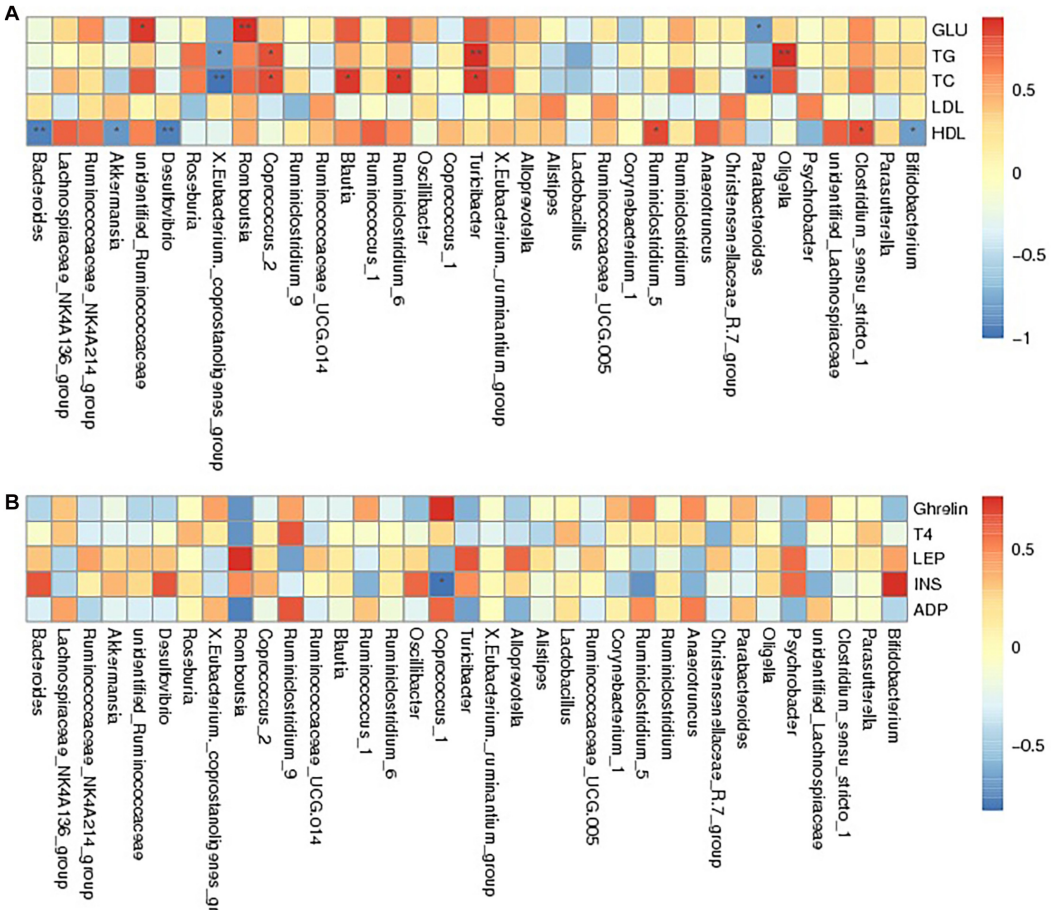


FIGURE 5 | Spearman association analysis between gut microbiota and metabolic parameters at the genus level. The depth of the color corresponded the extent of relevance between gut microbiota and metabolic parameters, red meant positive correlation and blue meant negative correlation. **(A)** Glucolipid metabolism parameters and **(B)** Serum hormone. *Correlation was significant at the 0.05 level, **Correlation was significant at the 0.01 level.

that the BRS-induced weight loss may partly root in the impact on intestinal flora.

DISCUSSION

Our former researches showed that BRS could play a role in the prevention and control of obesity, and the dietary supplement of BRS could reduce body fat and weight (Wang et al., 2019). Increasing evidence showed that the levels of serum parameters and the relative abundance of gut microbiota will change dramatically with the development of obesity. However, the relationship between biological parameters and gut microbiota, and the anti-obesity mechanism of BRS have not yet been investigated. This study showed the effects of BRS on glucose and lipid metabolism, gut bacteria and their correlation, in order to understand the anti-obesity mechanism of BRS. Therefore, this study could provide the evidence and data for banana functional food development.

In this experiment, the concentrations of GLU, TG and TC were notably increased in high-fat diet-induced obese rats, and studies have demonstrated that the accumulation of serum lipids is considered to be the major factor in the risk of cardiovascular disease accompanied by an increase in GLU, TG, TC, and LDL-C levels and a decrease in HDL-C levels (Dyrbus et al., 2018). BRS significantly reduced GLU, TC, TG, and LDL-C levels in serum compared to the MC group during 6 weeks of BRS supplementation, which is consistent with previous literature (Lee et al., 2018), suggesting that BRS may play an effective role in ameliorating abnormal blood glucose and lipid metabolism induced by obesity.

Obesity is associated with changes in hormones that can send signals to the brain to modulate energy balance, including reducing energy consumption and increasing energy intake (Hariri and Thibault, 2010). High levels of insulin are a sign of insulin resistance caused by obesity; an abundance of adipose tissue reduces the sensitivity of cells to insulin, and islet cells are then stimulated to produce more insulin, resulting in hyperinsulinaemia. Generally, insulin resistance will elevate the level of free fatty acids (FFAs), decrease the transportation of glucose and then store it as glycogen, resulting in damaged

glucose tolerance and an increase in blood glucose levels (Chen et al., 2018). As expected, the rats in the MC group had a significantly higher concentration of INS than those in the NC group, revealing that obese rats had severe insulin resistance. The research also showed that BRS could reduce INS significantly, which is consistent with previous studies (Maki et al., 2012). Meanwhile, with the increase in BRS dose, the insulin concentration gradually decreased in serum. Similarly, other hormones, including LEP, ADP, T4, and ghrelin, were also detected. LEP and ghrelin were associated with appetite and host energy balance. Similar to insulin resistance, there was resistance to leptin in obesity caused by excessive leptin content in serum and impaired energy homeostasis, leading to increased food intake and weight gain (Scarpace et al., 2005). In addition, the level of ghrelin was downregulated in obesity, and previous results indicated that ghrelin resistance may exist in obesity (Luna-Moreno et al., 2017). A low level of adiponectin in serum was related to chronic inflammation, and increasing adiponectin levels was conducive to preventing the occurrence of cardiovascular diseases through anti-inflammatory effects (Ohashi et al., 2010). According to the results, BRS treatment markedly decreased serum LEP levels and increased ADP, T4 and ghrelin levels compared with the MC group, which is consistent with previous research (Robertson et al., 2005; Shen et al., 2014). The data implied that BRS had potent effects on improving obesity-related hormone levels; furthermore, BRS could not only enhance the absorption and utilization of glucose and inhibit the rise in fasting blood glucose but also suppress the excessive secretion of insulin and improve the insulin sensitivity of cells.

The intestinal flora has a crucial influence on the human body, affecting the health and physiological functions of the host (Nicholson et al., 2012). Abundant evidence has indicated that gut microflora could be altered, including composition and diversity, in response to obesity caused by a high-fat diet (Damms-Machado et al., 2015; Liu et al., 2017). When the microbiome is distorted, dysbiosis may result in a disease state either by an excessive inflammatory response or poor immune system (Lumeng and Saltiel, 2011). Currently, diet is regarded as the key modulator in regulating disorders of the gut microbiota (Sonnenburg and Backhed, 2016). Therefore, long-term dietary intervention could be a potential, safe, and effective approach in the prevention and treatment of obesity. In the present report, alpha diversity was significantly enhanced by BRS treatment, especially in the MD group compared to the MC group, and the overall gut microbiota structure was distinctly shifted, as evidenced by PCA.

At the phylum level, all groups had the same species composition but different relative abundances. *Bacteroidetes* and *Firmicutes*, to our knowledge, co-exist in the human gut. The obese gut showed a tendency to reduce *Bacteroidetes* levels and increase *Firmicutes* levels, which was associated with host pathology (Ley et al., 2006). There was an outstanding decrease in the levels of *Bacteroidetes* in the MC group, and BRS could increase the *Bacteroidetes* levels and the ratios of B/F, which is consistent with a previous report (Parnell and Reimer, 2012). It is worth noting that in the MC group, the relative abundances of *Deferribacteres* and *Tenericutes* were sharply increased, while the relative abundance of *Cyanobacteria* was decreased. Although the difference was not significant in comparison with the NC group, the condition was significantly reversed after BRS treatment, which insinuated that BRS could improve gut bacterial structure. The above three bacteria belong to low-abundance bacteria (relative abundance $< 1\%$), while the dominant bacteria provide an overview of healthy or diseased states. Some key organisms with low abundance are also essential (Benjamino et al., 2018). Recent studies have shown that *Cyanobacteria* have anti-inflammatory effects due to heightened IL-10 levels, and exogenous *Cyanobacteria* supplementation could retard blood glucose levels and lipid peroxidation (Pandurangan and Kim, 2016; Li et al., 2019). The enrichment of the *Deferribacteres* and *Tenericutes* population is a common phenomenon in obesity, which is positively linked with the pro-inflammatory factors IL-6, TNF-α, and IL-17A, causing aggravation of inflammation in obesity (Wang et al., 2017; Li et al., 2019). BRS may have a protective effect on the integrity of the intestinal barrier by upregulating the B/F ratio, inhibiting the overgrowth of inflammation-related bacteria (*Deferribacteres* and *Tenericutes*) and repairing adverse changes in intestinal flora caused by a high-fat diet.

At the genus level, BRS administration enriched the relative abundance of *Alistipes*, *Parabacteroides*, and *Akkermansia* and decreased the levels of *Turicibacter*, *Romboutsia*, and *Oligella*, which were dramatically increased in the MC group. The increasing proportion of *Turicibacter* was illustrated to have a side effect on lipid metabolism, which was positively correlated with TG, TC, and LDL-C levels and negatively correlated with HDL-C levels (Wan et al., 2018). *Romboutsia*, the characteristic microbes in HFD-fed rats, exhibited a positive relationship with indicators of body weight (waistline and BMI) and lipid levels (TG, TC, and LDL-C) (Zeng et al., 2019). The current research on *Oligella* mainly focused on the urinary tract, but it could be isolated from wounds, making it an opportunistic pathogen (Demir and Celenk, 2014; Wang et al., 2018). *Alistipes* played a vital role in the improvement in obesity-related clinical indicators, including body weight, blood pressure, glucose homoeostasis, and uric acid (Wan et al., 2018). Antagonistic substances produced by *Parabacteroides* could defend against the colonization of pathogenic bacteria and prevent the development of infectious diseases (Nakano et al., 2013). *Akkermansia*, a mucin-degrading bacterium, belongs to the *Verrucomicrobia* phylum, which has been shown to play an important role in maintaining a healthy mucus layer in the human gut by degrading mucus to produce oligosaccharides and SCFAs (Belzer and de Vos, 2012). On the other hand, the higher species abundance of *Akkermansia* trended toward a healthier metabolic status in overweight and obese people, alleviating the progression of obesity (Dao et al., 2016). Increased levels of *Alistipes*, *Parabacteroides*, and *Akkermansia* and decreased levels of *Turicibacter*, *Romboutsia*, and *Oligella* were considered to have beneficial potential in the healthy state of the human intestine. The results showed that BRS had the

ability to regulate gut microbial disorders and improve the host's metabolic function.

It was essential to confirm the characteristic bacteria in different groups, finding the key phylotypes of gut bacteria modulated by BRS. *Bacteroides* facilitated the degradation of various complex carbohydrates, such as glycans and starch, and generated SCFAs, such as acetic acid, propionic acid, and succinic acid (Roberfroid et al., 2010). In addition, the commensal factor (polysaccharide A) originating from *Bacteroides* could promote host immune function (Telesford et al., 2015). *Ruminococcaceae* was abundant in the large bowel and cecum of animals and humans and enriched in response to a high-RS diet (Abell et al., 2008). Numerous studies have demonstrated that *Ruminococcaceae* contribute to the degradation and fermentation of carbohydrates, favoring the production of SCFAs (Hooda et al., 2012; Shang et al., 2016). *Lachnospiraceae*, a butyrate-producing taxonomic core in healthy colons, dominated most individuals and synthesized butyrate through the acetyl-coenzyme A (CoA) pathway, which was supported by a meta-genomic data analysis (Vital et al., 2014).

It is widely believed that SCFAs have a beneficial effect on maintaining the health of colon cells and providing energy for the body (Topping and Clifton, 2001; Brahe et al., 2013). The abundance of *Bacteroides*, *Ruminococcaceae*, and *Lachnospiraceae*, as members of SCFA producers, was elevated in response to BRS. Based on this, a supposition was put forward that BRS was primarily fermented by *Bacteroides*, *Ruminococcaceae*, and *Lachnospiraceae* and subsequently produced SCFAs, which then had beneficial effects on its growth and colonization. It can be seen that obvious shifts in the populations of bacteria in the intestinal tract, on the one hand, inhibit the proliferation of *Turicibacter*, *Romboutsia*, and *Oligella*, and on the other hand, promote the growth of *Alistipes*, *Parabacteroides*, *Akkermansia*, *Bacteroides*, *Ruminococcaceae*, and *Lachnospiraceae*. In summary, BRS intervention may repair the imbalance of intestinal flora and be responsible for protecting the gut steady state.

This research provided further evidence that the gut microbiome participates in a variety of host metabolic processes, especially lipid and hormone levels, in obese rats. Combined with the shifted gut microbiota profile, a speculation was proposed that the healthy glycolipid metabolism cycle and hormone homeostasis were linked to the upregulation of beneficial bacteria and downregulation of harmful bacteria. Spearman correlation analysis confirmed that *Turicibacter*, *Romboutsia*, and *Oligella* (downregulated by BRS) were positively related to GLU, TG, TC, LEP, and INS and negatively related to ghrelin and ADP; *Bacteroides*, *Akkermansia*, and *Parabacteroides* (upregulated by BRS) were negatively related to GLU, TG, and TC. Generally, these key metabolites hold the potential to forecast obesity-related disease, and improvement based on these biomarkers might be beneficial to weight control and reduce the risk of dysmetabolism. Therefore, it is surmised that BRS could ameliorate host metabolism and relieve obesity by altering gut microbiota structure.

CONCLUSION

(1) BRS reversed dyslipidaemia, controlled blood glucose stability, improved insulin sensitivity, and maintained hormone homeostasis in HFD-induced rats; in particular, BRS dose and hormone level showed a dose-effect relationship.
(2) BRS improved the diversity of gut microbiota and was responsible for the transformation in the overall structure of gut microbes, leading to a higher ratio of *Bacteroidetes*/*Firmicutes*; lower population of *Deferribacteres*, *Tenericutes*, *Turicibacter*, *Romboutsia*, and *Oligella*; and higher population of *Cyanobacteria*, *Alistipes*, *Parabacteroides*, *Bacteroides*, *Ruminococcaceae*, *Lachnospiraceae*, and *Akkermansia*.
(3) The change in gut microbiota induced by BRS was linked with obesity-related indicators (serum lipid, blood glucose, and hormone levels). *Turicibacter*, *Romboutsia*, and *Oligella* were positively related to GLU, TG, TC, LEP, and INS, while *Bacteroides*, *Akkermansia*, and *Parabacteroides* were negatively related to GLU, TG, and TC.
(4) The mechanism of BRS against obesity may be attributed to the manipulation of the intestinal microbiota that then improves glucolipid metabolism and guarantees hormone homeostasis, leading to a promotion in the state of host health and alleviation of obesity.

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: NCBI Sequence Read Archive accession numbers SRR13077928-SRR13077945.

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Ethics Review Committee of South China Agricultural University, and the approval number was 2017-B13. The ethical care and use of laboratory animals followed the guidelines for Animal Experimentation in the animal research laboratories.

AUTHOR CONTRIBUTIONS

JF and YW made an equal contribution to this manuscript. JF analyzed and interpreted the data and contributed to writing the manuscript. YW contributed to the investigation and data curation. ST contributed to the investigation. JW contributed to the project administration, funding acquisition,

writing—reviewing, and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by Natural Science Foundation of Guangdong Province (2018A030313026), the Special Funds for Rural Revitalization Strategy from Department of Agriculture and Rural Affairs of Guangdong Province, and the Project of Joint Research on Improved Banana Cultivation from Department of Agriculture and Rural Affairs of Guangdong Province.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.575724/full#supplementary-material

REFERENCES

Abell, G. C., Cooke, C. M., Bennett, C. N., Conlon, M. A., and McOrist, A. L. (2008). Phylotypes related to Ruminococcus bromii are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. *FEMS Microbiol. Ecol.* 66, 505–515. doi: 10.1111/j.1574-6941.2008.00527.x

Addante, F., Sancarlo, D., Copetti, M., Scarcelli, C., Longo, M. G., Niro, V., et al. (2011). Effect of obesity, serum lipoproteins, and apolipoprotein E genotypes on mortality in hospitalized elderly patients. *Rejuvenation. Res.* 14, 111–118. doi: 10.1089/rej.2010.1058

Alvarado-Jasso, G. M., Camacho-Díaz, B. H., Arenas Ocampo, M. L., Jiménez-Ferrer, J. E., Mora-Escobedo, R., and Osorio-Díaz, P. (2020). Prebiotic effects of a mixture of agavins and green banana flour in a mouse model of obesity. *J. Funct. Foods* 64:103685. doi: 10.1016/j.jff.2019.103685

Anonymous (1991). Resistant Starch. Proceedings for the 2nd plenary meeting of EURESTA: European FLAIR Concerted Action No. 11 on physiological implications of the consumption of resistant starch in man. *Eur.J. Clin. Nutr.* 46(Suppl. 2), S1–S148.

Belzer, C., and de Vos, W. M. (2012). Microbes inside–from diversity to function: the case of Akkermansia. *ISME J.* 6, 1449–1458. doi: 10.1038/ismej.2012.6

Benjamino, J., Lincoln, S., Srivastava, R., and Graf, J. (2018). Low-abundant bacteria drive compositional changes in the gut microbiota after dietary alteration. *Microbiome* 6:86. doi: 10.1186/s40168-018-0469-5

Brahe, L. K., Astrup, A., and Larsen, L. H. (2013). Is butyrate the link between diet, intestinal microbiota and obesity-related metabolic diseases? *Obes Rev.* 14, 950–959. doi: 10.1111/obr.12068

Chen, Y., Jin, L., Li, Y., Xia, G., Chen, C., and Zhang, Y. (2018). Bamboo-shaving polysaccharide protects against high-diet induced obesity and modulates the gut microbiota of mice. *J.Funct. Foods* 49, 20–31. doi: 10.1016/j.jff.2018.08.015

Chooi, Y. C., Ding, C., and Magkos, F. (2019). The epidemiology of obesity. *Metabolism* 92, 6–10. doi: 10.1016/j.metabol.2018.09.005

Collaborators, G. B. D. O., Afshin, A., Forouzanfar, M. H., Reitsma, M. B., Sur, P., Estep, K., et al. (2017). Health effects of overweight and obesity in 195 countries over 25 Years. *N. Engl. J. Med.* 377, 13–27. doi: 10.1056/NEJMoa1614362

Damms-Machado, A., Mitra, S., Schollenberger, A. E., Kramer, K. M., Meile, T., Konigsrainer, A., et al. (2015). Effects of surgical and dietary weight loss therapy for obesity on gut microbiota composition and nutrient absorption. *Biomed. Res. Int.* 2015:806248. doi: 10.1155/2015/806248

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., et al. (2016). Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut* 65, 426–436. doi: 10.1136/gutjnl-2014-308778

Delzenne, N. M., and Cani, P. D. (2011). Interaction between obesity and the gut microbiota: relevance in nutrition. *Annu. Rev. Nutr.* 31, 15–31. doi: 10.1146/annurev-nutr-072610-145146

Demir, T., and Celenk, N. (2014). Bloodstream infection with Oligella ureolytica in a newborn infant: a case report and review of the literature. *J. Infect. Dev. Ctries* 8, 793–795. doi: 10.3855/jidc.3260

Dong, J. L., Zhu, Y. Y., Ma, Y. L., Xiang, Q. S., Shen, R. L., and Liu, Y. Q. (2016). Oat products modulate the gut microbiota and produce anti-obesity effects in obese rats. *J. Funct. Foods* 25, 408–420. doi: 10.1016/j.jff.2016.06.025

Dyrbus, K., Osadnik, T., Desperak, P., Desperak, A., Gasior, M., and Banach, M. (2018). Evaluation of dyslipidaemia and the impact of hypolipidemic therapy on prognosis in high and very high risk patients through the Hyperlipidaemia Therapy in tERtiary Cardiological cEnTer (TERCET) Registry. *Pharmacol. Res.* 132, 204–210. doi: 10.1016/j.phrs.2017.12.015

Hariri, N., and Thibault, L. (2010). High-fat diet-induced obesity in animal models. *Nutr. Res. Rev.* 23, 270–299. doi: 10.1017/s0954422410000168

Hooda, S., Boler, B. M., Serao, M. C., Brulc, J. M., Staeger, M. A., Boileau, T. W., et al. (2012). 454 pyrosequencing reveals a shift in fecal microbiota of healthy adult men consuming polydextrose or soluble corn fiber. *J. Nutr.* 142, 1259–1265. doi: 10.3945/jn.112.158766

Laparra, J. M., and Sanz, Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacol. Res.* 61, 219–225. doi: 10.1016/j.phrs.2009.11.001

Lavie, C. J., McAuley, P. A., Church, T. S., Milani, R. V., and Blair, S. N. (2014). Obesity and cardiovascular diseases: implications regarding fitness, fatness, and severity in the obesity paradox. *J. Am. Coll. Cardiol.* 63, 1345–1354. doi: 10.1016/j.jacc.2014.01.022

Lee, E. S., Lee, B. H., Shin, D. U., Lim, M. Y., Chung, W. H., Park, C. S., et al. (2018). Amelioration of obesity in high-fat diet-fed mice by chestnut starch modified by amylosucrase from Deinococcus geothermalis. *Food Hydrocolloids* 75, 22–32. doi: 10.1016/j.foodhyd.2017.09.019

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology - Human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Li, K., Zhang, L., Xue, J., Yang, X., Dong, X., Sha, L., et al. (2019). Dietary inulin alleviates diverse stages of type 2 diabetes mellitus via anti-inflammation and modulating gut microbiota in db/db mice. *Food Funct.* 10, 1915–1927. doi: 10.1039/c8fo02265h

Li, Y. L., Chen, D., Zhang, F., Lin, Y. P., Ma, Y. G., Zhao, S. L., et al. (2020). Preventive effect of pressed degreased walnut meal extracts on T2DM rats by regulating glucolipid metabolism and modulating gut bacteria flora. *J. Funct. Foods* 64:103694. doi: 10.1016/j.jff.2019.103694

Liu, F., Wang, X., Shi, H., Wang, Y., Xue, C., and Tang, Q. J. (2017). Polymannuronic acid ameliorated obesity and inflammation associated with a high-fat and high-sucrose diet by modulating the gut microbiome in a murine model. *Br. J. Nutr.* 117, 1332–1342. doi: 10.1017/S0007114517000964

Lumeng, C. N., and Saltiel, A. R. (2011). Inflammatory links between obesity and metabolic disease. *J. Clin. Invest.* 121, 2111–2117. doi: 10.1172/JCI57132

Luna-Moreno, D., Pérez-Mendoza, M., Carmona-Castro, A., and Miranda-Anaya, M. (2017). Daily profile in ghrelin and hypothalamic ghrelin receptors in obese and lean mice Neotomodon alstoni. *Biol. Rhythm Res.* 49, 515–520. doi: 10.1080/09291016.2017.1385979

Maki, K. C., Pelkman, C. L., Finocchiaro, E. T., Kelley, K. M., Lawless, A. L., Schild, A. L., et al. (2012). Resistant starch from high-amylose maize increases insulin sensitivity in overweight and obese men. *J. Nutr.* 142, 717–723. doi: 10.3945/jn.111.152975

Malik, V. S., Popkin, B. M., Bray, G. A., Despres, J. P., and Hu, F. B. (2010). Sugar-sweetened beverages, obesity, type 2 diabetes mellitus, and cardiovascular disease risk. *Circulation* 121, 1356–1364. doi: 10.1161/CIRCULATIONAHA.109.876185

Meenu, M., and Xu, B. (2019). A critical review on anti-diabetic and anti-obesity effects of dietary resistant starch. *Crit. Rev. Food Sci. Nutr.* 59, 3019–3031. doi: 10.1080/10408398.2018.1481360

Nakano, V., Ignacio, A., Fernandes, M. R., Fugukaiti, M. H., and Avila-Campos, M. J. (2013). Intestinal *Bacteroides* and *Parabacteroides* species producing antagonistic substances. *Curr. Trends Immunol.* 1, 1–4.

Nam, S. Y. (2017). Obesity-related digestive diseases and their pathophysiology. *Gut Liver* 11, 323–334. doi: 10.5009/gnl15557

Niba, L. L. (2002). Resistant starch: a potential functional food ingredient. *Nutr. Food Sci.* 32, 62–67. doi: 10.1108/00346650210416985

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Ohashi, K., Parker, J. L., Ouchi, N., Higuchi, A., Vita, J. A., Gokce, N., et al. (2010). Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J. Biol. Chem.* 285, 6153–6160. doi: 10.1074/jbc.M109.088708

Pandurangan, M., and Kim, D. H. (2016). Therapeutic potential of cyanobacteria against streptozotocin-induced diabetic rats. *3 Biotech* 6:94. doi: 10.1007/s13205-016-0411-0

Parnell, J. A., and Reimer, R. A. (2012). Prebiotic fibres dose-dependently increase satiety hormones and alter Bacteroidetes and Firmicutes in lean and obese JCR:LA-cp rats. *Br. J. Nutr.* 107, 601–613. doi: 10.1017/S0007114511003163

Portune, K. J., Benitez-Paez, A., Del Pulgar, E. M., Cerrudo, V., and Sanz, Y. (2017). Gut microbiota, diet, and obesity-related disorders-The good, the bad, and the future challenges. *Mol. Nutr. Food Res.* 61, 1–38. doi: 10.1002/mnfr.201600252

Prospective Studies Collaboration (2009). Body-mass index and cause-specific mortality in 900000 adults: collaborative analyses of 57 prospective studies. *Lancet* 373, 1083–1096. doi: 10.1016/s0140-6736(09)60318-4

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., et al. (2010). Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* 104(Suppl. 2), S1–S63. doi: 10.1017/S0007114510003363

Robertson, M. D., Bickerton, A. S., Dennis, A. L., Vidal, H., and Frayn, K. N. (2005). Insulin-sensitizing effects of dietary resistant starch and effects on skeletal muscle and adipose tissue metabolism. *Am. J. Clin. Nutr.* 82, 559–567.

Saad, M. J., Santos, A., and Prada, P. O. (2016). Linking gut microbiota and inflammation to obesity and insulin resistance. *Physiology* 31, 283–293. doi: 10.1152/physiol.00041.2015

Sajilata, M. G., Singhal, R. S., and Kulkarni, P. R. (2006). Resistant starch - A review. *Compr. Rev. Food Sci. Food Saf.* 5, 1–17. doi: 10.1111/j.1541-4337.2006.tb00076.x

Sanchez-Tapia, M., Hernandez-Velazquez, I., Pichardo-Ontiveros, E., Granados-Portillo, O., Galvez, A., R Tovar, A., et al. (2020). Consumption of cooked black beans stimulates a cluster of some clostridia class bacteria decreasing inflammatory response and improving insulin sensitivity. *Nutrients* 12:1182. doi: 10.3390/nu12041182

Scarpace, P. J., Matheny, M., Tumer, N., Cheng, K. Y., and Zhang, Y. (2005). Leptin resistance exacerbates diet-induced obesity and is associated with diminished maximal leptin signalling capacity in rats. *Diabetologia* 48, 1075–1083. doi: 10.1007/s00125-005-1763-x

Shang, Q., Shan, X., Cai, C., Hao, J., Li, G., and Yu, G. (2016). Dietary fucoidan modulates the gut microbiota in mice by increasing the abundance of *Lactobacillus* and Ruminococcaceae. *Food Funct.* 7, 3224–3232. doi: 10.1039/c6fo00309e

Shen, R.-L., Zhang, W.-L., Dong, J.-L., Ren, G.-X., and Chen, M. (2014). Sorghum resistant starch reduces adiposity in high-fat diet-induced overweight and obese rats via mechanisms involving adipokines and intestinal flora. *Food Agric. Immunol.* 26, 120–130. doi: 10.1080/09540105.2013.876976

Sonnenburg, J. L., and Backhed, F. (2016). Diet-microbiota interactions as moderators of human metabolism. *Nature* 535, 56–64. doi: 10.1038/nature18846

Tachon, S., Zhou, J., Keenan, M., Martin, R., and Marco, M. L. (2013). The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses. *FEMS Microbiol. Ecol.* 83, 299–309. doi: 10.1111/j.1574-6941.2012.01475.x

Tan, S. M., and Wang, J. (2018). Weight losing function of five products including banana powder. *Food Sci. Technol.* 43, 61–66. doi: 10.13684/j.cnki.spkj.2018.12.012

Telesford, K. M., Yan, W., Ochoa-Reparaz, J., Pant, A., Kircher, C., Christy, M. A., et al. (2015). A commensal symbiotic factor derived from *Bacteroides* fragilis promotes human CD39(+)Foxp3(+) T cells and Treg function. *Gut Microbes* 6, 234–242. doi: 10.1080/19490976.2015.1056973

Topping, D. L., and Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* 81, 1031–1064.

Tremmel, M., Gerdtham, U. G., Nilsson, P. M., and Saha, S. (2017). Economic burden of obesity: a systematic literature review. *Int. J. Environ. Res. Public Health* 14:435. doi: 10.3390/ijerph14040435

Upadhyaya, B., McCormack, L., Fardin-Kia, A. R., Juenemann, R., Nichenametla, S., Clapper, J., et al. (2016). Impact of dietary resistant starch type 4 on human gut microbiota and immunometabolic functions. *Sci. Rep.* 6:28797. doi: 10.1038/srep28797

Vekic, J., Zeljkovic, A., Stefanovic, A., Jelic-Ivanovic, Z., and Spasojevic-Kalimanovska, V. (2019). Obesity and dyslipidemia. *Metabolism* 92, 71–81. doi: 10.1016/j.metabol.2018.11.005

Vital, M., Howe, A. C., and Tiedje, J. M. (2014). Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. *mBio* 5:e00889. doi: 10.1128/mBio.00889-14

Wan, X., Li, T., Liu, D., Chen, Y., Liu, Y., Liu, B., et al. (2018). Effect of marine microalga chlorella pyrenoidosa ethanol extract on lipid metabolism and gut microbiota composition in high-fat diet-fed rats. *Mar. Drugs* 16:498. doi: 10.3390/md16120498

Wang, C. C., Yen, J. H., Cheng, Y. C., Lin, C. Y., Hsieh, C. T., Gau, R. J., et al. (2017). Polygala tenuifolia extract inhibits lipid accumulation in 3T3-L1 adipocytes and high-fat diet-induced obese mouse model and affects hepatic transcriptome and gut microbiota profiles. *Food Nutr. Res.* 61:1379861. doi: 10.1080/16546628.2017.1379861

Wang, J., Huang, H. H., and Chen, P. S. (2016). Structural and physicochemical properties of banana resistant starch from four cultivars. *Int. J. Food Prop.* 20, 1338–1347. doi: 10.1080/10942912.2016.1209517

Wang, J., Tang, X. J., Chen, P. S., and Huang, H. H. (2014). Changes in resistant starch from two banana cultivars during postharvest storage. *Food Chem.* 156, 319–325. doi: 10.1016/j.foodchem.2014.02.012

Wang, M., Chen, Y., Wang, Y., Li, Y., Zhang, X., Zheng, H., et al. (2018). Beneficial changes of gut microbiota and metabolism in weaned rats with *Lactobacillus acidophilus* NCFM and Bifidobacterium lactis Bi-07 supplementation. *J. Funct. Foods* 48, 252–265. doi: 10.1016/j.jff.2018.07.008

Wang, Y. T. (2018). *Evaluation on Anti-Obesity Function of Banana Resistant Starch and Study on their Effects on the Intestinal Microflora*. Guangzhou: South China University of Technology.

Wang, J., Sheng, O., Xiao, J. X., and Fu, J. F. (2019). "Weight loss function of banana resistant starch and its mechanism," in *Conference Proceedings, Chinese Institute of Food Science and Technology*, Wuhan.

Wu, T. Y., Tsai, S. J., Sun, N. N., Dai, F. J., Yu, P. H., Chen, Y. C., et al. (2020). Enhanced thermal stability of green banana starch by heat-moisture treatment and its ability to reduce body fat accumulation and modulate gut microbiota. *Int. J. Biol. Macromol.* 160, 915–924. doi: 10.1016/j.ijbiomac.2020.05.271

Yildiz, B. O., Suchard, M. A., Wong, M. L., McCann, S. M., and Licinio, J. (2004). Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10434–10439. doi: 10.1073/pnas.0403465101

Zeng, Q., Li, D., He, Y., Li, Y., Yang, Z., Zhao, X., et al. (2019). Discrepant gut microbiota markers for the classification of obesity-related metabolic abnormalities. *Sci. Rep.* 9:13424. doi: 10.1038/s41598-019-49462-w

Zhao, L. (2013). The gut microbiota and obesity: from correlation to causality. *Nat. Rev. Microbiol.* 11, 639–647. doi: 10.1038/nrmicro3089

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 Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 19 February 2021
doi: 10.3389/fbioe.2021.635858

Natural Melanin-Based Nanoparticles With Combined Chemo/Photothermal/Photodynamic Effect Induce Immunogenic Cell Death (ICD) on Tumor

Ding Liu[1†], Huilin Huang[2†], Bingxia Zhao[3] and Weihong Guo[2*]*

[1] Department of Organ Transplantation, Zhujiang Hospital, Southern Medical University, Guangzhou, China, [2] Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China, [3] Guangzhou Key Laboratory of Tumor Immunology Research, School of Basic Medical Sciences, Cancer Research Institute, Southern Medical University, Guangzhou, China

OPEN ACCESS

Edited by:
Xian Wu,
Miami University, United States

Reviewed by:
Xiaoqiong Cao,
Harvard University, United States
Jinjun Shao,
Nanjing Tech University, China

***Correspondence:**
Bingxia Zhao
bingxiaz@gmail.com;
bingxiaz@foxmail.com
Weihong Guo
drguowh@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: 30 November 2020
Accepted: 28 January 2021
Published: 19 February 2021

Citation:
Liu D, Huang H, Zhao B and Guo W (2021) Natural Melanin-Based Nanoparticles With Combined Chemo/Photothermal/Photodynamic Effect Induce Immunogenic Cell Death (ICD) on Tumor. Front. Bioeng. Biotechnol. 9:635858. doi: 10.3389/fbioe.2021.635858

Melanin, as a natural product, has been used as an extraordinary ingredient for nanomedicine due to its great biocompatibility and light responsive property. In this study, polydopamine (PDA), an analog of melanin, was extracted from dopamine and encapsulated with doxorubicin (DOX). The as-prepared nanoparticles (NPs) with good stability, great biosafety and high near infrared (NIR) responsive property ameliorated the cell uptake of DOX in OS-RC-2/ADR cells, exhibited synergistic chemo/photothermal (PTT)/photodynamic (PDT) effects, induced the release of damage associated molecular patterns (DAMPs), and finally, led to immunogenic cell death (ICD). In general, it was suggested that PDA-DOX NPs with NIR irradiation could serve as a promising agent for tumor therapy.

Keywords: immunogenic cell death, melanin, natural product, photothermal, photodynamic, ROS, DOX

INTRODUCTION

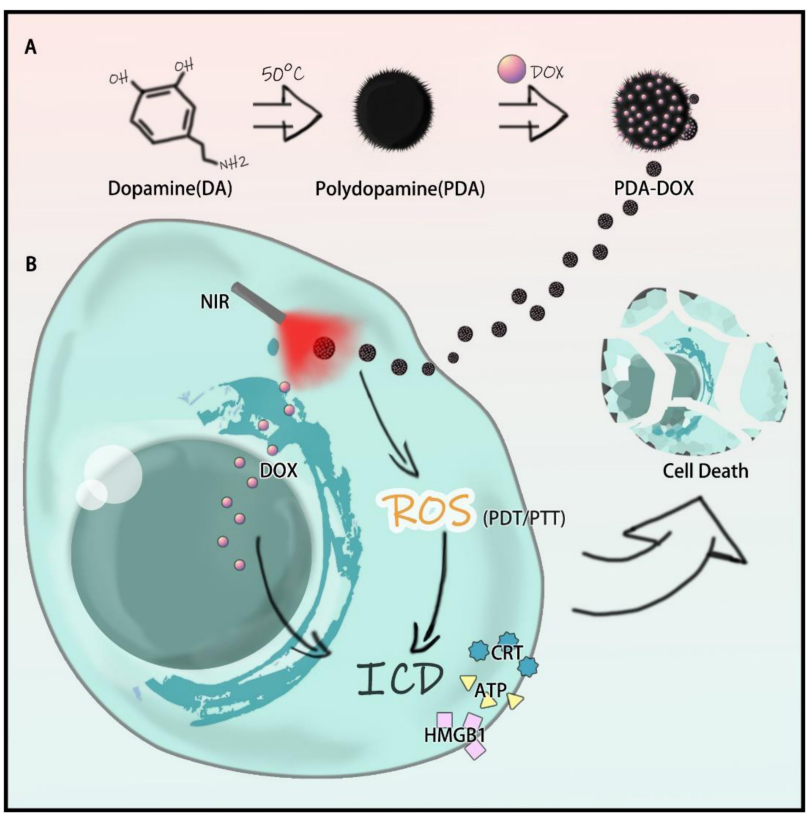
Cancer is a challenging health issues for human beings, with 14 million new cases and over eight million deaths worldwide every year (Bray et al., 2018). As the investigation of tumor characteristics continues, a number of potent cancer fighting strategies have been successfully adopted in clinical practice. Among these combating methods, chemotherapy remains the most preferred and remarkable treatment. For example, doxorubicin (DOX) can not only restrain the proliferation and metastasis of tumor cells, but can also simultaneously lead to immunogenic cell death (ICD) (Casares et al., 2005). ICD, as a promising treatment, aims at enhancing anti-tumor immunity, controlling and damaging cancer cells, and sensitizing therapy by immune system activation. Antineoplastic chemotherapeutic agents can alter the tumor microenvironment that has been infiltrated by various immune cells; whose characteristics usually determine the therapeutic outcome. So, it is prospectively used to facilitate antitumor chemo-immunotherapy (Casares et al., 2005; Obeid et al., 2007; Lu et al., 2018). However, their clinical use is greatly compromised by their adverse systemic effects arising from poor specificity on cancerous tissue. Furthermore, the therapeutic effect could be dramatically reduced owing to drug resistance and an adverse tumor microenvironment. Clinicians often choose to increase the dosage of drugs or change the therapy scheme when topical drug concentration in the cancer region is reduced. Nevertheless,

this method could also lead to systemic toxicity, such as liver damage, bone marrow suppression, neuritis, and other unknown adverse events. As a result, there is an urgent need to explore the deeper mechanisms of drug resistance and to find other ways of improving therapeutic efficiency.

Numerous DOX-based nanomedicines have been synthesized to enhance the absorption of DOX at the cancer region, by taking advantage of the enhanced permeability and retention (EPR) effect. However, the therapeutic performance of these nanomedicines remains unsatisfactory. Whether chemotherapeutic resistance is inherent or acquired (Dean et al., 2005; Iyer et al., 2013; Pérez-Herrero and Fernández-Medarde, 2015; Liang et al., 2016) is one of the greatest challenges of effective therapy. Consequently, nanomedicines incorporated into additional therapeutic modalities have the potential to yield better clinical benefits. In recent years, phototherapy based on near infrared (NIR) coupled with chemotherapy, has been developed as a desirable treatment strategy due to its precise tumor localization, highly efficient ablating capability, and better biocompatibility (Zhu et al., 2018). Phototherapy can accelerate the release of drugs to a deeper part of the tumor, by increased vascular permeability and reverse drug resistance (Li et al., 2015, Li F. et al., 2017; Huang et al., 2019). On the other hand, the integrity and permeability of the cancer cell membrane would be damaged by phototherapy, which can also stimulate the release of tumor-related antigens and can activate the immune response, namely ICD (Sweeney et al., 2018; Li et al., 2019; Shang et al., 2020; Wang et al., 2020). Detecting a proper photosensitive agent for cementing between chemotherapy and phototherapy to achieve ICD and therapy sensitization is therefore of great urgency.

Melanin and its analogs are distributed in many creatures, and widely utilized as ubiquitous biomaterials, owing to their optical absorption property, photoconversion, and affinity. They are wonderful nanocarriers applied to the field of biological imaging, phototherapy, antioxidant therapy, and drug delivery systems, etc. (Solano, 2017; Huang et al., 2018). Moreover, it is worth noting that melanin-like nanoparticles (NPs) can modulate an immune response, as it has been elaborated that NPs from cuttlefish ink mediated the repolarization of M2 TAMs to M1 (Ye et al., 2017; Deng et al., 2019; Rong et al., 2019). As a result, melanin and melanin-like nanoparticles are suitable nanoplatform for drug loading, phototherapy, and immune activation.

Polydopamine (PDA) was synthesized, as one kind of melanin-like NPs, while the characteristics and the biocompatibility of PDA and DOX loading PDA (PDA-DOX) NPs were also detected. Synergistic antitumor efficiency of PDA-DOX NPs with NIR was investigated on cancer cells. More importantly, it was found that PDA-DOX NPs could induce the release of damage associated molecular patterns (DAMPs) leading to ICD (as shown in **Scheme 1**).



SCHEME 1 | The mechanism of PDA-DOX with NIR inducing ICD in OS-RC-2/ADR cells.

MATERIALS AND METHODS

Materials and Characterization

Dopamine hydrochloride (98%), Sodium hydrate (99%), and Poly-(ethylene glycol) (NH_2-mPEG-NH_2 MW 2000, 98%) were obtained from Guangzhou Tanshui Co., Ltd (Guangzhou, China). Deionized (DI) water (18.2 MΩ cm), obtained from a water purification system (Synergy, Millipore, MA), was used in all preparation processes. Transmission electron microscope (TEM) images were taken by a JEOL JEM-2100F TEM. Zeta potential and hydrodynamic diameter measurement was performed by Zetasizer Nano ZS (Malvern). Shimadzu UV-2600 UV–vis spectrophotometer was used to acquire UV–vis absorption spectra. Nicolet/Nexus 670 Fourier transform infrared (FTIR) Analyzer (Thermo Nicolet, United States) was used to obtain FTIR spectra.

Synthesis of Different Sizes of PDA NPs

300 mg of dopamine hydrochloride (1.95 mmol) (Aladdin) were dissolved in 216 mL of deionized water. 1,700 μL of 1 mol/L NaOH solution was added to a dopamine hydrochloride solution at 50°C under vigorous stirring. When the solution's color turned to pale yellow, NaOH was added into the solution, and gradually the color changed to dark brown. After stirring for 6 h, the solution was further centrifuged with a centrifugal-filter (Amicon centrifugal filter device, MWCO = 10 kDa) and washed with deionized water; this was repeated three times.

Surface Modification of PDA NPs With NH2-PEG_{5000}-NH_2 (PEG-Melanin-Like NPs)

1 mol/L NaOH solution was added to 5 mL of melanin-like aqueous solution (5 mg/mL of water) to adjust the pH of the solution to 9. This mixed solution was added dropwise into a 25 mg NH_2-PEG_{2000}-NH_2 aqueous solution with pH = 9. After vigorous stirring for 8 h, PEG-modified PDA NPs was retrieved by centrifugation with a centrifugal-filter (Amicon centrifugal filter device, MWCO = 10 kDa), followed by washing with deionized water several times to remove the unreacted NH_2-PEG_{2000}-NH_2. Finally, the aqueous solvent was removed by freeze-drying, and a PEG-PDA-like powder was obtained.

Photothermal (PTT)/Photodynamic (PDT) Effects of PDA-DOX NPs

PDA-DOX NPs were treated by 808 nm wavelength laser irradiation (0.7 W/cm^2, 5 min), as a thermal probe was used to detect the temperature changes at different time-points, while an equivalent amount of PBS with the same laser irradiation was chosen as the negative control. The images of temperature changes were recorded by an infrared imaging device (ThermaCAMSC3000, Flirsystem Incorporation, United States) at 0.5 min internals for a total of 5 min. To further validate PDT potentials, the yield of ROS produced by PDA-DOX NPs under NIR (0.7 W/cm^2) was quantitatively analyzed by DPBF. The absorption value of DPBF and PDA-DOX NPs mixed solution at 410 nm was detected every 1 min.

Drug Loading Efficiency of PDA-DOX NPs

Doxorubicin and PDA NPs was mixed at ratios of 1:0.125, 1:0.25, 1:0.5, and stirred in the dark at room temperature for 24 h. The formed NPs were centrifuged with a centrifugal-filter (Amicon centrifugal filter device, MWCO = 30 kDa) and washed with deionized water to remove unloaded DOX. Unloaded DOX was collected and analyzed using a UV–vis–NIR spectrophotometer. The loading content [weight of loaded DOX/(weight of loaded DOX + weight of NPs) × 100%] and loading efficiency (weight of loaded DOX/weight of added DOX × 100%) of DOX on the NPs was calculated.

pH-Responsive Drug Release of PDA-DOX NPs

10 mg PDA NPs loaded with DOX were resuspended in 10 mL deionized water. The samples were transferred into a dialysis membrane bag with a MWCO of 3,500, which was immersed in 30 mL buffer at 5.5, 6.5 and 7.4, respectively. At predetermined time points, 3 mL of release medium was taken out and 3 mL fresh buffer was added. The content of released DOX was measured by a PerkinElmer UV750 spectrophotometer (PE Co., United States) at a wavelength of 480 nm.

Cell Culture and Preparation

Human renal proximal tubule epithelial cell (HK-2) cells and OS-RC-2/ADR cells (American Type Culture Collection) were bought, cultured, and maintained by Dulbecco's modified Eagle's medium (DMEM; Gibco, Langley, OK, United States) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics (100 U/mL) at 37°C in 5% CO_2. For the preparation of experiments, cells were seeded into 6-well plates or 96-well plates, respectively, and incubated with appropriate DMEM added with 10% FBS. The prepared cells were exposed to the DMEM with 10% FBS (*the blank control*), DOX (*the DOX group*), PDA (*the PDA group*), and PDA-DOX (*the PDA-DOX group*) at different concentrations. After incubation with these nanomaterials, half of the treated cells were irradiated with an 808 nm laser (*NIR group*) (0.7 W/cm^2, 5 min), and all continued to incubate for further experiments.

Confocal Laser Scanning Microscope Imaging

OS-RC-2/ADR cells were seeded in confocal laser scanning microscope (CLSM) dishes, cultured for 24 h in DMEM supplemented with 10% FBS. Cells were then incubated with PDA-DOX NPs for 1–4 h. The cells were then washed, fixed by 4% paraformaldehyde, and stained with DAPI. Finally, the dishes were imaged by CLSM (Olympus, Japan).

Flow Cytometry Assay

OS-RC-2/ADR cells were seeded in 6-well plates, cultured for 24 h in DMEM supplemented with 10% FBS. Cells were then incubated with PDA-DOX NPs for 1–4 h, harvested, suspended,

and analyzed by flow cytometry. Furthermore, in our study, the fluorescence channel of DOX and PE is similar so that the PE-positive cells were considered to have internalized DOX, and flow cytometry assays were also used to assess the amount of Reactive Oxygen Species (ROS).

Cell Counting Kit-8 Assay

HK-2 cells were cultured for 24 h in DMEM supplemented with 10% FBS before the incubation with PDA-DOX, at the varying concentrations for 24 h. Then a Cell Counting Kit-8 (CCK-8) detection kit was applied to prove the biosafety of PDA-DOX NPs following the protocol. The biosafety was assumed by the cell viability ratio of exposed groups to the blank control (cells exposed to the DMEM with 10% FBS).

To detect the anti-tumor effect of PDA-DOX NPs, OS-RC-2/ADR cells were seeded into 96-plates, cultured for 24 h in DMEM supplemented with 10% FBS. After that, cells were exposed to different treatments, and cell viabilities were analyzed using CCK-8 assay.

Live and Death Assay

The antitumor effect of PDA-DOX with/without NIR was assessed by Live and Death Staining Kit (KeyGen, Nanjing, China). OS-RC-2/ADR cells were plated and cultured overnight. Then, cells were incubated with different concentrations of DOX, PDA, and PDA-DOX, respectively. Following the above treatment, cells were subsequently treated with the absence or presence of NIR irradiation (0.7 W/cm^2, 5 min). Next, as per the manufacturer's instruction of Live and Death Staining Kit, treated cells were labeled as green (live) or red (dead), and finally monitored by a confocal laser scanning microscope (CLSM, Olympus).

5-Ethinyl-2′ DNA Nucleoside Uracil (EdU) Assay

The antitumor effect of PDA-DOX with/without NIR was assessed by 5-ethinyl-2′ DNA nucleoside uracil (EdU) assay (KeyGen, Nanjing, China). OS-RC-2/ADR cells were plated and cultured overnight. Cells were then incubated with the same concentration of DOX, PDA, and PDA-DOX, respectively. Following the above treatment, cells were subsequently treated with or without NIR irradiation (0.7 W/cm^2, 5 min). Next, as per the manufacturer's instruction of EdU assay, treated cells were labeled, fixed, stained, and finally monitored by a confocal laser scanning microscope (CLSM, Olympus). The three random zones were captured to calculate the number of EdU-positive cells.

ROS Assay

OS-RC-2/ADR cells were seeded into a 6-well plate and cultured for 24 h (37°C, 5% CO2). After that, cells were incubated with the same concentrations of DOX, PDA, and PDA-DOX for 48 h respectively, with or without the presence of NIR irradiation (0.7 W/cm^2, 5 min). Finally, cells were incubated with ROS Assay Kit (KeyGen, Nanjing, China).

In vitro Detection of ICD Biomarkers

The treated OS-RC-2/ADR cells were collected and fixed with 4% paraformaldehyde for 10 min. After being blocked for 1 h at room temperature by 5% BSA, the cells were further incubated with rabbit anti-Rabbit chaperone calreticulin (CRT) or large amounts of high-mobility group box 1 (HMGB1) primary antibodies, respectively, at 4°C overnight. The cells were subsequently incubated with PE or FITC-labeled goat anti-rabbit secondary antibodies away from light for an additional 1 h. Finally, the nuclei were stained with DAPI and the CRT and HMGB1 expression levels were observed under CLSM (Olympus, Japan). Additionally, the HMGB1 ELISA kit was used to detect the release of HMGB1 in the supernatant, while the Luminescent ATP Detection Assay was applied to evaluate the release of adenosine triphosphate (ATP) in the supernatant.

Statistical Analysis

Mean ± standard deviation (SD) was used to value the data. All experiments were repeated at least three times, unless indicated otherwise. The unpaired Student's *t*-test or the analysis of variance (ANOVA) followed by Scheffe's *post hoc* test was applied to value the data. A $P < 0.05$ was considered to be a significant difference.

RESULTS AND DISCUSSION

Synthesis and Characterizations of PDA-DOX

Melanin, as a natural product, has been used for cancer phototherapy due to its great biocompatibility and NIR light responsive properties. In this study, a cancer phototherapy nanoplatform was obtained based on PDA, by oxidation–polymerization of dopamine monomers in alkaline environments. The different sizes were synthesized from ∼80 to 370 nm (**Supplementary Figure 1**) by simply adjusting the pH value, as the size of PDA would decrease with the increase of the pH value (Hawley et al., 1967; Ju et al., 2011). The antitumor drug, DOX was loaded into PDA NPs (named as PDA-DOX) for the purpose of synergetic chemotherapy, as DOX can be loaded onto PDA by means of π-π conjugation and coordination (Li W. Q. et al., 2017). The DOX loading capacity (DLC) increased with the amount of feeding DOX, and a DLC as high as 67% was obtained when the feeding DOX vs. PDA (w/w) was 1:0.125 (**Supplementary Figure 2A**). However, the DOX loading efficiency (DLE) gradually decreased as the feeding DOX/PDA mass ratios increased (**Supplementary Figure 2B**). Therefore, considering the economical utilization efficiency, we chose the feeding ratio of 1:0.25 (DOX *vs.* PDA) for the following experiments. To further improve the water solubility, PEG was applied to modify the surface of PDA NPs. The successful synthesis of PDA-DOX NPs was characterized by TEM, zeta potential, UV–vis spectra analysis, and FTIR analysis. PDA-DOX NPs exhibited an average size distribution of 79.21 ± 27.11 nm with a negative charged surface zeta potential of −41.3 ± 4.2 eV, as demonstrated by the TEM, zeta potential and DLS results

(**Figures 1A–C**). The broad absorption bands shown in the ATR-FTIR analysis of PDA-DOX NPs between 3,690 and 3,000 cm^{-1} are characteristics of the O–H or N–H stretching vibration modes. These broad absorption bands were resulted by carboxylic acid, phenolic, and aromatic amino functions present in the indolic and pyrrolic systems (Ozlu et al., 2019). At 1,612 cm^{-1}, NH_2 scissoring could be seen, representing the successful modification of PEG on PDA-DOX NPs (**Figure 1D**).

Apart from that, several studies have reported that melanin and its analogs could respond well to NIR light (650–900 nm), in most conditions transferring light into heat (Baldea et al., 2018; Deng et al., 2019; Rong et al., 2019; Xiong et al., 2019).

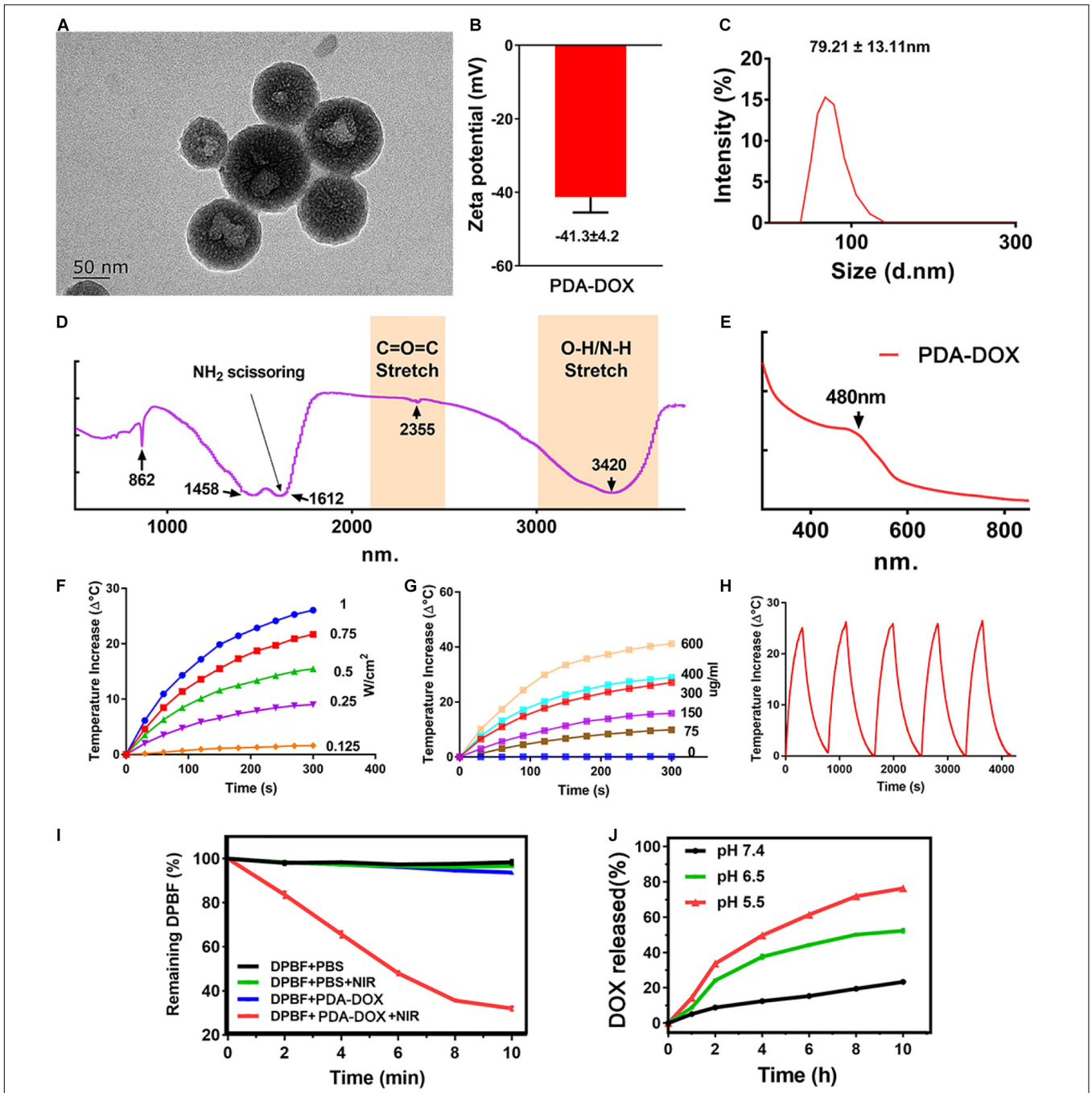


FIGURE 1 | (A) TEM image, **(B)** Zeta potentials, **(C)** DLS, **(D)** FT-IR, and **(E)** UV results of PDA-DOX NPs. **(F)** The temperature increase curve induced under different powers of NIR irradiation. **(G)** The temperature increase curve induced by different concentrations of PDA-DOX NPs and PBS. **(H)** PTT stability of PDA-DOX NPs under NIR irradiation (0.7 W/cm^2). **(I)** The production of $^1O_2$ by PDA-DOX NPs with or without NIR irradiation (0.7 W/cm^2). **(J)** The pH-responsive release curve of PDA-DOX.

The NIR-responsive properties of PDA-DOX NPs were then measured by irradiating with a NIR laser (808 nm). It could be seen that the temperature increases of the PDA-DOX NPs solution showed both concentration and laser power density dependent properties, indicating their good photothermal effect (**Figures 1F,G**). Moreover, the temperature increase remained almost the same even after five laser on/off cycles, demonstrating their good photothermal stability (**Figure 1H**). Furthermore, in our previous works (Hou et al., 2018; Chen et al., 2019; Guo et al., 2020) it has been found that some photothermal agents also possess the ability to generate ROS under NIR irradiation and could be used as photosensitizers for photodynamic therapy. Herein, ROS generation ability of our as-prepared NPs was also investigated using a ROS probe DPBF. As seen from **Figure 1I**, the absorption of DPBF decreased most in the PDA-DOX-treated group than in the other groups, suggesting that more $^1O_2$ could be produced when the NPs were irradiated by NIR laser. The above results indicate that the PDA-DOX NPs could be applied as a promising PTT and photodynamic therapy (PDT) agent.

The encapsulation of DOX was characterized by UV–vis spectra. It could be seen that a distinctive absorbance peak appeared at approximately 480 nm of the PDA-DOX NP compared with PDA NPs only, indicating the successful encapsulation of DOX (**Figure 1E**). The loading efficiency of DOX was calculated as 30%. The drug release property of PDA-DOX NPs was also evaluated under different pH values (pH 7.4, pH 6.5, and pH 5.5). It could be seen that only a small amount of DOX would be released at a neutral pH value, which could decrease the side effects to the normal tissues. The drug was released more rapidly at lower pH values and exhibited an obvious pH-dependent drug release performance (**Figure 1J**). Considering the acid tumor microenvironments, the pH-sensitive dox release makes the PDA-DOX NPs a promising DOX delivery system.

Cellular Uptake and Biosafety of PDA-DOX *in vitro*

As a natural product, it has already been proven that melanin and its analogs can easily enter cancer cells, making them a widely used drug delivery platform (Ju et al., 2013; Jiang et al., 2017). It is therefore expected that cellular uptake of DOX in cancer cells could increase greatly using PDA nanocarriers. To confirm the synergistic uptake of PDA-DOX, OS-RC-2/ADR cells were exposed to DOX and PDA-DOX separately from 0 to 4 h and analyzed with CLSM and flow cytometry. CLSM showed that red fluorescence intensity from DOX increased as time passed, both in DOX and PDA-DOX groups. It could be observed that the fluorescence intensities in PDA-DOX group were stronger than those in the free DOX group at different time points (**Figure 2A**), indicating that PDA indeed helps to gain a more desired cell uptake ratio of DOX. Correspondingly, the accumulation of DOX in OS-RC-2/ADR cells treated with free DOX and PDA-DOX was also analyzed by flow cytometry. The result revealed that cellular uptake of DOX at 4 h was threefold that of free DOX (**Figure 2B**), indicating that the as-prepared PDA-DOX NPs could significantly enhance the cellular uptake of DOX.

In addition, a prerequisite of nanoparticles for therapy is that they are less toxic to normal cells (Huang et al., 2018). Hence, the CCK-8 assay was used to evaluate the biosafety of PDA-DOX to normal HK-2 cells, while over 85% cell viability was observed, after HK-2 cells were exposed to PDA-DOX for 24 h with the increasing PDA concentrations up to 200 μg/mL (**Supplementary Figure 3**). Overall, these results revealed that PDA-DOX NPs have great biocompatibility and could be used to delivery DOX into renal cancer cells for treatment.

PDA-DOX NPs With Combined Chemo/PTT/PDT Effect on Killing Cancer Cells

As mentioned above, PDA gives rise to high cellular uptake of DOX and exhibit a pH-sensitive drug release property, which could theoretically increase the chemo-therapeutic effect of DOX. Therefore, better antitumor effects could be achieved when PDA NPs were used as the nanocarrier with the same DOX concentration (**Figure 3A**). As proven above, the intrinsic NIR-responsive properties of the PDA NPs make them a potential PTT/PDT agent. For example, it has been reported that PDA, functioned with arginine-glycine-aspartic-cysteine acid (RGDC) peptide and loaded with DOX, could be released and induces chemo-photothermal effect availably (Li Y. et al., 2017). It could therefore be hypothesized that PDA-DOX with NIR irradiation can cause not only a chemical lesion but also thermal injury and oxidative stress. To further validate the synergistic chemo/PTT/PDT effect of our as-prepared NPs, OS-RC-2/ADR cells were incubated with the PDA, DOX, and PDA-DOX at different concentrations, respectively, with or without the presence of NIR (0.7 W/cm^2, 5min). After being cultured for 24 h, treated OS-RC-2/ADR cells were analyzed by CCK-8, EdU and Live/Dead Cell Double Staining Kit. As illustrated in **Figure 3B**, cell viabilities decreased with the increasing concentrations of DOX and PDA-DOX, while PDA-DOX with NIR-irradiation could achieve the best therapeutic effect compared with PDA-and-NIR-irradiation-treated cells, PDA-DOX-treated cells, and the control group. Moreover, EdU assay revealed that PDA-DOX-and-NIR-irradiation-treated, PDA-and-NIR-irradiation-treated, and PDA-DOX-treated cells were shown to be less EdU-positive in comparison with the control and DOX-treated groups (**Figure 3C**). A similar tendency was also observed in Live/Dead Cell Double Staining Kit results (**Figure 3D**). These results suggested that PDA-DOX could facilitate the chemo-effectiveness of DOX, and in combination with NIR would stimulate better efficiency than mono chemotherapy or phototherapy, implying a PDA-DOX induced synergetic chemo/PTT/PDT effect against OS-RC-2/ADR cells.

Immunogenic Cell Death Induced by Synergetic Chemo/PTT/PDT Effect of PDA-DOX *in vitro*

Immunogenic Cell Death is a kind of regulated cell death and is able to distinguish from other cell death so that it can activate the immune system against tumor cells (Ahmed and Tait, 2020; Fumet et al., 2020). It has been reported that DOX has the

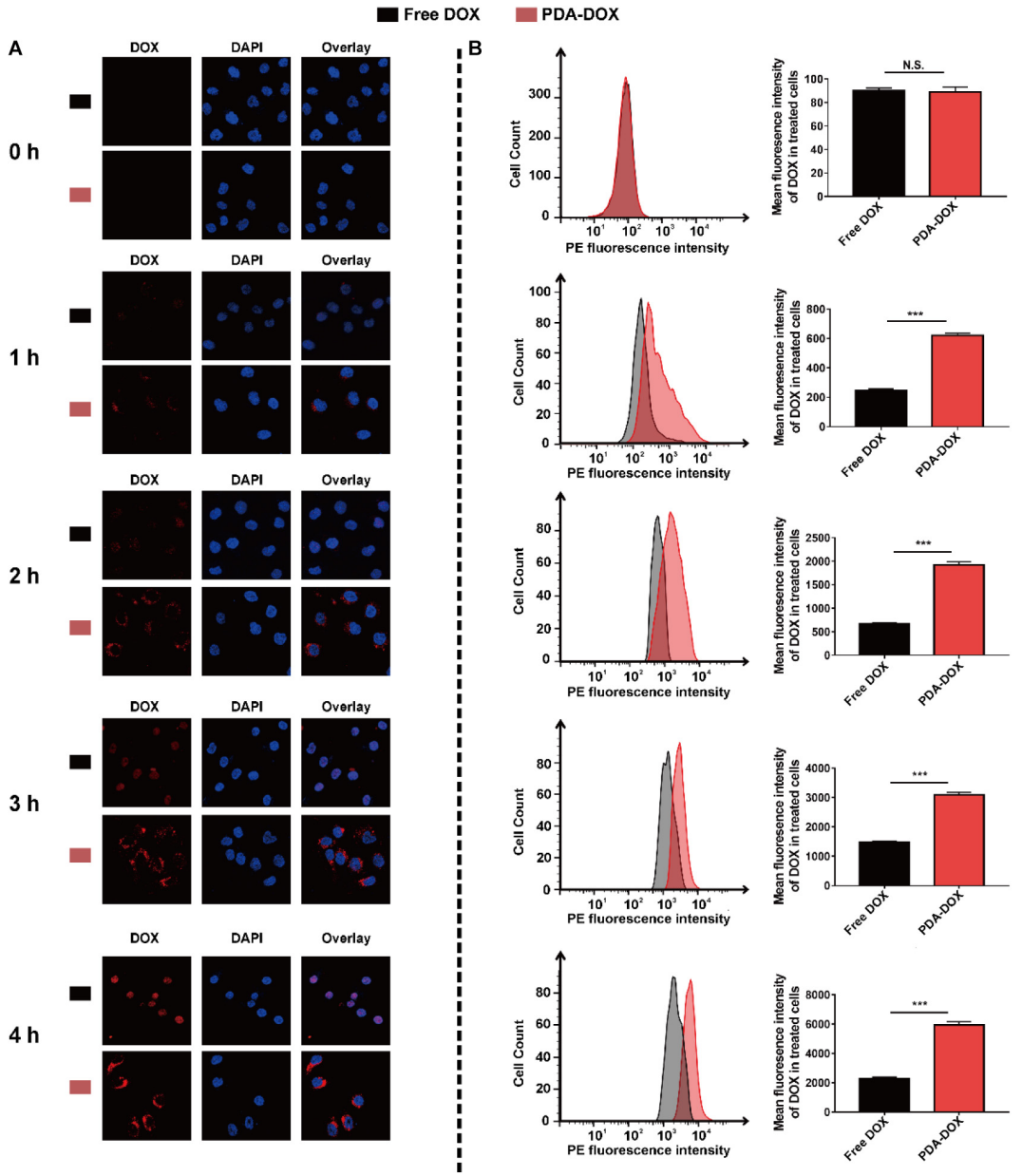


FIGURE 2 | Cell uptake of PDA-DOX analyzed by **(A)** CLSM and **(B)** flow cytometry. *** indicates $P < 0.001$. N.S. means no significance.

immunogenicity to recruit immune cells according to the ICD, but the efficiency of mono DOX is not strong enough (Casares et al., 2005). Recent studies found that phototherapy based on melanin and its derivatives might cause ICD, consecutively reinforcing immune response and more tumor cell death (Yan et al., 2019; Li et al., 2020). During this process, ICD plays an important initial role and DAMPs are provoked into release to promote the immunostimulatory effect. Three typical DAMPs have been recognized in virtually all ICD inducers, including CRT, ATP, and HMGB1 (Kepp et al., 2014). Moreover, it has been confirmed that the release of DAMPs, especially CRT's translocating into membrane, results from endoplasmic reticulum (ER) stress related to the generation of extra ROS (Garg et al., 2011; Galluzzi et al., 2012; Gomes-da-Silva et al., 2018; Deng, 2020). Considering this, we assumed that increasing ROS might be key in leading to ICD effects of PDA-DOX under NIR irradiation. Interestingly, it conformed with our hypothesis that stronger ROS means that fluorescence intensity could be observed in the PDA-DOX-and-NIR-irradiation-treated group rather than in other groups (**Figures 4A,B**). The extra generation of ROS might be rooted in the combined chemo/PTT/PDT effect of PDA-DOX due to its high light absorption.

Consequently, we attempted to clarify whether the combination of PDA-DOX and NIR can stimulate ROS

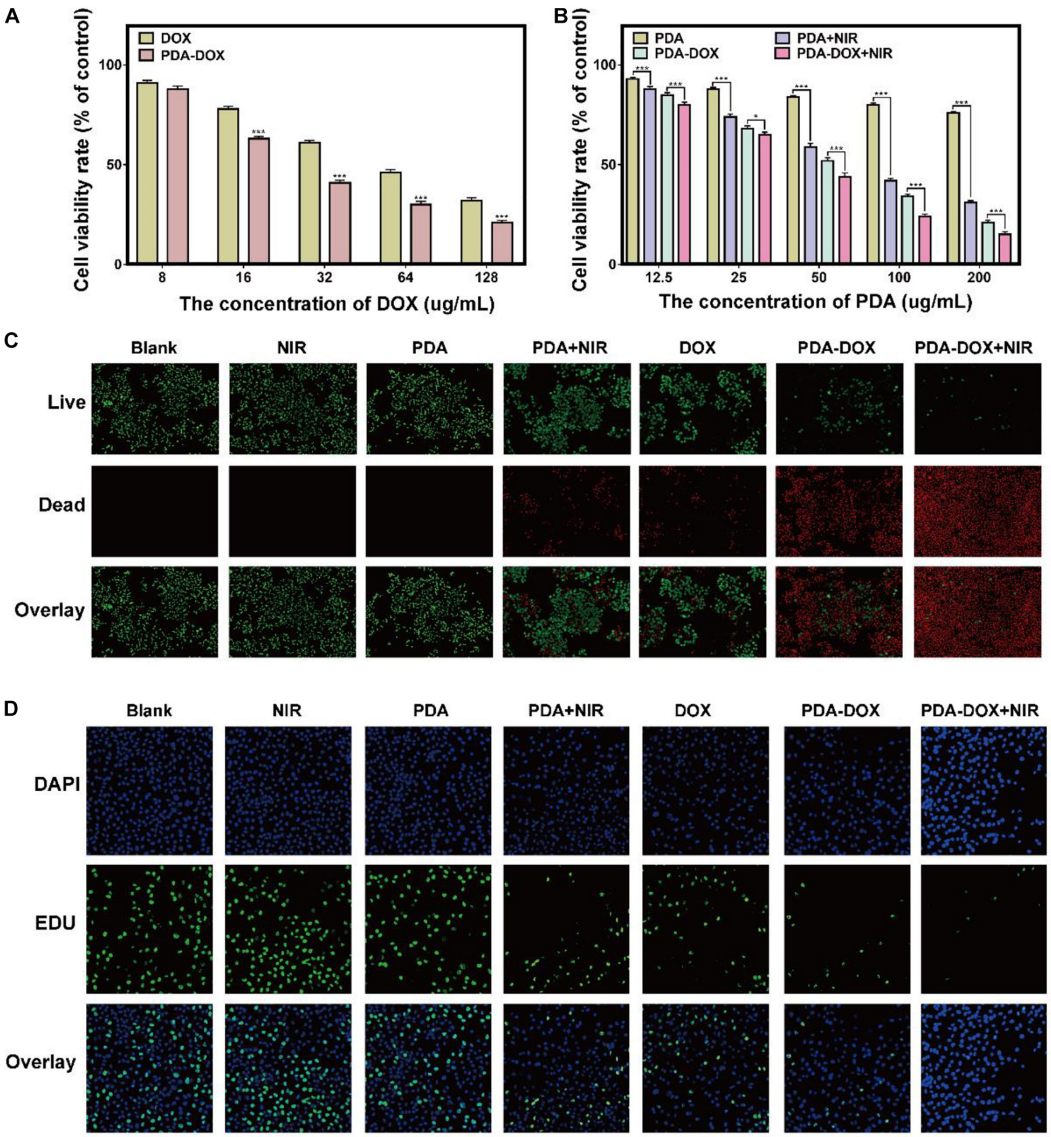


FIGURE 3 | The antitumor effect of PDA-DOX *in vitro*. **(A)** Viabilities of OS-RC-2/ADR cells after incubation with DOX and PDA-DOX at different concentrations of DOX. **(B)** Viabilities of OS-RC-2/ADR cells after incubation with PDA and PDA-DOX, with or without NIR at different concentrations of PDA, while the DMEM with 10% FBS was used as the negative control (blank) group. **(C)** Live/Dead Cell Double Staining images of OS-RC-2/ADR cells after the incubation with PDA, DOX, and PDA-DOX, at presence or absence of NIR. **(D)** EdU images of OS-RC-2/ADR cells after the incubation with PDA, DOX, and PDA-DOX, upon NIR or not. Data are shown as the mean ± SD, $n = 3$. *** indicates $P < 0.001$. *indicates $P < 0.05$.

production to boost the release of the above DAMPs and ICD of DOX or PDA with NIR alone. CLSM imaging then revealed that DOX, PDA-DOX, PDA plus DOX, and PDA-DOX plus NIR, could induce translocation of CRT into the cell membrane, while the PDA-DOX plus NIR group led to more obvious CRT translocation (**Figure 4E**). In addition, we observed the downregulated expression level of HMGB1 inside the cell (**Figure 4C**), and more HMGB1 release and ATP secretion in the supernatant after treatment with PDA-DOX plus NIR (**Figure 4D,F**), indicating that ICD could be elicited by PDA-DOX plus NIR. Taken together, these results suggest that PDA-DOX with NIR irradiation do not only facilitate the release of DOX to attract tumor cell death, but also results in more ICD effects in contrast to DOX or PDA with NIR. It is the same as previous research, where DOX combined with NIR irradiation resulting in ICD. It is presumed that high-efficient ICD is attributed not only to a high concentration of local DOX but also phototherapy (Phung et al., 2019; Wen et al., 2019; Xia et al., 2019; Zhang et al., 2019). In summary, this shows that the synergetic chemo/PTT/PDT effect of PDA-DOX and NIR irradiation could lead to the ICD *via* the generation of ROS, providing prospects for the combination with tumor immunotherapy.

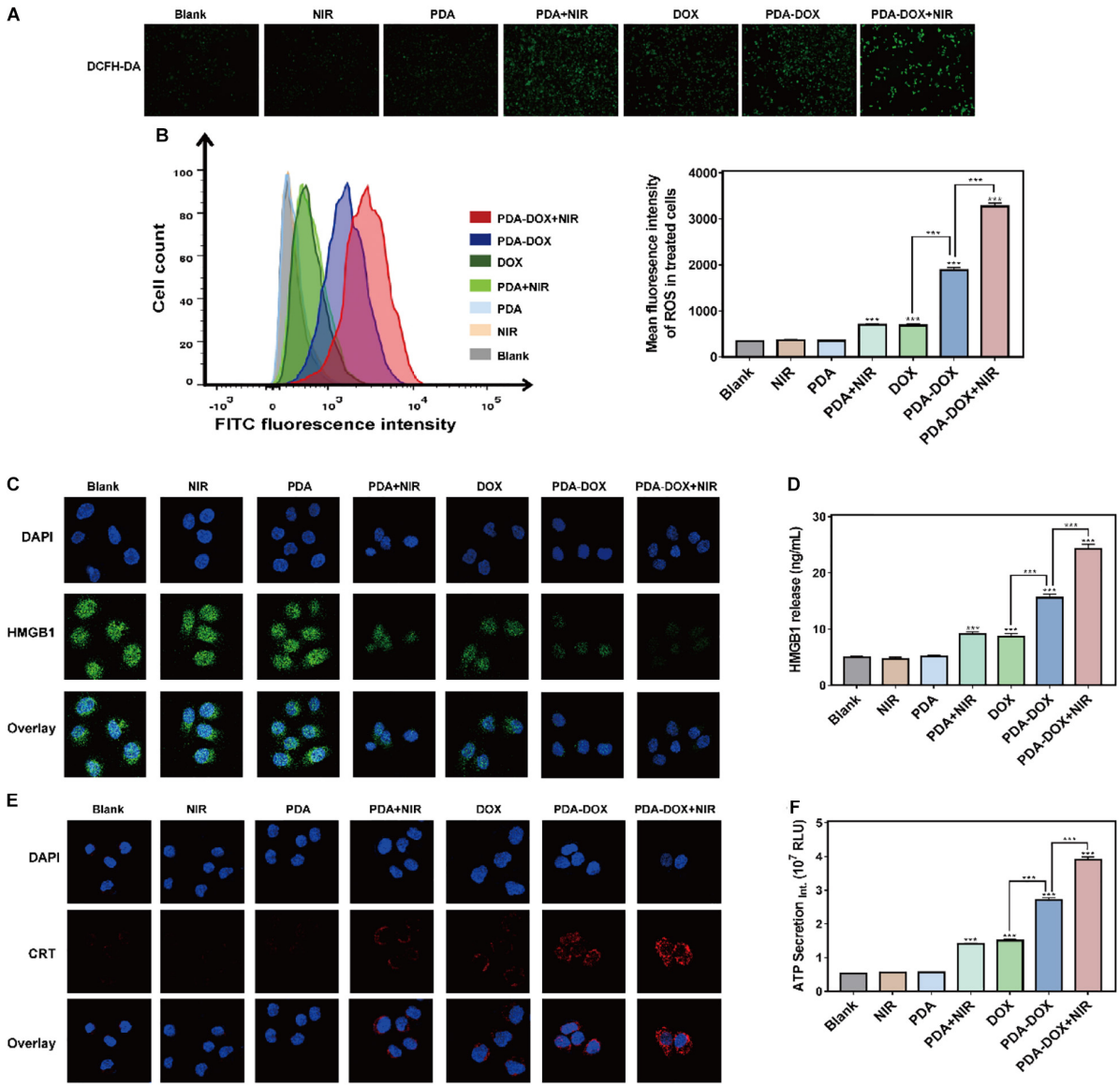


FIGURE 4 | ICD induced by synergetic chemo/PTT/PDT effect of PDA-DOX *in vitro*. **(A,B)** The generation of ROS in OS-RC-2/ADR cells after the incubation with PDA, DOX, and PDA-DOX with or without NIR. **(C)** HMGB1 images of OS-RC-2/ADR cells after the incubation with PDA, DOX, and PDA-DOX with or without NIR. **(D)** The HMGB1 release of OS-RC-2/ADR cells after incubation with PDA, DOX, and PDA-DOX with or without NIR. **(E)** CRT images of OS-RC-2/ADR cells after the incubation with PDA, DOX, and PDA-DOX with or without NIR. **(F)** The ATP secretion of OS-RC-2/ADR cells after incubation with PDA, DOX, and PDA-DOX with or without NIR. Data are shown as the mean ± SD, $n = 3$. *** indicates $P < 0.001$.

CONCLUSION

In this work, we developed PDA-DOX NPs, the natural product used for chemotherapy drug loading, to improve the effect of DOX. It not only inherited great stability and sound biocompatibility of the natural product, but also exhibited strong photothermal conversion ability and a simultaneous ROS generation effect, which is appropriate for phototherapy. Considering this, PDA-DOX plus proper NIR irradiation *in vitro* showed good synergistic chemo/PTT/PDT effects against OS-RC-2/ADR cells, compared to DOX or PDA with NIR alone. Furthermore, boosting ROS is a key process to mediating the release of DAMPs, involving CRT, HMGB1, and ATP. In conclusion, PDA-DOX might be a dramatic drug for renal carcinoma chemotherapy and phototherapy, and is promising for tumor immunotherapy.

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/s.

AUTHOR CONTRIBUTIONS

WG and BZ designed the study, revised the figures and tables, and contributed to drafting the manuscript. DL and HH collated the data, carried out data analyses, and produced the initial draft of the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (82001948), the China Postdoctoral Science Foundation (2019M662977), the Medical Scientific Research Foundation of Guangdong Province of China (A2020297), and Special Funds for Cultivation of Guangdong College Students' Scientific and Technological Innovation (pdjh2020a0108).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.635858/full#supplementary-material

REFERENCES

Ahmed, A., and Tait, S. W. G. (2020). Targeting immunogenic cell death in cancer. *Mol. oncol.* 14, 2994–3006. doi: 10.1002/1878-0261.12851

Baldea, I., Giurgiu, L., Teacoe, I. D., Olteanu, D. E., Olteanu, F. C., Clichici, S., et al. (2018). Photodynamic therapy in melanoma – where do we stand? *Curr. Med. Chem.* 25, 5540–5563. doi: 10.2174/0929867325666171226115626

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Casares, N., Pequignot, M. O., Tesniere, A., Ghiringhelli, F., Roux, S., Chaput, N., et al. (2005). Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J. Exp. Med.* 202, 1691–1701. doi: 10.1084/jem.20050915

Chen, W., Wang, X., Zhao, B., Zhang, R., Xie, Z., He, Y., et al. (2019). CuS-MnS_2 nano-flowers for magnetic resonance imaging guided photothermal/photodynamic therapy of ovarian cancer through necroptosis. *Nanoscale* 11, 12983–12989. doi: 10.1039/c9nr03114f

Dean, M., Fojo, T., and Bates, S. (2005). Tumour stem cells and drug resistance. *Nat. Rev. Cancer* 5, 275–284. doi: 10.1038/nrc1590

Deng, H., Zhou, Z., Yang, W., Lin, L.-S., Wang, S., Niu, G., et al. (2020). Endoplasmic reticulum targeting to amplify immunogenic cell death for cancer immunotherapy. *Nano Lett.* 20, 1928–1933. doi: 10.1021/acs.nanolett.9b05210

Deng, R.-H., Zou, M.-Z., Zheng, D., Peng, S.-Y., Liu W, Bai, X.-F., et al. (2019). Nanoparticles from cuttlefish ink inhibit tumor growth by synergizing immunotherapy and photothermal therapy. *ACS Nano* 13, 8618–8629. doi: 10.1021/acsnano.9b02993

Fumet, J.-D., Limagne, E., Thibaudin, M., and Ghiringhelli, F. (2020). Immunogenic cell death and elimination of immunosuppressive cells: a double-edged sword of chemotherapy. *Cancers* 12:2637. doi: 10.3390/cancers12092637

Galluzzi, L., Kepp, O., and Kroemer, G. (2012). Enlightening the impact of immunogenic cell death in photodynamic cancer therapy. *EMBO J.* 31, 1055–1057. doi: 10.1038/emboj.2012.2

Garg, A. D., Krysko, D. V., Vandenabeele, P., and Agostinis, P. (2011). DAMPs and PDT-mediated photo-oxidative stress: exploring the unknown. *Photochem. Photobiol. Sci.* 10, 670–680. doi: 10.1039/c0pp00294a

Gomes-da-Silva, L. C., Zhao, L., Bezu, L., Zhou, H., Sauvat, A., Liu, P., et al. (2018). Photodynamic therapy with redaporfin targets the endoplasmic reticulum and Golgi apparatus. *EMBO J.* 37:e98354. doi: 10.15252/embj.201798354

Guo, W., Chen, Z., Chen, J., Feng, X., Yang, Y., Huang, H., et al. (2020). Biodegradable hollow mesoporous organosilica nanotheranostics (HMON) for multi-mode imaging and mild photo-therapeutic-induced mitochondrial damage on gastric cancer. *J. Nanobiotechnol.* 18:99. doi: 10.1186/s12951-020-00653-y

Hawley, M. D., Tatawawadi, S. V., Piekarski, S., and Adams, R. N. (1967). Electrochemical studies of the oxidation pathways of catecholamines. *J. Am. Chem. Soc.* 89, 447–450. doi: 10.1021/ja00978a051

Hou, M., Yan, C., Chen, Z., Zhao, Q., Yuan, M., Xu, Y., et al. (2018). Multifunctional NIR-responsive poly(vinylpyrrolidone)-Cu-Sb-S nanotheranostic agent for photoacoustic imaging and photothermal/photodynamic therapy. *Acta Biomater.* 74, 334–343. doi: 10.1016/j.actbio.2018.05.011

Huang, L., Liu, M., Huang, H., Wen, Y., Zhang, X., and Wei, Y. (2018). Recent advances and progress on melanin-like materials and their biomedical applications. *Biomacromolecules* 19, 1858–1868. doi: 10.1021/acs.biomac.8b00437

Huang, W., Zhao, H., Wan, J., Zhou, Y., Xu, Q., Zhao, Y., et al. (2019). pH- and photothermal-driven multistage delivery nanoplatform for overcoming cancer drug resistance. *Theranostics* 9, 3825–3839. doi: 10.7150/thno.33958

Iyer, A. K., Singh, A., Ganta, S., and Amiji, M. M. (2013). Role of integrated cancer nanomedicine in overcoming drug resistance. *Adv. Drug Deliv. Rev.* 65, 1784–1802. doi: 10.1016/j.addr.2013.07.012

Jiang, Q., Luo, Z., Men, Y., Yang, P., Peng, H., Guo, R., et al. (2017). Red blood cell membrane-camouflaged melanin nanoparticles for enhanced photothermal therapy. *Biomaterials* 143, 29–45. doi: 10.1016/j.biomaterials.2017.07.027

Ju, K. Y., Lee, J. W., Im, G. H., Lee, S., Pyo, J., Park, S. B., et al. (2013). Bio-inspired, melanin-like nanoparticles as a highly efficient contrast agent for T1-weighted magnetic resonance imaging. *Biomacromolecules* 14, 3491–3497. doi: 10.1021/bm4008138

Ju, K.-Y., Lee, Y., Lee, S., Park, S. B., and Lee, J.-K. (2011). Bioinspired polymerization of dopamine to generate melanin-like nanoparticles having an excellent free-radical-scavenging property. *Biomacromolecules* 12, 625–632. doi: 10.1021/bm101281b

Kepp, O., Senovilla, L., Vitale, I., Vacchelli, E., Adjemian, S., Agostinis, P., et al. (2014). Consensus guidelines for the detection of immunogenic cell death. *Oncoimmunol.* 3:e955691. doi: 10.4161/21624011.2014.955691

Li, F., Yang, H., Bie, N., Xu, Q., Yong, T., Wang, Q., et al. (2017). Zwitterionic Temperature/Redox-Sensitive nanogels for near-infrared light-triggered synergistic thermo-chemotherapy. *ACS Appl. Mater. Interfaces* 9, 23564–23573. doi: 10.1021/acsami.7b08047

Li, W., Yang, J., Luo, L., Jiang, M., Qin, B., Yin, H., et al. (2019). Targeting photodynamic and photothermal therapy to the endoplasmic reticulum enhances immunogenic cancer cell death. *Nat. Commun.* 10:3349.

Li, W. Q., Wang, Z., Hao, S., He, H., Wan, Y., Zhu, C., et al. (2017). Mitochondria-Targeting polydopamine nanoparticles to deliver doxorubicin for overcoming drug resistance. *ACS Appl. Mater. Interfaces* 9, 16793–16802. doi: 10.1021/acsami.7b01540

Li, Y., Deng, Y., Tian, X., Ke, H., Guo, M., Zhu, A., et al. (2015). Multipronged design of light-triggered nanoparticles to overcome cisplatin resistance for efficient ablation of resistant tumor. *ACS Nano* 9, 9626–9637. doi: 10.1021/acsnano.5b05097

Li, Y., Jiang, C., Zhang, D., Wang, Y., Ren, X., Ai, K., et al. (2017). Targeted polydopamine nanoparticles enable photoacoustic imaging guided chemo-photothermal synergistic therapy of tumor. *Acta Biomater.* 47, 124–134. doi: 10.1016/j.actbio.2016.10.010

Li, Y., Liu, X., Pan, W., Li, N., and Tang, B. (2020). Photothermal therapy-induced immunogenic cell death based on natural melanin nanoparticles against breast cancer. *Chem. Comm.* 56, 1389–1392. doi: 10.1039/c9cc08447a

Liang, C., Xu, L., Song, G., and Liu, Z. (2016). Emerging nanomedicine approaches fighting tumor metastasis: animal models, metastasis-targeted drug delivery, phototherapy, and immunotherapy. *Chem. Soc. Rev.* 45, 6250–6269. doi: 10.1039/c6cs00458j

Lu, J., Liu, X., Liao, Y. P., Wang, X., Ahmed, A., Jiang, W., et al. (2018). Breast cancer chemo-immunotherapy through Liposomal delivery of an immunogenic cell death stimulus plus interference in the IDO-1 pathway. ACS Nano 12, 11041–11061. doi: 10.1021/acsnano.8b05189

Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J. L., et al. (2007). Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat. Med.* 13, 54–61. doi: 10.1038/nm1523

Ozlu, B., Kabay, G., Bocek, I., Yilmaz, M., Piskin, A. K., Shim, B. S., et al. (2019). Controlled release of doxorubicin from polyethylene glycol functionalized melanin nanoparticles for breast cancer therapy: part I. Production and drug release performance of the melanin nanoparticles. *Int. J. Pharmaceut.* 570:118613. doi: 10.1016/j.ijpharm.2019.118613

Pérez-Herrero, E., and Fernández-Medarde, A. (2015). Advanced targeted therapies in cancer: drug nanocarriers, the future of chemotherapy. *Eur. J. Pharmaceut. Biopharmaceut.* 93, 52–79. doi: 10.1016/j.ejpb.2015.03.018

Phung, C. D., Nguyen, H. T., Choi, J. Y., Pham, T. T., Acharya, S., Timilshina, M., et al. (2019). Reprogramming the T cell response to cancer by simultaneous, nanoparticle-mediated PD-L1 inhibition and immunogenic cell death. *J. Controll. Release* 315, 126–138. doi: 10.1016/j.jconrel.2019.10.047

Rong, L., Zhang, Y., Li, W. S., Su, Z., Fadhil, J. I., and Zhang, C. (2019). Iron chelated melanin-like nanoparticles for tumor-associated macrophage repolarization and cancer therapy. *Sci. Immunol.* 225:119515. doi: 10.1016/j.biomaterials.2019.119515

Shang, T., Yu, X., Han, S., and Yang, B. (2020). Nanomedicine-based tumor photothermal therapy synergized immunotherapy. *Biomater. Sci.* 8, 5241–5259. doi: 10.1039/d0bm01158d

Solano, F. (2017). Melanin and melanin-related polymers as materials with biomedical and biotechnological applications-cuttlefish ink and mussel foot proteins as inspired biomolecules. *Int. J. Mol. Sci.* 18:1561. doi: 10.3390/ijms18071561

Sweeney, E. E., Cano-Mejia, J., and Fernandes, R. (2018). Photothermal therapy generates a thermal window of immunogenic cell death in neuroblastoma. *Small* 14:e1800678. doi: 10.1002/smll.201800678

Wang, L., Ding, K., Zheng, C., Xiao, H., Liu, X., Sun, L., et al. (2020). Detachable nanoparticle-enhanced chemoimmunotherapy based on precise killing of tumor seeds and normalizing the growing soil strategy. *Nano Lett.* 20, 6272–6280. doi: 10.1021/acs.nanolett.0c01415

Wen, Y., Chen, X., Zhu, X., Gong, Y., Yuan, G., Qin, X., et al. (2019). Photothermal-chemotherapy integrated nanoparticles with tumor microenvironment response enhanced the induction of immunogenic cell death for colorectal cancer efficient treatment. *ACS Appl. Mater. Interfaces* 11, 43393–43408. doi: 10.1021/acsami.9b17137

Xia, C., Yin, S., Xu, S., Ran, G., Deng, M., Mei, L., et al. (2019). Low molecular weight heparin-coated and dendrimer-based core-shell nanoplatform with enhanced immune activation and multiple anti-metastatic effects for melanoma treatment. *Theranostics* 9, 337–354. doi: 10.7150/thno.29026

Xiong, Y., Sun, F., Zhang, Y., Yang, Z., Liu, P., Zou, Y., et al. (2019). Polydopamine-mediated bio-inspired synthesis of copper sulfide nanoparticles for T(1)-weighted magnetic resonance imaging guided photothermal cancer therapy. *Colloids Surfaces B, Biointerfaces* 173, 607–615. doi: 10.1016/j.colsurfb.2018.10.023

Yan, S., Zeng, X., Tang, Y., Liu, B.-F., Wang, Y., and Liu, X. (2019). Activating antitumor immunity and antimetastatic effect through polydopamine-encapsulated core-shell upconversion nanoparticles. *Adv. Mater. (Deerfield Beach, Fla).* 31:e1905825. doi: 10.1002/adma.201905825

Ye, Y., Wang, C., Zhang, X., Hu, Q., Zhang, Y., Liu, Q., et al. (2017). A melanin-mediated cancer immunotherapy patch. *Sci. Immunol.* 2:eaan5692. doi: 10.1126/sciimmunol.aan5692

Zhang, J., Zhang, D., Li, Q., Jiang, Y., Song, A., Li, Z., et al. (2019). Task-specific design of immune-augmented nanoplatform to enable high-efficiency tumor immunotherapy. *ACS Appl. Mater. Interfaces* 11, 42904–42916. doi: 10.1021/acsami.9b13556

Zhu, H., Cheng, P., Chen, P., and Pu, K. (2018), Recent progress in the development of near-infrared organic photothermal and photodynamic nanotherapeutics. *Biomater. Sci.* 6, 746–765. doi: 10.1039/C7BM01210A

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 Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 13 April 2021
doi: 10.3389/fbioe.2021.662432

The Synthesis and Antitumor Activity of 1,8-Naphthalimide Derivatives Linked 1,2,3-Triazole

Zhong-jie Xu[1], Ying-jie Zhou[2], Jia-hao Wang[2], Long-fei Mao[2], Wei Li[2]* and Gui-qing Xu[2]**

[1] College of Life Science and Technology, Xinxiang Medical University, Xinxiang, China, [2] Henan Engineering Research Center of Chiral Hydroxyl Pharmaceutical, School of Chemistry and Chemical Engineering, Henan Normal University, Xinxiang, China

In this study, acenaphthylene was used as the raw material, and a series of novel 1,8-naphthalimide-1,2,3-triazole derivatives was obtained through oxidation, acylation, alkylation, and click reactions, and subsequently, their anti-tumor activities were tested. After screening, we found that Compound **5e** showed good activity against H1975 lung cancer cells, with the half maximal inhibitory concentration (IC_{50}) reaching 16.56 μM.

Keywords: acenaphthylene, synthesis, 1,8-naphthalimide-1,2,3-triazole derivatives, anti-proliferative activities, H1975

OPEN ACCESS

Edited by:
Wenzhen Liao, Southern Medical University, China

Reviewed by:
Ana Margarida Goncalves Carvalho Dias, New University of Lisbon, Portugal
Angel León-Buitimea, Universidad Autonoma de Nuevo Leon, Mexico

****Correspondence:***
Wei Li
liweigq@163.com
Longfei Mao
longfeimao1988@163.com
Gui-qing Xu
guiqingxu@163.com

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *01 February 2021*
Accepted: *22 March 2021*
Published: *13 April 2021*

Citation:
Xu Z-j, Zhou Y-j, Wang J-h, Mao L-f, Li W and Xu G-q (2021) The Synthesis and Antitumor Activity of 1,8-Naphthalimide Derivatives Linked 1,2,3-Triazole. Front. Bioeng. Biotechnol. 9:662432. doi: 10.3389/fbioe.2021.662432

INTRODUCTION

Cancer is the leading cause of death worldwide. At present, surgery is still the first choice of treatment for many cancers, but easy recurrence and metastasis after surgery greatly affect the efficacy and prognosis (Gaitanis et al., 2018). For patients with local advanced tumors or distant metastases that are not suitable for surgery, traditional chemotherapy and radiotherapy show poor efficacy, and finding active and effective cancer treatments has become a main focus for researchers, especially the development of safe anti-cancer drugs. Based on DNA intercalators, small molecule drugs are being developed as anti-tumor drugs. Due to differences between the DNA of cancerous cells and normal cells, DNA intercalators play a significant role in treating tumors (Jiang, 2013). Naphthalimide derivatives have a special rigid planar structure, which gives them a strong ability to intercalate into DNA (Finney, 2006; Takahashi et al., 2007), so they have attracted extensive attention in the field of anti-tumor drug research and development. Some mononaphthalimides, such as amonafide (Wang et al., 2017; Johnson et al., 2019) and mitonafide (Sinha et al., 1985; Rosell et al., 1992), and bisnaphthalimides have entered the clinical research stage. Amonafide and mitonafide can not only intercalate into DNA and inhibit the synthesis of DNA and RNA, but also inhibit the activity of topoisomerase II, thereby inhibiting tumor cell division (Nazari and Ghandi, 2015). In addition, bisnaphthalimide compounds can bis-intercalate into DNA, have specificity to G-C bases, and exhibit stronger DNA binding and greater cytotoxicity than mononaphthalimides (Laquindanum et al., 1996; Wang and Yu, 2003; Langhals, 2004; Li and Wonneberger, 2012; Verma et al., 2013). For example, DMP-840 is a novel DNA-interactive chemical entity with excellent activityagainst human tumor colony-formingunits and its IC50 to Leukemia and other cell lines is 2.3–53 nM (Kirshenbaum et al., 1994; Nitiss et al., 1998). LU-79553, a bisnaphthalimides, exhibited dramatic antitumor activity in a variety of xenograft models and activity over a broad spectrum of tumor types made it an excellent candidate (Bousquet et al., 1995). For example, IC50 of LU-79553

is 18.0 μM to ovarian cell (Braña et al., 2004; Gonzalez-Bulnes and Gallego, 2009; Ferri et al., 2011; González-Bulnes and Gallego, 2012). Yet, the activity of naphthalimide derivatives on lung cancer is rarely reported (**Figure 1**).

Herein, a series of novel 1,8-naphthalimide-linked 1,2,3-triazole compounds were synthesized and tested for their antilung-cancer activity. From the raw material acenaphthylene (Compound **1**), 1,8-naphthalic anhydride (Compound **2**) was obtained by an oxidation reaction, and then the intermediate (Compound **3**) was obtained by an acylation reaction. Compound **3** underwent alkylation with 4-bromo-1-butyne to yield Compound **4**, which underwent a 1,3-dipolar cycloaddition reaction with different substituted azides to obtain target Compound **5** with a 1,2,3-triazole structure. The structures of the synthesized compounds **Scheme 1**) were confirmed by nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR).

RESULTS AND DISCUSSION

Effect of the Amount of Potassium Dichromate on the Yield of 1,8-Naphthalic Anhydride (Compound 2)

The key to this reaction is to use a strong oxidant to oxidize the carbon–carbon double bond to yield acid anhydride. We chose sodium dichromate as the oxidant and screened the amount of added sodium dichromate (**Table 1**). It can be seen from the table that under the condition that the reaction conditions remained unchanged, the reaction yield gradually increased with increasing amount of sodium dichromate. When the molar ratio of compound **1** to sodium dichromate is 1:2, the yield reached 80%. With the increase of the oxidant, the yield did not increase obviously. Therefore, the ratio of 1:2 was chosen as the optimal ratio to prepare compound **2**.

Influence of Reaction Temperature on the Yield of 1,8-Naphthalimide (Compound 3)

This reaction is an acylation reaction, and its temperature has a great influence on the yield of the reaction product. If the reaction is not complete, the raw materials will precipitate with the product during the cooling and crystallization process, which will affect the purity of the product. Therefore, we maintained the other reaction conditions unchanged and studied the reaction temperature (**Table 2**). With the increase of temperature, the yield increased. But the yield remained unchanged with the increase of temperature until 70°C. So we chose 70°C as the optimal reaction temperature.

Inhibitory Activity of Compounds on Tumor Cells

The inhibitory effect of Compounds **5a**–**5o** on H1975 lung cancer cells is shown in **Table 3**. Most of the compounds in this series had poor inhibitory effects on this cell type. Only compounds **5e**, **5g**, **5h**, and **5j** had a certain activity. Among them, **5e** showed the strongest effect with an IC_{50} of 16.56 μM. We then studied the structure-activity relationship. By comparing compounds **5a** and **5j**, **5c** and **5f**, **5k** and **5o**, and **5e** and **5m**, we clearly found that compounds with substituents at the *ortho* position of the benzene ring were more active than those with substituents at the *para* position. For compounds **5j** and **5k**, and **5h** and **5m**, we found that the activity of 1,2,3-triazole linked with the phenyl structure was generally stronger than those with 1,2,3-triazole linked with the benzyl structure. The results of **5e** and **5h** showed that when an electron withdrawing group with steric hindrance is at the *para* position of the benzene ring, the activity was obviously improved. The reason is worthy of further exploring.

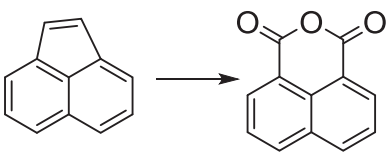
CONCLUSION

In summary, we designed and synthesized a series of novel 1,8-naphthalimide-1,2,3-triazole derivatives through simple and efficient methods, conducted anti-tumor activity studies on them, and found that compound **5e** had a good inhibitory effect on H1975 lung cancer cells. The results showed that compounds **5e** deserves further research about its anti-tumor activity and mechanism of action in order to find better compounds. Meanwhile, because the introduced substituents of our compounds are lipophilic, the solubility in water of our compounds is less than DMP. Our next step is to improve the water solubility of **5e**.

MATERIALS AND METHODS

1,8-naphthalimide-1,2,3-triazole derivatives were in-house synthesized. All reagents were obtained from commercial sources and used without further purification. All reactions were monitored by thin-layer chromatography (TLC). ^{1}H and ^{13}C spectra were recorded on a Bruker Avance 400 or 600 MHz spectrometer, respectively. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at room temperature (20 ± 2 °C). ^{1}H and ^{13}C chemical shifts are quoted in parts per million downfield from TMS. **H1975** lung cancer cell line, DMEM medium and fetal bovine serum were purchased from ATCC (Virginia, United States). MTT powder and Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Morris Plains, NJ, United States).

General Procedure for the Compound 2



In a reaction flask, acenaphthylene (Compound **1**, 15 g, 0.1 mol) was added into 500 mL of glacial acetic acid, followed by adding 55 g (0.2 mol) of sodium dichromate. The mixture was

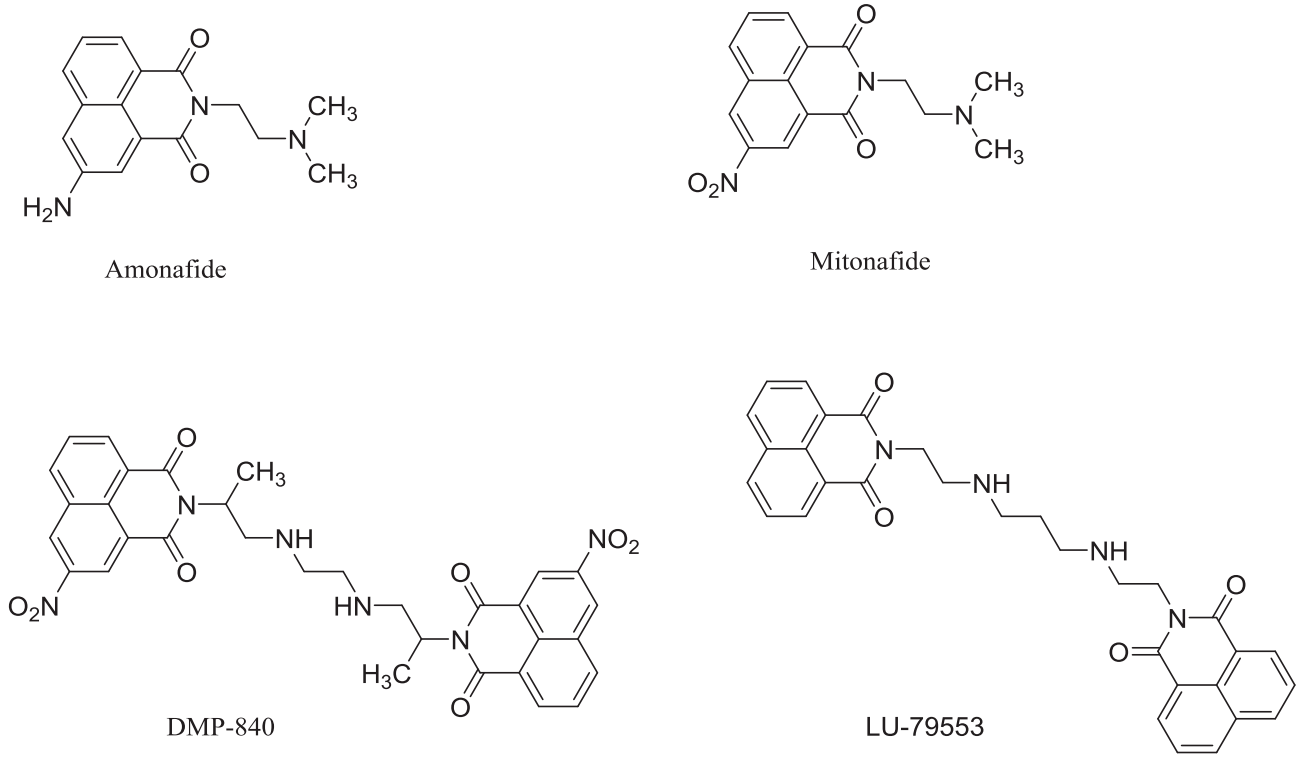
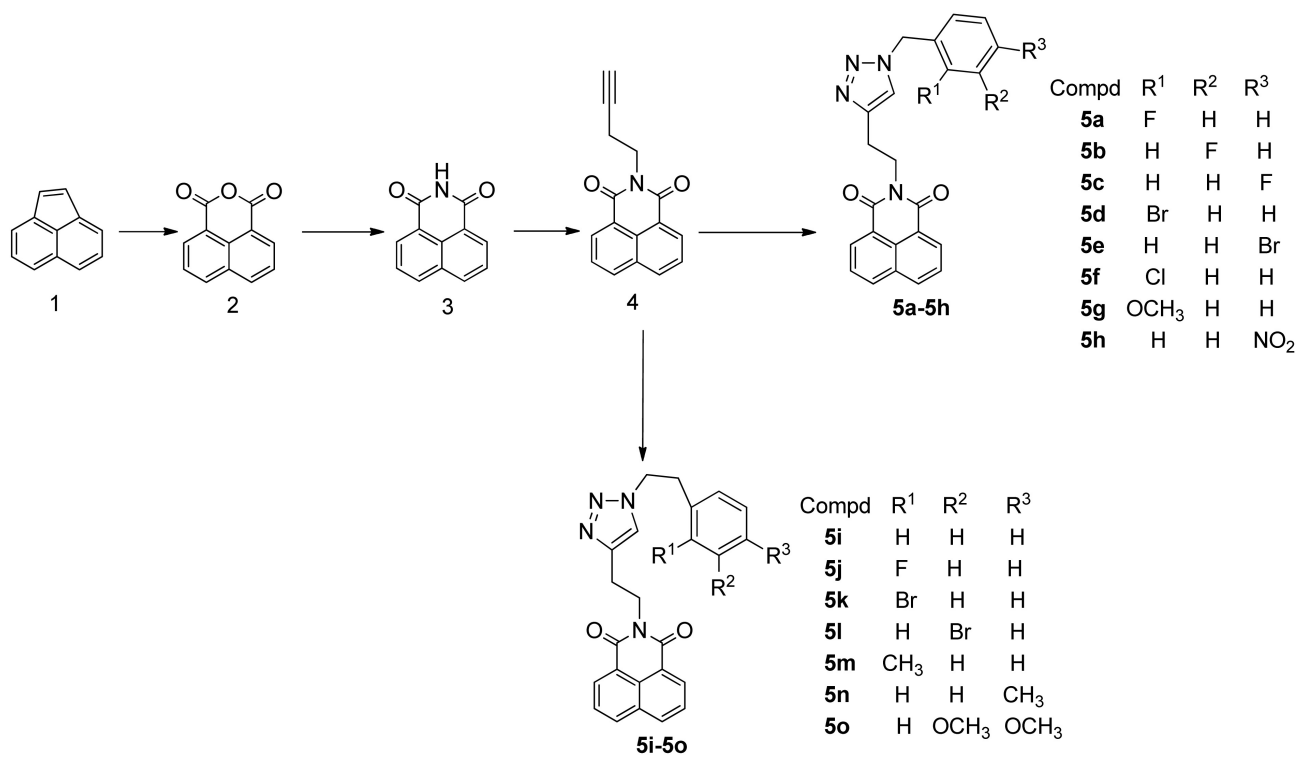


FIGURE 1 | The reported naphthalimide derivatives.



SCHEME 1 | Reaction routes for the production of the target compounds.

TABLE 1 | Ratio optimization of sodiumdichromate to yield compound **2**.

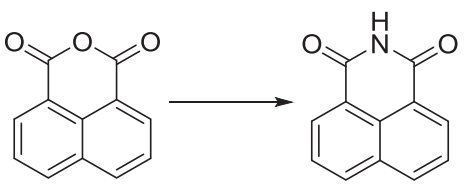
Number	n (Compound 1):n (sodium dichromate)	Temperature (°C)	Time (h)	Yield (%)
1	1:1.0	80	6	59
2	1:1.2	80	6	63
3	1:1.4	80	6	69
4	1:1.6	80	6	74
5	1:1.8	80	6	78
6	1:2.0	80	6	80
7	1:2.2	80	6	77
8	1:2.4	80	6	81
9	1:2.6	80	6	79

TABLE 2 | Optimization of the temperature of reaction to produce compound **3**.

Number	Temperature (°C)	Time (h)	Yield (%)
1	30	1.5	32
2	40	1.5	39
3	50	1.5	56
4	60	1.5	78
5	70	1.5	88
6	80	1.5	86
7	90	1.5	84
8	100	1.5	87

stirred evenly at room temperature, and then the temperature was slowly increased to 80 °C, after which the reaction was carried out for 6 h. Thin layer chromatography (TLC) was used to monitor the progress of the reaction. When the reaction was complete, the reaction mixture was poured into 2,000 mL of ice water while it was hot. Solid precipitates appeared, and the reaction mixture was filtered, and the filter cake was dried to obtain 1,8-naphthalic anhydride (Compound **2**, 16 g), and the yield was 80%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.55 (dd, $J_1 = 8.0$ Hz, $J_1 = 4.0$ Hz, 4H), 7.93 (t, $J_1 = 4.0$ Hz, $J_1 = 8.0$ Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): 161.19, 135.86, 132.93, 130.22, 128.03, 119.54 ppm.

General Procedure for the Compound 3



2 3

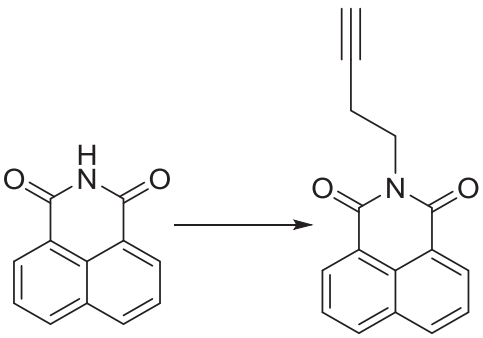
In a reaction flask, 1,8-naphthalic anhydride (Compound **2**, 50 g, 0.25 mol) was added to 1,000 mL of saturated ammonia water, and the mixture was stirred at room temperature for 10 min to obtain a yellow mixed liquid, which was slowly heated to 70°C. At this temperature, the reaction was carried out for 90 min. TLC was used to monitor the progress of the reaction. When the reaction was complete, heating was stopped. The flask was slowly cooled down to room temperature, solid precipitates appeared, and the reaction mixture was filtered. The filter cake was washed with 500 mL of water to neutralize it, and then dried at 60 °C to obtain 1,8-naphthalimide (Compound **3**,44 g), with a yield of 88%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.42 (d, $J = 4.0$ Hz, 4H), 7.83 (t, $J_1 = 8.0$ Hz, $J_2 = 4.0$ Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): 164.56, 134.78, 132.03, 130.42, 127.53, 122.93ppm.

TABLE 3 | Data of the inhibitory effect of compounds **5a**–**5o** on lung cancer cell line.

Compound	IC_{50}, μ M	Compound	IC_{50}, μ M
5a	169.3 ± 1.38	**5i**	123 ± 1.24
5b	151.4 ± 1.35	**5j**	89.15 ± 1.27
5c	>200	**5k**	190.4 ± 1.47
5d	>200	**5l**	102.1 ± 1.26
5e	**16.56 ± 1.14**	**5m**	121.6 ± 1.21
5f	172.9 ± 1.47	**5n**	137.2 ± 1.30
5g	94.16 ± 1.47	**5o**	>200
5h	80.63 ± 1.27		

The bold value indicates that the activity of the compound 5e is the best.

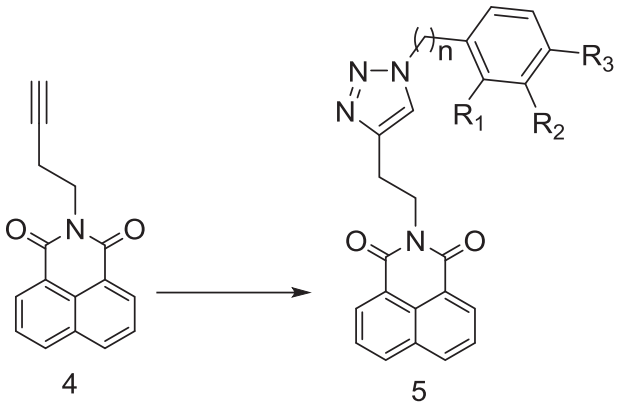
General Procedure for the Preparation of Compound 4



3 4

Compound **3** (10.00 g, 50.71 mmol) was added to a reaction flask and dissolved in N,N-dimethylformamide (DMF) (150 mL), and 4-bromo-1-butyne (7.42 g, 55.78 mmol) and potassium carbonate (21.02 g, 152.13 mmol) were added successively. Under nitrogen protection, the reaction was carried out at 100 °C overnight, and TLC was used to monitor the progress of the reaction. When the reaction was completed, the reaction mixture was filtered while it was hot, the filtrate was cooled at room temperature, and solid precipitates appeared. The filter cake was washed with a small amount of DMF and dried to obtain a white solid Compound **4** (9.65 g, 38.71 mmol), with a yield of 76.3%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.50 (d, $J = 7.2$ Hz, 2H), 8.46 (d, $J = 7.8$ Hz, 2H), 7.86 (t, $J = 7.8$ Hz, 2H), 4.45 (t, $J = 7.2$ Hz, 2H), 3.18 (t, $J = 7.2$ Hz, 2H), 2.89 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.85, 143.96, 134.65, 131.68, 128.63, 127.84, 124.26, 80.95, 56.53, 44.18, 24.92.

General Procedure for the Preparation of Compound 5a–5o



4 5

Aryl-azido (1.2 mmol) and compound **4** (1.0 mmol) were added to 15 mL mixed solvent (water/*tert*-butanol = 2:1). The reaction was catalyzed with CuI (0.1 mmol) at 80 °C. After completion of the reaction (monitored by TLC), the mixture was extracted with dichloromethane (15 mL × 3). The combined organic phase was washed successively with water and brine, dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by through column chromatography (CH_2Cl_2/MeOH) to obtain the desired compound **5** as a crystalline powder.

2-(2-(1-(2-fluorophenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione(5a)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.50 (d, J = 7.2 Hz, 2H), 8.49 (d, J = 1.8 Hz, 1H), 8.47 (d, J = 7.8 Hz, 2H), 7.88 (t, J = 7.8 Hz, 2H), 7.80 (td, J_1 = 7.8 Hz, J_2 = 1.2Hz, 1H), 7.61–7.58 (m, 1H), 7.57–7.53 (m, 1H), 7.43 (t, J = 8.4 Hz, 1H), 4.40 (t, J = 7.2 Hz, 2H), 3.14 (t, J = 7.8 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.88, 144.94, 134.83, 131.80, 131.55, 131.49, 131.21, 127.90, 127.71, 126.28, 126.00, 124.60, 124.57, 122.56, 117.68, 117.55, 24.00.

2-(2-(1-(3-fluorophenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5b)

ield:69.5%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (s, 1H), 8.48 (dd, J = 7.2, 1.2 Hz, 2H), 8.45 (dd, J_1 = 8.0 Hz, J_2 = 1.2Hz, 2H), 7.88–7.84 (m, 2H), 7.81–7.74 (m, 2H), 7.66–7.59 (m, 1H), 7.35–7.28 (m, 1H), 4.38 (t, J = 8.0 Hz, 2H), 3.11 (t, J = 7.6 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.86, 161.68, 145.63, 134.81, 132.33, 132.24, 131.78, 131.19, 127.88, 127.67, 122.52, 121.49, 116.14, 115.68, 115.47, 107.82, 107.55, 39.70, 24.10.

2-(2-(1-(4-fluorophenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5c)

ield: 35.9%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (s, 1H), 8.48 (dd, J_1 = 7.2 Hz, J_2 = 1.2 Hz, 2H), 8.45 (dd, J_1 = 8.4 Hz, J_2 = 1.2 Hz, 2H), 7.93–7.88 (m, 2H), 7.86 (m, 2H), 7.43 (t, J = 8.8 Hz, 2H), 4.38 (t, J = 7.6 Hz, 2H), 3.14–3.07 (t, J = 7.6 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.89, 145.47, 134.81, 133.79, 131.77, 131.22, 127.71, 122.65, 122.61, 122.57, 122.53, 121.55, 117.27, 117.07, 24.13.

2-(2-(1-(2-bromophenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5d)

ield:36.1%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.50 (dd, J_1 = 7.2 Hz, J_2 = 1.2Hz, 2H), 8.48–8.45 (m, 2H), 8.37 (s, 1H), 7.90–7.86 (m, 3H), 7.62–7.59 (m, 1H), 7.58 (dd, J_1 = 7.8 Hz, J_2 = 1.8Hz, 1H), 7.54–7.52 (m, 1H), 4.41 (t, J = 7.2 Hz, 2H), 3.14 (t, J = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.84, 144.16, 136.84, 134.82, 134.02, 132.27, 131.80, 131.23, 129.38, 129.09, 127.91, 127.70, 125.30, 122.59, 119.28, 39.65, 23.95.

2-(2-(1-(4-bromophenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5e)

ield29.2%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.76 (s, 1H), 8.49 (d, J = 7.2 Hz, 2H), 8.46 (d, J = 8.4 Hz, 2H), 7.89–7.83 (m, 4H), 7.78 (d, J = 8.8 Hz, 2H), 4.38 (d, J = 7.6 Hz, 2H), 3.11 (d, J = 7.6 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.91, 145.65, 136.38, 134.83, 133.22, 131.79, 131.20, 127.89, 127.69, 122.53, 122.20, 122.17, 121.48, 121.39, 24.11.

2-(2-(1-(2-chlorophenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5f)

Yield17.4%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.54 (d, J = 7.2 Hz, 2H), 8.51 (d, J = 8.0 Hz, 2H), 8.44 (s, 1H), 7.93 (t, J = 7.8 Hz, 2H), 7.78 (d, J = 8.4 Hz, 1H), 7.68–7.64 (m, 2H), 7.62 (t, J = 7.8 Hz, 1H), 4.46 (t, J = 7.2 Hz, 2H), 3.20 (t, J = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.84, 144.27, 135.16, 134.81, 131.92, 131.79, 131.21, 130.95, 128.96, 128.87, 128.76, 127.90, 127.68, 125.30, 122.57, 39.65, 23.97.

2-(2-(1-(2-methoxyphenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5g)

ield7.5%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.51 (d, J = 7.2 Hz, 2H), 8.47 (d, J = 8.4 Hz, 2H), 8.31 (s, 1H), 7.89 (t, J = 7.8 Hz, 2H), 7.58 (dd, J_1 = 7.8 Hz, J_2 = 1.8 Hz, 1H), 7.53–7.48 (m, 1H), 7.30 (d, J = 8.3 Hz, 1H), 7.13 (t, J = 7.6 Hz, 1H), 4.40 (t, J = 7.8 Hz, 2H), 3.78 (s, 3H), 3.12 (t, J = 7.2 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.87, 152.00, 144.01, 134.82, 131.80, 131.22, 130.95, 127.90, 127.71, 126.34, 126.07, 124.91, 122.58, 121.32, 113.47, 56.48, 24.05.

2-(2-(1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5h)

ield30.5%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.97 (s, 1H), 8.52 (d, J = 7.2 Hz, 2H), 8.49 (d, J = 8.4 Hz, 2H), 8.45 (d, J = 8.4 Hz, 2H), 8.20 (d, J = 8.4 Hz, 2H), 7.90 (t, J = 7.8 Hz, 2H), 4.42 (t, J = 7.8 Hz, 2H), 3.15 (t, J = 7.8 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.94, 147.01, 146.19, 141.42,

134.88, 131.84, 131.26, 127.96, 127.74, 126.10, 122.60, 121.84, 120.77, 40.53, 24.09.

2-(2-(1-benzyl-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5i)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.46 (t, J = 7.2 Hz, 4H), 8.02 (s, 1H), 7.87 (t, J = 7.8 Hz, 2H), 7.34 (t, J = 7.2 Hz, 2H), 7.31 (t, J = 6.6 Hz, 1H), 7.21 (d, J = 6.6 Hz, 2H), 5.54 (s, 2H), 4.31 (t, J = 7.2 Hz, 2H), 3.03 (t, J = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.81, 136.77, 134.80, 131.78, 131.20, 129.13, 128.39, 128.00, 127.85, 127.69, 123.38, 122.50, 53.04, 24.06.

2-(2-(1-(2-fluorobenzyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5j)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.49–8.41 (m, 4H), 8.00 (s, 1H), 7.86 (t, J = 7.2 Hz, 2H), 7.43–7.38 (m, 1H), 7.23 (t, J = 9.0 Hz, 1H), 7.20–7.17 (m, 2H), 5.60 (s, 2H), 4.31 (t, J = 7.2 Hz, 2H), 3.03 (t, J = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.79, 134.77, 131.76, 131.17, 130.96, 130.91, 130.66, 130.64, 127.66, 125.24, 125.22, 123.63, 123.53, 123.43, 122.47, 116.03, 115.89, 47.11, 24.03.

2-(2-(1-(2-bromobenzyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5k)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.46 (t, J = 7.8 Hz, 4H), 7.99 (s, 1H), 7.87 (t, J = 7.8 Hz, 2H), 7.65 (d, J = 7.8 Hz, 1H), 7.37 (t, J = 7.2 Hz, 1H), 7.29 (t, J = 7.8 Hz, 1H), 6.95 (d, J = 7.8 Hz, 1H), 5.60 (s, 2H), 4.33 (t, J = 7.2 Hz, 2H), 3.05 (t, J = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.84, 144.40, 135.67, 134.81, 133.21, 131.78, 131.21, 130.61, 130.15, 128.67, 127.86, 127.68, 123.74, 122.89, 122.50, 53.16, 39.80, 24.02.

2-(2-(1-(3-bromobenzyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5l)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.46 (t, J = 7.2 Hz, 4H), 8.05 (s, 1H), 7.86 (t, J = 7.8 Hz, 2H), 7.53 (d, J = 8.4 Hz, 1H), 7.49 (s, 1H), 7.31 (t, J = 7.8 Hz, 1H), 7.20 (d, J = 7.8 Hz, 1H), 5.55 (s, 2H), 4.31 (t, J = 7.2 Hz, 2H), 3.03 (t, J = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.81, 144.61, 139.39, 134.79, 131.78, 131.34, 131.18, 130.89, 127.85, 127.67, 127.15, 123.47, 122.50, 122.28, 52.22, 39.81, 24.06.

2-(2-(1-(2-methylbenzyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5m)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.46 (d, J = 1.8 Hz, 2H), 8.45 (d, J = 3.0 Hz, 2H), 7.87 (t, J = 7.8 Hz, 3H), 7.22 (t, J = 7.2 Hz, 1H), 7.18 (d, J = 7.2 Hz, 1H), 7.14 (t, J = 7.2 Hz, 1H), 6.94 (d, J = 7.2 Hz, 1H), 5.52 (s, 2H), 4.31 (t, J = 7.2 Hz, 2H), 3.03 (t, J = 7.2 Hz, 2H), 2.24 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.81, 144.36, 136.55, 134.78, 131.78, 131.18, 130.73, 128.64, 128.55, 127.84, 127.67, 126.63, 123.28, 122.49, 51.27, 39.79, 24.02, 19.02.

2-(2-(1-(4-methylbenzyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5n)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.45 (dd, J_1 = 7.2 Hz, J_2 = 4.8 Hz, 4H), 7.97 (s, 1H), 7.87 (t, J = 7.8 Hz, 2H), 7.14–7.10 (m, 4H), 5.47 (s, 2H), 4.30 (t, J = 7.2 Hz, 2H), 3.01 (t, J = 7.2 Hz, 2H), 2.28 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.79, 137.68, 134.78, 133.71, 131.77, 131.19, 129.65, 128.09, 127.84, 127.66, 123.12, 122.49, 52.87, 39.78, 24.05, 21.16.

2-(2-(1-(3,4-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5o).

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.67 (s, 1H), 8.50 (d, J = 7.2 Hz, 2H), 8.47 (d, J = 8.4 Hz, 2H), 7.88 (t, J = 7.8 Hz, 2H), 7.42 (d, J = 2.4 Hz, 1H), 7.37 (dd, J_1 = 8.4 Hz, J_2 = 2.4 Hz, 1H), 7.11 (d, J = 9.0 Hz, 1H), 4.39 (t, J = 7.8 Hz, 2H), 3.85 (s, 3H), 3.82 (s, 3H), 3.10 (t, J = 7.8 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.88, 149.76, 149.14, 145.12, 134.83, 131.80, 131.21, 130.69, 127.91, 127.70, 122.58, 121.36, 112.49, 112.29, 104.88, 56.31, 56.27, 39.79, 24.15.

MTT Assay for Cell Proliferation and Cytotoxicity

Cells (approximately 3,000–5,000 cells/well) were seeded in 96-well plates and cultured for 16 h until the cells were adherent. The concentration of test compounds was varied between 2, 4, 8, 16, and 32 μM, 0.1% DMSO was added to cells as control five multiple wells. Plates were cultured at 37 °C in 5% CO_2 environment for 72 h. After treatment, 10 μL of MTT (5 mg/mL) was added to each well and the plates were incubated at 37 °C and 5% CO_2 for 4 h. The reaction mixture containing 10% SDS and 0.1 mM of HCL was then added to each well before plates were incubated at 37 °C for another 4 h. The absorbance was measured on a microplate reader at a wavelength of 570 nm (Wang et al., 2020).

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/s.

AUTHOR CONTRIBUTIONS

L-FM and WL conceived the study, designed the experiments, and supervised all research. Z-JX prepared the draft of the manuscript. Z-JX and Y-JZ synthesized all compounds. J-HW carried out the experiments and analyzed

the data. G-QX contributed to the preliminary activity test and article collation of this paper. All authors reviewed the manuscript.

ACKNOWLEDGMENTS

This study was supported by the Scientific and Technological Project of Henan Province (No. 192102310142) and Key scientific research projects of colleges and universities in Henan Province (No. 21B180010).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.662432/full#supplementary-material

REFERENCES

Bousquet, P. F., Braña, M. F., Conlon, D., Fitzgerald, K. M., Perron, D., Cocchiaro, C., et al. (1995). Preclinical evaluation of LU 79553: a novel bis-naphthalimide with potent antitumor activity. *Cancer Res.* 55, 1176–1180. doi: 10.1007/BF01517352

Braña, M. F., Cacho, M., García, M., Pascual-Teresa, B. D., Ramos, A., Domínguez, M., et al. (2004). New analogues of amonafide and elinafide, containing aromatic heterocycles: synthesis, antitumor activity, molecular modeling, and DNA binding properties. *J. Med. Chem.* 47, 1391–1399. doi: 10.1021/jm0308850

Ferri, N., Radice, T., Antonino, M., Beccalli, E. M., Tinelli, S., Zunino, F., et al. (2011). Synthesis, structural, and biological evaluation of bis-heteroarylmaleimides and bis-heterofused imides. *Bioorg. Med. Chem.* 19, 5291–5299. doi: 10.1016/j.bmc.2011.08.016

Finney, N. S. (2006). Combinatorial discovery of fluorophores and fluorescent probes. *Curr. Opin. Chem. Biol.* 10, 238–245. doi: 10.1016/j.cbpa.2006.04.025

Gaitanis, G., Magiatis, P., Velegraki, A., and Bassukas, I. D. (2018). A traditional Chinese remedy points to a natural skin habitat: indirubin (indigo naturalis) for psoriasis and the *Malassezia metabolome*. *Br. J. Dermatol.* 179:800. doi: 10.1111/bjd.16807

González-Bulnes, L., and Gallego, J. (2012). Analysis of mixed DNA-bisnaphthalimide interactions involving groove association and intercalation with surface-based and solution methodologies. *Biopolymers* 97, 974–987. doi: 10.1002/bip.22114

Gonzalez-Bulnes, L., and Gallego, J. (2009). Indirect effects modulating the interaction between DNA and a cytotoxic bisnaphthalimide reveal a two-step binding process. *J. Am. Chem. Soc.* 131:7781. doi: 10.1021/ja901505p

Jiang, Z. C. (2013). Wavelength conversion perylene diester chromophores and luminescent films. *U.S.Pat. Appl. Publ.* 20130284265. vol,

Johnson, A. D., Zammit, R., Vella, J., Valentino, M., Buhagiar, J. A., and Magri, D. C. (2019). Aminonaphthalimide hybrids of mitoxantrone and amonafide as anticancer and fluorescent cellular imaging agents. *Bioorg. Chem.* 93:103287. doi: 10.1016/j.bioorg.2019.103287

Kirshenbaum, M. R., Chen, S. F., Behrens, C. H., Papp, L. M., Stafford, M. M., Sun, J. H., et al. (1994). (R,R)-2,2'-[1,2-ethanediylbis[imino(1-methyl-2,1-ethanediyl)]]-bis[5-nitro-1Hbenz[de] isoquinoline-1,342H)-dione] dimethanesulfonate (DMP 840), a novel Bis-naphthalimide with potent nonselective tumoricidal activity in *Vitro*. *Cancer Res.* 54, 2199–2206.

Langhals, H. (2004). Properties and applications of organic dyes and pigments. *Angew. Chem. Int. Ed.* 43, 5291–5293. doi: 10.1002/anie.200385122

Laquindanum, J. G., Katz, H. E., Dodabalapur, A., and Lovinger, A. J. (1996). N-channel organic transistor materials based on naphthalene frameworks. *J. Am. Chem. Soc.* 45, 11331–11332. doi: 10.1021/ja962461j

Li, C., and Wonneberger, H. (2012). Perylene imides for organic photovoltaics: yesterday, today, and tomorrow. *Adv. Mater.* 24, 613–636. doi: 10.1002/adma.201104447

Nazari, S., and Ghandi, K. (2015). Solvent and microwave effects on oxidation of aromatic α-diketones. *J. Ind. Eng. Chem.* 21, 198–205. doi: 10.1016/j.jiec.2014.02.025

Nitiss, J. L., Zhou, J., Rose, A., Hsiung, Y., Gale, K. C., and Osheroff, N. (1998). The bis(naphthalimide) DMP-840 causes cytotoxicity by its action against eukaryotic topoisomerase II. *Biochemistry* 37, 3078–3085. doi: 10.1021/bi9723257

Rosell, R., Carles, J., Abad, A., Ribelles, N., Barnadas, A., Benavides, A., et al. (1992). Phase i study of mitonafide in 120 hour continuous infusion in non-small cell lung cancer. *Invest. New Drugs* 10, 171–175. doi: 10.1007/bf00877242

Sinha, B. K., Strong, J., Gibson, N. W., and Kalyanaraman, B. (1985). Mechanism of DNA strand breaks by mitonafide, an imide derivative of 3-nitro-1,8-naphthalic acid. *Biochem. Pharmacol.* 34, 3845–3852. doi: 10.1016/0006-2952(85)90433-2

Takahashi, M., Suzuki, Y., Ichihashi, Y., Yamashita, M., and Kawai, H. (2007). 1,3,8,10-tetrahydro-2,9-diazadibenzo[cd, lm]perylenes: synthesis of reduced perylene bisimide analogues. *Tetrahedron. Lett.* 3, 357–359. doi: 10.1016/j.tetlet.2006.11.100

Verma, M., Luxami, V., and Paul, K. (2013). Synthesis, in vitro evaluation and molecular modelling of naphthalimide analogue as anticancer agents. *Eur. J. Med. Chem.* 68, 352–360. doi: 10.1016/j.ejmech.2013.07.027

Wang, H., and Yu, G. (2003). Copolymers having tunable energy levels and color of emission. *PCT Int. Appl.* 2003103070. vol,

Wang, W. J., Mao, L. F., Lai, H. L., Wang, Y. W., Jiang, Z. B., Li, W., et al. (2020). Dolutegravir derivative inhibits proliferation and induces apoptosis of non-small cell lung cancer cells via calcium signaling pathway. *Pharmacol. Res.* 161:105129. doi: 10.1016/j.phrs.2020.105129

Wang, Y., Zhang, J., Li, M., Li, M., Xie, S., and Wang, C. (2017). Synthesis and evaluation of novel amonafide-polyamine conjugates as anticancer agents. *Chem. Biol. Drug Des.* 89, 670–680. doi: 10.1111/cbdd.12888

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 Bioengineering and Biotechnology

REVIEW
published: 19 April 2021
doi: 10.3389/fbioe.2021.635504

The Relationship Between Nutrition and Atherosclerosis

Taotao Wei[1], Junnan Liu[2], Demei Zhang[2], Xiaomei Wang[2], Guangling Li[2], Ruchao Ma[1], Gang Chen[1], Xin Lin[1†] and Xueya Guo[1,2†]*

[1] Department of Cardiology, Lanzhou University Second Hospital, Lanzhou University, Lanzhou, China, [2] Lanzhou University Second Hospital, The Second Clinical Medical College of Lanzhou University, Lanzhou, China

Atherosclerosis is the basic pathological process of many diseases, such as coronary atherosclerosis and stroke. Nutrients can affect the occurrence and development of atherosclerosis. At present, in nutrition science, the research on atherosclerosis focuses on which nutrients play an important role in its prevention strategy, and what are the possible mechanisms of its action. In the current study, the process of atherosclerosis can be affected by adjusting the proportion of nutrients in the diet. In this review, we pay attention to the effects of phytosterols, omega-3-polyunsaturated fatty acids, polyphenol, vitamin, and other nutrients on atherosclerosis, pay attention to their current epidemiological status, current nutritional research results, and prevention or a possible mechanism to reduce the risk of development of atherosclerosis. So that more personalized nutritional approaches may be more effective in terms of nutritional intervention responses to atherosclerosis.

Keywords: atherosclerosis, nutrients, antioxidants, vitamin, omega-3

OPEN ACCESS

Edited by:
Miaomiao Yuan,
Sun Yat-sen University, China

Reviewed by:
Yongsheng Chen,
Jinan University, China
Yingbin Shen,
Guangzhou University, China

****Correspondence:***
Xueya Guo
guoxueya2006@126.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *30 November 2020*
Accepted: *19 March 2021*
Published: *19 April 2021*

Citation:
Wei T, Liu J, Zhang D, Wang X, Li G, Ma R, Chen G, Lin X and Guo X (2021) The Relationship Between Nutrition and Atherosclerosis. Front. Bioeng. Biotechnol. 9:635504. doi: 10.3389/fbioe.2021.635504

INTRODUCTION

Due to population growth, population aging, and disease epidemiological changes, the number of deaths from cardiovascular diseases (CVDs) is increasing in worldwide. From 1990 to 2013, the number of deaths due to CVD increased by 41% in the worldwide. In 2016, CVD caused about 17.6 million deaths worldwide, an increase of 14.5% compared with 2006. CVD cause huge health and economic burdens in the United States and globally (Benjamin et al., 2019).

Atherosclerosis is defined as a chronic inflammatory disease. The infiltration and retention of lipoproteins in the arterial wall is a key initiation event that triggers an inflammatory response and promotes the development of atherosclerosis. Blood lipids are transported in the form of lipoproteins in the blood circulation. Studies have found that the increase in plasma low-density lipoprotein (LDL) levels and the decrease in high-density lipoprotein (HDL) levels are positively correlated with the incidence of atherosclerosis. After being oxidized and modified by arterial wall cells, LDL can promote the formation of atherosclerotic plaques. At present, oxidized LDL (ox-LDL) is considered to be an important atherosclerotic factor and a major factor that causes damage to endothelial cells and smooth muscle cells. Ox-LDL cannot be recognized by normal LDL receptors, but easily recognized by scavenger receptors of macrophages and quickly taken up, which promotes the formation of foam cells by macrophages. On the contrary, HDL remove cholesterol from the arterial wall through the cholesterol reverse transport mechanism, and prevent the occurrence of atherosclerosis. In addition, HDL also has antioxidant effects, can prevent the oxidation of LDL, and can competitively inhibit the receptors of LDL and endothelial cells.

The pathogenesis of atherosclerosis has not been elucidated. The injure of endothelial cells caused by various reasons leads to endothelial dysfunction, promotes the modification of

lipoproteins and the infiltration of monocytes into inner subcutaneous space. The increased plasma lipoprotein is deposited on the arterial intima, causing connective tissue hyperplasia, thickening and hardening of the arterial wall, and then necrosis of the connective tissue to form atherosclerosis. Endothelial cells are damaged due to various reasons, so that plasma components include lipoprotein deposits on the inner membrane, causing platelets to adhere, aggregate, release various active substances [monocyte chemoattractant protein-1, interleukin (IL)-8, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin], attract monocytes to aggregate, adhere to the endothelium, and migrate to the subendothelial tissue of the blood vessel, and combine with oxidized lipoproteins to form mononuclear cells. At the same time, the active substance activates the smooth muscle cells of the arterial media to migrate into the intima to form smooth muscle-derived foam cells. Finally, the proliferating smooth muscle cells synthesize extracellular matrix such as collagen and proteoglycan to thicken and harden the intima of the disease, promote plaque formation, and accelerate the development of atherosclerosis.

Studies have found that NO can prevent the expression of pro-inflammatory factors, such as nuclear factor NF-κB (NF-kB) and adhesion molecules (monocyte chemoattractant protein-1, ICAM-1, VCAM-1, E-selectin, and P-selectin) (Badimón et al., 2009; Zhu et al., 2018). There are also studies show a large amount of reactive oxygen species (ROS) can mediate vascular endothelial dysfunction by promoting the action of superoxide dismutase, weaken the antioxidant capacity in cells, cause lipid peroxidation and DNA damage, and cause atherosclerosis (Förstermann et al., 2017; Zhu et al., 2018).

In this review, we summarize the nutrients that have been studied in the study of atherosclerosis. Such as phytosterols, omega-3-polyunsaturated fatty acids, polyphenol, and vitamin, we summarize the active components of nutrients in foods and their effects on atherosclerosis (**Figure 1**).

NUTRIENT AND ATHEROSCLEROSIS

Diet is an inseparable part of our lives. It is generally believed that good eating habits have a certain inhibitory effect on the development of atherosclerosis. In recent years, some nutrients [such as polyunsaturated fatty acids (PUFAs), vitamins, and polyphenols], it can stabilize atherosclerotic plaque or reduce the level of biomarkers related to inflammation (Casas et al., 2018).

Phytosterols

Phytosterols are biologically active compounds found in food, mainly derived from plants. Phytosterols can be divided into plant sterols and plant stanols. The chemical structure of phytosterols is similar to that of cholesterol. The only difference is that an extra ethyl group at the C-24 position (Gylling and Simonen, 2015), while cholesterol is only found in food which is animal origin. More than 250 types of plant sterols have been confirmed (Cohn et al., 2010). The food sources of plant sterols are mainly vegetable oils, including corn, sunflower, and soybeans, and olives, almonds, grains such as wheat germ, also fruits and vegetables, such as passion fruit, oranges, and cauliflower.

According to some guidelines and social consensus in different countries around the world (Cohn et al., 2010; Simão et al., 2013; Expert Dyslipidemia Panel of the International Atherosclerosis Society Panel Members, 2014; Gylling et al., 2014; Catapano et al., 2016). There is similar evidence that the intake of plant sterols and stanols (2 g/day) can significantly reduce the level of low-density lipoprotein-cholesterol (LDL-c) (8–10%). A study compared yogurt with phytostanol esters (4 g/day) and low-dose (2 g/day) yogurt (Vásquez-Trespalacios and Romero-Palacio, 2014). After 4 weeks of observation, it was found that it effectively reduced 10.3% LDL-c level in patients with hypercholesterolemia. A meta-analysis included 124 studies with an average phytosterol dose of 2.1 g/day (range 0.2–9.0 g/day) (Ras et al., 2014). It indicated that daily intake of 0.6–3.3 g of phytosterols, as the dose increases, LDL-c concentration gradually decreases by 6–12%.

The main mechanism for phytosterols to reduce LDL-c levels is to reduce the cholesterol absorbed through the intestine. The main mechanism is to reduce the amount of cholesterol absorbed through the intestinal lumen by competing with cholesterol to dissolve the mixed micelles in the intestinal lumen. Another mechanism is to modify the protein that encodes sterols, such as Niemann-Pick C1-like 1 (NPC1-L1) protein, to reduce cholesterol transport to intestinal epithelial cells, or intestinal epithelial cell ATP-binding cassette transporter to promote cholesterol from Intestinal epithelial cells flow out to the intestinal lumen. It can also reduce cholesterol levels through the transintestinal cholesterol excretion (Gylling and Simonen, 2015).

OMEGA-3-POLYUNSATURATED FATTY ACIDS

Polyunsaturated fatty acids are straight-chain fatty acids with two or more double bonds and a carbon chain length of 18–22 carbon atoms. In the PUFA molecule, the double bond that is the most distant from the carboxyl group on the third carbon atom is called omega-3 PUFAs. The two most important unsaturated fatty acids of omega-3 PUFAs to the human body are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Omega-3 PUFAs represents the most important PUFA in biology. The current research on them is mainly about their role in CVDs, inflammatory diseases, and metabolic diseases (Ander et al., 2003; Khandelwal et al., 2013; Tortosa-Caparrós et al., 2017; Schunck et al., 2018).

The REDUCE-IT trial is a randomized controlled trial that found that high-dose omega-3 PUFAs can significantly improve the prognosis of CVD, especially the use of 4 g/day of ethyl eicosapentaenoate can reduce the occurrence of cardiovascular events and reduce the risk of CVD by 25% compared with the control group (Bhatt et al., 2019). A meta-analysis conducted by Sekikawa et al. (2019) etc. included six studies, used the following criteria: adult subjects, omega-3 PUFAs (defined as ≥3.0 g/day,

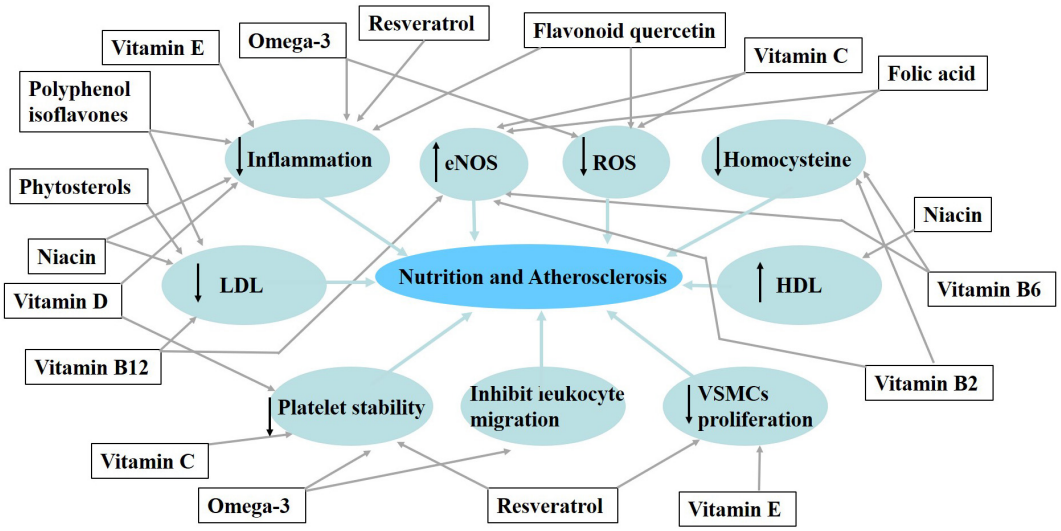


FIGURE 1 | The influence of some nutrients and bioactive compounds on the process of atherosclerosis.

or 1.8 g/day in Japan), take changes in atherosclerosis as a result and perform RCT intervention time ≥6 months. Found a large dose of omega-3 PUFAs can slows down the progression of atherosclerosis and has anti-atherosclerotic effects.

Omega-3 PUFAs can regulate lipid and lipoprotein profile, and down regulate leukocyte expression and the concentration of various pro-inflammatory biomarkers related to the development of atherosclerosis. It can also reduce oxidative stress and inhibit platelets. Its activity improves the function of blood vessels (Burke et al., 2017; Innes and Calder, 2018). Studies have confirmed that in subjects suffering from MetS, supplementation with omega-3 PUFA can improve arterial vascular endothelial function and stiffness, and has parallel anti-inflammatory effects (Tousoulis et al., 2014). In an intervention study on patients waiting for carotid endarterectomy, Thies et al. (2003) found that atherosclerotic plaques can easily incorporate omega-3 PUFAs from fish oil supplements, and the induced changes can enhance atherosclerotic plaques the stability of the block. Plaque stability may be related to the reduction of non-fatal and fatal cardiovascular events caused by increased omega-3 PUFA intake. Studies have also confirmed that higher levels of EPA in plaques are associated with a decrease in the number of foam cells and T cells, leading to reduced inflammation and increased stability (Cawood et al., 2010). The results of studies also support the potential benefit of fish oil supplements in reducing the risk of atherosclerotic thrombosis in stable coronary artery disease (sCAD), and the greatest benefit for patients who have not received lipid-lowering therapy (Franzese et al., 2015).

POLYPHENOL

Polyphenols are the most abundant antioxidants in the human diet and are usually found in fruits, vegetables, green tea, red wine, nuts, spices, and extra virgin olive oil. The most common polyphenols include resveratrol and flavonoids, the latter can divided into six subcategories: flavanols, flavonoids, flavanones, anthocyanins, flavonols, and isoflavones (Korakas et al., 2018). We will focus on three types of polyphenols, polyphenol isoflavones, resveratrol, and flavonoid quercetin.

Polyphenol Isoflavones

The polyphenol isoflavones in soybeans have anti-atherosclerotic properties because their structure is similar to estrogen and binds to estrogen receptors. Consumption of soybean products can reduce the serum levels of LDL-c and triglycerides (Anderson et al., 1995). Tokede et al. (2015) analyzed a total of 35 studies (50 comparisons). The duration of treatment ranges from 4 weeks to 1 year. Ingestion of soy products resulted in a significant decrease in serum LDL-c concentration, which was −4.83 mg/dl, triglycerides was −4.92 mg/dl, and total cholesterol concentration was −5.33 mg/dl. The serum high-density lipoprotein-cholesterol (HDL-c) concentration also increased significantly, at 1.40 mg/dl, and the LDL of patients with hypercholesterolemia decreased more significantly, at −7.47 mg/dl. The results are obviously heterogeneous. However, in a community-based cross-sectional study involving 2939 subjects (2135 women and 804 men) aged 50–75 years old, it was found that greater soy consumption was related to decreased serum TC levels, dyslipidemia, hyperuricemia and less frequent cardiometabolic disorders in women (Liu et al., 2014).

Studies have shown that the isoflavones in soy protein isolate will not change the plasma cholesterol level of LDLr-null mice, but it will reduce the plasma cholesterol level of C57BL/6 mice by 30% and reduce the atherosclerotic lesion area by 50%. At the same time, studies have shown that the cholesterol clearance mechanism mediated by LDL receptors can reduce the consumption of total plasma cholesterol in C57BL/6 mice (Kirk et al., 1998). Proteomics analysis showed that soybean extract or genistein/daidzein mixture can

reverse the changes in protein expression profile induced by stressors. The two application forms of isoflavones only jointly regulate protein entities related to mitochondrial dysfunction. Proteins identified through proteomics analysis indicate that soy isoflavones may enhance the anti-inflammatory response of monocytes in the blood, thereby promoting atherosclerosis prevention activities in a soy-rich diet (Fuchs et al., 2006; Wenzel et al., 2008).

Resveratrol

Resveratrol is a natural non-flavonoid polyphenol, which is found in wine, peanuts, red wine, etc. The current research has confirmed that it has anti-oxidant (da Silva et al., 2019), anti-platelet (Bertelli et al., 1995; Baur and Sinclair, 2006), and anti-inflammatory effects (Frémont, 2000), which play an important role in the process of atherosclerosis.

A large number of epidemiological studies have reported that resveratrol can improve high blood pressure, atherosclerosis, and ischemic heart disease (Zordoky et al., 2015). A three-blind, randomized, placebo-controlled trial study found that seventy-five patients (three parallel arms) took one capsule (350 mg) per day for 6 months, which contained grape extracts rich in resveratrol, grape extract without resveratrol (similar in polyphenol content) or placebo (maltodextrin). After 6 months, only LDL-c in the GE group was reduced by 2.9% (p = 0.013). Conversely, LDL-c, ApoB, LDLox, and LDLox/ApoB, decreased in the Stilvid® group, the ratio of non-HDL-c (total atherosclerotic cholesterol load)/ApoB increased, confirming that resveratrol reduced atherosclerosis markers and may have other cardioprotective effects beyond the gold standard drugs (Tomé-Carneiro et al., 2012). However, data indicate that resveratrol may prevent atherosclerosis in individuals who are not currently at high risk, indicating that resveratrol can be considered as the main atherosclerosis preventive agent (Agarwal et al., 2013).

The inflammatory response associated with atherosclerosis is largely regulated by the NF-κB pathway (Wang et al., 2016). NF-κB is connected with various signaling agents, which can trigger an inflammatory cascade. Animal studies have shown that the process of upregulation of SIRT-1 may have a significant impact on the activation and homeostasis of endothelial cells (Brandes, 2008; Ota et al., 2010). In endothelial cells, SIRT1 controls angiogenesis through multiple transcriptional regulators. Experimental studies have shown that the use of resveratrol can increase the serum SIRT1 concentration. Pre-treatment of human vascular smooth muscle cells (VSMCs) at a dose of 3–100 μM can significantly increase the expression of SIRT1 (Kao et al., 2010; Thompson et al., 2014). SIRT-1 inhibits NF-κB signaling pathway can inhibit the synthesis of a variety of pro-inflammatory cytokines, including: TNF-α, IL-1β, IL-6, and MCP-1.

Another important mechanism of resveratrol's anti-atherosclerosis effect is antiplatelet activity. Its antiplatelet activity mechanism mainly focuses on inhibiting COX-1. Selective inhibition of COX-1 leads to TXA2 (thromboxane A2) synthesis reduced, which is an effective trigger for platelet aggregation (FitzGerald, 1991).

Flavonoid Quercetin

Flavonoid quercetin is an important food antioxidant, found in vegetables and fruits, especially onions, apples and berries, wine and tea. The quercetin can be used as a valuable protective agent in a variety of diseases including cardiovascular inflammatory diseases (D'Andrea, 2015). Quercetin can prevent obesity induced by high-fat diet, and its anti-obesity effect may be related to the regulation of adipogenesis at the transcriptional level (D'Andrea, 2015). A randomized, double-blind, placebo-controlled crossover trial involving 37 apparently healthy (hypertensive) hypertensive men and women (40–80 years old) confirmed that quercetin may provide the heart by improving endothelial function and reducing inflammation protective effects (Dower et al., 2015). A meta-analysis of randomized controlled trials (RCT) showed that supplementation with quercetin had a significant effect on C-reactive protein-especially in patients with doses greater than 500 mg/day and CRP <3 mg/l. Other polyphenols, such as cocoa flavanols, have been found to improve endothelial function and protect the cardiovascular system (Lin et al., 2016; Mozaffarian and Wu, 2018).

Studies have found that the polyphenol flavonoid quercetin reduces the inflammatory response induced by high cholesterol levels and regulates the inflammatory process of atherosclerosis by affecting the TLR-NF-κB signaling pathway. In addition, the increase of quercetin can significantly reduce serum inflammatory mediators expression of mRNA such as COX, 5-LOX, MPO, CRP, and NOS (Bhaskar et al., 2016). Thereby reducing the atherosclerosis process related to endothelial dysfunction. Mechanism studies have shown that dietary hyperquercetin can significantly reduce the expression of p47phox in the aorta of ApoE−/− mice fed a high-fat diet and inhibit NADPH oxidase-derived oxidative stress, however, the expression and activity of the antioxidant enzyme heme oxygenase-1 (HO-1) are enhanced. *In vitro*, quercetin significantly reduced the formation of NADPH oxidase-derived O2·-in endothelial cells by inducing HO-1. It is proved that quercetin has indirect antioxidant properties in the process of atherosclerosis with NADPH oxidase and HO-1 (Luo et al., 2020).

VITAMIN

The diet involved in some micronutrients in atherosclerosis role is recognized. Vitamins, in particular. Studies demonstrate that increase the intake of vitamins in patients with subclinical atherosclerosis can reduce and slow down the incidence of CVD events, thereby preventing the development of pathological events (Hansson et al., 2006). However, with the population ages and the diet diversifies, vitamin deficiency is not uncommon in the worldwide, which may be an additional explanation for the increasing rate of coronary heart disease and cerebrovascular disease caused by atherosclerosis. In order to further raise people's awareness, it is necessary to further emphasize the influence of vitamins on the development of atherosclerosis in order to better guide the prevention of CVD.

The current new research focuses on the effect of vitamins on atherosclerosis. Especially the antioxidant capacity, so that the damage can still be reversed or at least slowed down, thereby preventing or slowing down the occurrence of vascular events caused by atherosclerosis. It appears that vitamins which with antioxidant and anti-inflammatory properties, may play an important role in targeting the subclinical atherosclerosis by equilibrating the balance of oxidation and antioxidation in human metabolism (Apryatin et al., 2018). Different groups of vitamins may play different roles, such as improving endothelial function, improving metabolism, inhibiting the renin-angiotensin-aldosterone system, anti-inflammatory, antioxidant, lowering blood homocysteine acid levels, and reversal of arterial calcification. Serum Vitamin B, C, D, E levels are of great significance for assessing cardiovascular risk and early prevention of CVD.

VITAMIN E

Vitamin E is a type of vitamin with antioxidant effect and an essential micronutrient. It exists in plants, seeds and their derivatives. It contains eight different isomers: four tocopherols (T) (α, β, γ, and δ) and four tocotrienols (T3) (α, β, γ, and δ). Among them, tocopherol α-T is the most active isomer of vitamin E. It is precursor-free and has important antioxidant effects (Parker et al., 1993). A lot of research has been done on the prevention of atherosclerosis. Vitamin E appears to be found in fat deposits, lipoproteins, and lipid-rich tissues. It seems to be involved in multiple stages of inflammation and immunity by regulating cell functions and gene expression (Rasool et al., 2008). By eliminating oxygen freelance fundamentals in cells, tissues, or membranes, it protects CVD, metabolic disorders, and other diseases at risk of oxidative stress (Meganathan and Fu, 2016).

Several interventional studies have shown that the effect of vitamin E in the early subclinical atherosclerosis phase of atherosclerosis seems encouraging. A study that used vitamin E and placebo to intervene in 36 healthy men to assess arterial compliance by measuring pulse wave velocity (PWV) and enhancement index (AI) concluded that vitamin E supplementation for 2 months had a tendency to improve arterial compliance (Waniek et al., 2017). A population-based study of the effects of antioxidant vitamin supplementation in preventing atherosclerosis has shown for the first time that a reasonable dose of a combined dose of vitamin E and vitamin C can delay the progression of common cervical atherosclerosis in men (Rasool et al., 2008). This may be further evidence of the role of vitamins in atherosclerosis trials revealed that patients with subclinical atherosclerosis treated with vitamin E a significant improvement in peripheral-artery disease and a reduced incidence of pectoris. This may imply that the earlier prevention, even if the progression of atherosclerosis is not fully controlled, can delay the occurrence of CVD. Many observational and cohort studies suggested a negative association between dietary vitamin E supplementation intake and CV events (Mozos et al., 2017). However, a meta-analysis has shown that high doses of vitamin E have oxidative effects, when vitamin E dose ≥400 IU may increase the all-cause mortality rate. Therefore, the benefits of vitamin E supplementation need to be accompanied by consideration of the hazards of higher doses.

Vitamin E inhibits the expression of adhesion molecules on endothelial cells and ligands on monocytes, and reduces the adhesion interaction between them. This is an important early event that can trigger the formation of fat streaks and atherosclerosis (Devaraj et al., 1996; Freedman et al., 1996; Wu et al., 1999). It can regulate inflammation by inhibiting 5-lipoxygenase, thereby reducing the release of interleukin-1β released by monocytes. It may also reduce the adhesion of monocytes *in vitro* by inhibiting the activation of nuclear factor NF-κB (Devaraj and Jialal, 1998). α-tocopherol inhibits protein kinase C (PKC)-mediated monocyte superoxide production, SMC proliferation, and platelet aggregation and adhesion (Ricciarelli et al., 1998; Keaney et al., 1999). Studies also show that vitamin E, through its non-antioxidant properties, may inhibit smooth muscle cell proliferation (Ricciarelli et al., 1998) and platelet aggregation (Freedman et al., 1996), which are important processes in plaque formation and atherosclerosis.

VITAMIN C

Vitamin C is a polyhydroxy compound. The two adjacent enol hydroxyl groups at the 2nd and 3rd positions in the molecule are easily dissociated to release H+, so it has acid properties and is also called ascorbic acid. Dietary sources of vitamin C are widely found in fresh vegetables and fruits, such as tomatoes, cauliflower, bell peppers, dark leafy vegetables, bitter gourd, citrus, grapefruit, grapes, kiwi, oranges, etc. Vitamin C must be obtained from external sources (primarily fruits and vegetables) because humans cannot synthesize it internally.

A meta-analysis consisting of 13 independent cohorts included 278,459 people (including 9143 CHD events). They were followed up for an average of 11 years and found that the daily intake of fruits and vegetables increased from less than three to more than five. A 17% reduction in heart disease risk is related (He et al., 2007).

Previous studies have demonstrated that vitamin C being able to scavenge ROS. It may prevent the oxidation of LDL-c by reducing the α-tocopherol free radical. Inhibiting ROS-mediated direct damage to the vascular endothelium and oxidative stress-induced signaling pathways, which are involved in the occurrence and development of atherosclerosis (Haendeler et al., 1996). Therefore, vitamin C plays an important role in preventing atherosclerosis and delaying the progression of coronary heart disease. Vitamin C can also prevent activation of platelets and apoptosis. A study investigated the relationship between vitamin C in supplements and early atherosclerosis [detection of carotid intima-media thickness (IMT)] and found that vitamin C in food is associated with accelerated early atherosclerosis measured by carotid IMT (Meganathan and Fu, 2016). Many studies have shown that atherosclerosis is negatively related to the intake of antioxidants, in contrast, some clinical trials have found that vitamin C are not beneficial for supplementary treatment of CVD. However, these clinical trials are not without their

limitations. Antioxidant therapy for a relatively short period of time or treatment of patients with advanced disease may not provide information related to disease.

VITAMIN D

Vitamin D is a group of steroids, the most important of which are vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). They can be obtained from diet and various supplements. The human body can also synthesize vitamin D.

Vitamin D affects many cells involved in atherogenesis, such as immune cells, endothelial cells, smooth muscle cells, and cardiomyocytes (Brewer et al., 2011). Vitamin D may influence the pathophysiology of atherosclerosis through decreasing the expression of TNFα, IL-6, IL-1, and IL-8 in isolated blood monocytes (Giulietti et al., 2007; Kassi et al., 2013). It may regulate the expression of thromboregulatory proteins and tissue factors in monocytes, thereby affecting platelet aggregation and thrombosis activity (Koyama et al., 1998). Thereby possibly preventing luminal rupture and thrombosis due to plaque instability (Oh et al., 2009). In addition, there is conclusive evidence that VDR and 1α-hydroxylase are expressed in the heart and blood vessels. Vitamin D has been shown to delay the development of porcine coronary artery disease by inhibiting NF-κB activation, supporting that vitamin D is considered an important factor in CVD (Chen et al., 2016).

VITAMIN B

Vitamin B is an essential nutrient for all human tissues. It is all coenzymes and participates in the metabolism of sugar, protein and fat in the body, so it is classified as a family. There are more than twelve types in this category, nine of which are considered essential vitamins for the human body, all of which are water-soluble vitamins. They stay in the body for only a few hours and must be supplemented daily. Folate (B9), B12, B6, niacin (B3), and riboflavin (B2) in B vitamins are believed to be related to reducing the risk of CVD.

Niacin

Niacin, also known as vitamin B3, or vitamin PP, is a water-soluble vitamin belonging to the vitamin B family. Niacin is converted into nicotinamide in the human body. Niacinamide is a component of Coenzyme I and Coenzyme II and participates in lipid metabolism in the body. Food-derived niacin is widely found in arterial liver, lean meat, cereals, beans, and green leafy vegetables. In addition to being directly ingested from food, niacin can also be converted from tryptophan in the body, with an average of about 60 mg tryptophan converting 1 mg niacin.

In pharmacological doses, niacin can reduce serum levels of LDL-c, very VLDL-c and lipoprotein (a) (Lp a). In addition, it can significantly increase the serum level of HDL-c. The mechanism of niacin to lower blood lipids and prevent atherosclerosis may have the following two aspects. Niacin can up-regulate PPARγ by stimulating the ATP-binding cassette transporter A1 in monocytes and macrophages, and ultimately lead to reverse cholesterol transport (Rubic et al., 2004). On the other hand, niacin can inhibit inflammation to prevent atherosclerosis. An animal study showed that niacin inhibits vascular inflammation by down-regulating the NF-κB signaling pathway (Si et al., 2014). Niacin lowers can reduce the level of CRP and lipoprotein-related phospholipase A2 (an independent risk factor for CVD) (Kuvin et al., 2006). Studies have shown that niacin has been shown to inhibit the expression and release of chemokine induced by TNF-α (Digby et al., 2010).

Folic Acid

Folic acid is a water-soluble vitamin. The biologically active form of folic acid is tetrahydrofolate. Human intestinal bacteria can synthesize folic acid, which can cause folic acid deficiency when malabsorption, metabolic disorders or long-term use of intestinal antibacterial drugs. In addition, folic acid is also widely present in animal and plant foods, rich in: offal, eggs, fish and

TABLE 1 | Proposed mechanism of nutrients in preventing atherosclerosis.

		↓Inflammation	↓ROS	↓Homocysteine	↓LDL	↑HDL	↓Platelet stability	Inhibit leukocyte migration	↓VSMCs proliferation	↑eNOS
Phytosterols					•					
Omega-3		•	•				•	•		
Polyphenol isoflavones		•			•					
Resveratrol		•					•		•	
Flavonoid quercetin		•	•							
Vitamin E		•							•	
Vitamin C			•				•			•
Vitamin D		•					•			
Vitamin B	Niacin	•			•	•				
	Folic acid			•						•
	Vitamin B12			•						•
	Vitamin B6			•						•
	Vitamin B2			•						•

pears, broad beans, beets, spinach, cauliflower, celery, citrus, nuts, and soy foods.

Vitamin B12

Vitamin B12, also called cobalamin, is the only vitamin that needs the help of intestinal secretions (endogenous factors) to be absorbed. Vitamin B12 in nature is mainly synthesized by bacteria in the rumen and colon of herbivores. Therefore, its dietary sources are mainly animal foods. Among them, animal offal, meat, and eggs are rich sources of vitamin B12. Soy products will produce part of vitamin B12 after fermentation. Human intestinal bacteria can also synthesize part of it.

Vitamin B6

Vitamin B6 is also known as pitocin, which includes pyridoxine, pyridoxamine, and pyridoxal. It exists in the form of phosphate ester in the body and is a water-soluble vitamin. Vitamin B6 is higher in meat, cereal products (especially wheat), vegetables and nuts. Vitamin B6 is a component of some coenzymes in the human body and participates in various metabolic reactions, especially closely related to amino acid metabolism.

Vitamin B2

Vitamin B2, also called riboflavin, is slightly soluble in water and stable when heated in neutral or acidic solutions. It is a component of the prosthetic group of yellow enzymes in the body (yellow enzymes play a role of hydrogen transfer in biological redox). The storage of vitamin B2 in the body is very limited, so it must be provided by the diet every day. Vitamin B2 is widely present in various foods, but the content of animal foods is usually higher than that of plant foods, such as liver, kidney, heart, egg yolk, eel, and milk of various animals. Many green leafy vegetables and legumes are also high in content, while cereals and general vegetables are low in content.

Current studies have found that B vitamins can reduce homocysteine levels. Homocysteine can be methylated into methionine. Vitamin B12 and riboflavin are used as cofactors in this process. At the same time, folic acid can provide methyl groups to react. It can also be metabolized into cysteine with the help of vitamin B6 and removed from the circulation. In recent years, more and more studies have shown that high plasma concentration of homocysteine, enhances the risk of atherothrombotic vascular disease (Knapen et al., 2015; Fulton et al., 2016). High homocysteine levels are an independent risk factor for atherosclerosis. It can increase oxidative stress and vascular smooth muscle growth and can also cause endothelial cell damage (Mudd, 1985). Both folic acid and B12 are necessary components for the conversion of HCY to methionine and are significantly negatively correlated with HCY levels. It was found in hemodialysis patients that combined folic acid and vitamin b12 supplementation can reduce the degree of atherosclerosis (McCully, 1969). Although studies have confirmed that vitamin B12 and folic acid can reduce plasma high homocysteine levels and play a role in protecting atherosclerosis (Shargorodsky et al., 2009), the specific efficacy of preventing CVD remains to be further studied. But for patients with an increased risk of atherosclerosis, it is necessary to monitor serum B vitamins and appropriate supplements.

In conclusion, we have summarized the relevant research and possible mechanisms of nutrients in the occurrence and progression of atherosclerosis (**Table 1**). In this review, we outline the current research on nutrients in atherosclerosis and their effects on related diseases.

CONCLUSION

Atherosclerosis is one of the most important risk factors for CVD and stroke. At present, clinical doctor reducing the level of LDL-c delays the development of atherosclerosis. In recent years, some studies have emphasized the important role of good eating habits and living habits in the development of atherosclerosis. Including phytosterols, omega-3-polyunsaturated fatty acids, polyphenol, and vitamin, which mentioned in this article, can directly or indirectly act on the vascular system by reducing inflammation, reducing oxidative stress, or forming active metabolites. We need to pay attention to the nutritional status of patients with atherosclerosis, which is a controllable risk factor for atherosclerosis, which can improve the patient's nutritional status and thereby improve the prognosis of patients. Therefore, it is important to establish the consensus on nutrient intake in the field of nutrition to reduce the occurrence of atherosclerosis, thereby reducing the incidence of cardiovascular and cerebrovascular diseases. Diet is an inseparable part of our lives, and whether the nutrients in food can benefit our body is worthy of long-term research in the future.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

We wish to acknowledge the members of Department of Cardiology, Lanzhou University, and our corresponding author XG.

REFERENCES

Agarwal, B., Campen, M. J., Channell, M. M., Wherry, S. J., Varamini, B., Davis, J. G., et al. (2013). Resveratrol for primary prevention of atherosclerosis: clinical trial evidence for improved gene expression in vascular endothelium. *Int. J. Cardiol.* 166, 246–248. doi: 10.1016/j.ijcard.2012.09.027

Ander, B. P., Dupasquier, C. M., Prociuk, M. A., and Pierce, G. N. (2003). Polyunsaturated fatty acids and their effects on cardiovascular disease. *Exp. Clin. Cardiol.* 8, 164–172.

Anderson, J. W., Johnstone, B. M., and Cook-Newell, M. E. (1995). Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* 333, 276–282. doi: 10.1056/nejm199508033330502

Apryatin, S. A., Mzhel'skaya, K. V., Balakina, A. S., Soto, S. J., Beketova, N. A., Kosheleva, O. V., et al. (2018). [Sex and line differences in biochemical indices and fat soluble vitamins sufficiency in rats on in vivo model of metabolic syndrome]. *Vopr. Pitan.* 87, 51–62. doi: 10.24411/0042-8833-2018-10006

Badimón, L., Vilahur, G., and Padró, T. (2009). Lipoproteins, platelets and atherothrombosis. *Rev. Esp. Cardiol.* 62, 1161–1178. doi: 10.1016/s1885-5857(09)73331-6

Baur, J. A., and Sinclair, D. A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nat. Rev. Drug Discov.* 5, 493–506. doi: 10.1038/nrd2060

Benjamin, E. J., Muntner, P., Alonso, A., Bittencourt, M. S., Callaway, C. W., Carson, A. P., et al. (2019). Heart disease and stroke statistics-2019 update: a report from the american heart association. *Circulation* 139, e56–e528. doi: 10.1161/cir.0000000000000659

Bertelli, A. A., Giovannini, L., Giannessi, D., Migliori, M., Bernini, W., Fregoni, M., et al. (1995). Antiplatelet activity of synthetic and natural resveratrol in red wine. *Int. J. Tissue React.* 17, 1–3.

Bhaskar, S., Sudhakaran, P. R., and Helen, A. (2016). Quercetin attenuates atherosclerotic inflammation and adhesion molecule expression by modulating TLR-NF-κB signaling pathway. *Cell Immunol.* 310, 131–140. doi: 10.1016/j.cellimm.2016.08.011

Bhatt, D. L., Steg, P. G., Miller, M., Brinton, E. A., Jacobson, T. A., Ketchum, S. B., et al. (2019). Cardiovascular Risk Reduction with Icosapent Ethyl for Hypertriglyceridemia. *N. Engl. J. Med.* 380, 11–22. doi: 10.1056/NEJMoa1812792

Brandes, R. P. (2008). Activating SIRT1: a new strategy to prevent atherosclerosis? *Cardiovasc. Res.* 80, 163–164. doi: 10.1093/cvr/cvn245

Brewer, L. C., Michos, E. D., and Reis, J. P. (2011). Vitamin D in atherosclerosis, vascular disease, and endothelial function. *Curr. Drug Targets* 12, 54–60. doi: 10.2174/138945011793591617

Burke, M. F., Burke, F. M., and Soffer, D. E. (2017). Review of cardiometabolic effects of prescription Omega-3 fatty acids. *Curr. Atheroscler. Rep.* 19:60. doi: 10.1007/s11883-017-0700-z

Casas, R., Estruch, R., and Sacanella, E. (2018). Influence of bioactive nutrients on the atherosclerotic process: a review. *Nutrients* 10:1630. doi: 10.3390/nu10111630

Catapano, A. L., Graham, I., De Backer, G., Wiklund, O., Chapman, M. J., Drexel, H., et al. (2016). 2016 ESC/EAS guidelines for the management of dyslipidaemias. *Eur. Heart J.* 37, 2999–3058. doi: 10.1093/eurheartj/ehw272

Cawood, A. L., Ding, R., Napper, F. L., Young, R. H., Williams, J. A., Ward, M. J., et al. (2010). Eicosapentaenoic acid (EPA) from highly concentrated n-3 fatty acid ethyl esters is incorporated into advanced atherosclerotic plaques and higher plaque EPA is associated with decreased plaque inflammation and increased stability. *Atherosclerosis* 212, 252–259. doi: 10.1016/j.atherosclerosis.2010.05.022

Chen, S., Swier, V. J., Boosani, C. S., Radwan, M. M., and Agrawal, D. K. (2016). Vitamin D deficiency accelerates coronary artery disease progression in swine. *Arterioscler. Thromb. Vasc. Biol.* 36, 1651–1659. doi: 10.1161/atvbaha.116.307586

Cohn, J. S., Kamili, A., Wat, E., Chung, R. W., and Tandy, S. (2010). Reduction in intestinal cholesterol absorption by various food components: mechanisms and implications. *Atheroscler Suppl.* 11, 45–48. doi: 10.1016/j.atherosclerosissup.2010.04.004

da Silva, A. D., Dos Santos, J. A., Machado, P. A., Alves, L. A., Laque, L. C., de Souza, V. C., et al. (2019). Insights about resveratrol analogs against trypanothione reductase of Leishmania braziliensis: molecular modeling, computational docking and in vitro antileishmanial studies. *J. Biomol. Struct. Dyn.* 37, 2960–2969. doi: 10.1080/07391102.2018.1502096

D'Andrea, G. (2015). Quercetin: a flavonol with multifaceted therapeutic applications? *Fitoterapia* 106, 256–271. doi: 10.1016/j.fitote.2015.09.018

Devaraj, S., and Jialal, I. (1998). The effects of alpha-tocopherol on critical cells in atherogenesis. *Curr. Opin. Lipidol.* 9, 11–15. doi: 10.1097/00041433-199802000-00004

Devaraj, S., Li, D., and Jialal, I. (1996). The effects of alpha tocopherol supplementation on monocyte function. decreased lipid oxidation, interleukin 1 beta secretion, and monocyte adhesion to endothelium. *J. Clin. Invest.* 98, 756–763. doi: 10.1172/jci118848

Digby, J. E., McNeill, E., Dyar, O. J., Lam, V., Greaves, D. R., and Choudhury, R. P. (2010). Anti-inflammatory effects of nicotinic acid in adipocytes demonstrated by suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin. *Atherosclerosis* 209, 89–95. doi: 10.1016/j.atherosclerosis.2009.08.045

Dower, J. I., Geleijnse, J. M., Gijsbers, L., Schalkwijk, C., Kromhout, D., and Hollman, P. C. (2015). Supplementation of the pure flavonoids epicatechin and quercetin affects some biomarkers of endothelial dysfunction and inflammation in (Pre)hypertensive adults: a randomized double-blind, placebo-controlled, crossover trial. *J. Nutr.* 145, 1459–1463. doi: 10.3945/jn.115.211888

Expert Dyslipidemia Panel of the International Atherosclerosis Society Panel Members (2014). An International atherosclerosis society position paper: global recommendations for the management of dyslipidemia–full report. *J. Clin. Lipidol.* 8, 29–60. doi: 10.1016/j.jacl.2013.12.005

FitzGerald, G. A. (1991). Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. *Am. J. Cardiol.* 68, 11b–15b. doi: 10.1016/0002-9149(91)90379-y

Förstermann, U., Xia, N., and Li, H. (2017). Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. *Circ. Res.* 120, 713–735. doi: 10.1161/circresaha.116.309326

Franzese, C. J., Bliden, K. P., Gesheff, M. G., Pandya, S., Guyer, K. E., Singla, A., et al. (2015). Relation of fish oil supplementation to markers of atherothrombotic risk in patients with cardiovascular disease not receiving lipid-lowering therapy. *Am. J. Cardiol.* 115, 1204–1211. doi: 10.1016/j.amjcard.2015.02.002

Freedman, J. E., Farhat, J. H., Loscalzo, J., Keaney, J. F., and Jr. (1996). alpha-tocopherol inhibits aggregation of human platelets by a protein kinase C-dependent mechanism. *Circulation* 94, 2434–2440. doi: 10.1161/01.cir.94.10.2434

Frémont, L. (2000). Biological effects of resveratrol. *Life Sci.* 66, 663–673. doi: 10.1016/s0024-3205(99)00410-5

Fuchs, D., Dirscherl, B., Schroot, J. H., Daniel, H., and Wenzel, U. (2006). Soy extract has different effects compared with the isolated isoflavones on the proteome of homocysteine-stressed endothelial cells. *Mol. Nutr. Food Res.* 50, 58–69. doi: 10.1002/mnfr.200500133

Fulton, R. L., McMurdo, M. E., Hill, A., Abboud, R. J., Arnold, G. P., Struthers, A. D., et al. (2016). Effect of vitamin k on vascular health and physical function in older people with vascular disease–a randomised controlled trial. *J. Nutr. Health Aging* 20, 325–333. doi: 10.1007/s12603-015-0619-4

Giulietti, A., van Etten, E., Overbergh, L., Stoffels, K., Bouillon, R., and Mathieu, C. (2007). Monocytes from type 2 diabetic patients have a pro-inflammatory profile. 1,25-Dihydroxyvitamin D(3) works as anti-inflammatory. *Diabetes Res. Clin. Pract.* 77, 47–57. doi: 10.1016/j.diabres.2006.10.007

Gylling, H., Plat, J., Turley, S., Ginsberg, H. N., Ellegård, L., Jessup, W., et al. (2014). Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. *Atherosclerosis* 232, 346–360. doi: 10.1016/j.atherosclerosis.2013.11.043

Gylling, H., and Simonen, P. (2015). Phytosterols, phytostanols, and lipoprotein metabolism. *Nutrients* 7, 7965–7977. doi: 10.3390/nu7095374

Haendeler, J., Zeiher, A. M., and Dimmeler, S. (1996). Vitamin C and E prevent lipopolysaccharide-induced apoptosis in human endothelial cells by modulation of Bcl-2 and Bax. *Eur. J. Pharmacol.* 317, 407–411. doi: 10.1016/s0014-2999(96)00759-5

Hansson, G. K., Robertson, A. K., and Söderberg-Nauclér, C. (2006). Inflammation and atherosclerosis. *Annu. Rev. Pathol.* 1, 297–329. doi: 10.1146/annurev.pathol.1.110304.100100

He, F. J., Nowson, C. A., Lucas, M., and MacGregor, G. A. (2007). Increased consumption of fruit and vegetables is related to a reduced risk of coronary heart disease: meta-analysis of cohort studies. *J. Hum. Hypertens* 21, 717–728. doi: 10.1038/sj.jhh.1002212

Innes, J. K., and Calder, P. C. (2018). The differential effects of eicosapentaenoic acid and docosahexaenoic acid on cardiometabolic risk factors: a systematic review. *Int. J. Mol. Sci.* 19:532. doi: 10.3390/ijms19020532

Kao, C. L., Chen, L. K., Chang, Y. L., Yung, M. C., Hsu, C. C., Chen, Y. C., et al. (2010). Resveratrol protects human endothelium from H(2)O(2)-induced oxidative stress and senescence via SirT1 activation. *J. Atheroscler Thromb.* 17, 970–979. doi: 10.5551/jat.4333

Kassi, E., Adamopoulos, C., Basdra, E. K., and Papavassiliou, A. G. (2013). Role of vitamin D in atherosclerosis. *Circulation* 128, 2517–2531. doi: 10.1161/circulationaha.113.002654

Keaney, J. F. Jr., Simon, D. I., and Freedman, J. E. (1999). Vitamin E and vascular homeostasis: implications for atherosclerosis. *Faseb J.* 13, 965–975. doi: 10.1096/fasebj.13.9.965

Khandelwal, S., Kelly, L., Malik, R., Prabhakaran, D., and Reddy, S. (2013). Impact of omega-6 fatty acids on cardiovascular outcomes: a review. *J. Preventive Cardiol.* 2, 325–336.

Kirk, E. A., Sutherland, P., Wang, S. A., Chait, A., and LeBoeuf, R. C. (1998). Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *J. Nutr.* 128, 954–959. doi: 10.1093/jn/128.6.954

Knapen, M. H., Braam, L. A., Drummen, N. E., Bekers, O., Hoeks, A. P., and Vermeer, C. (2015). Menaquinone-7 supplementation improves arterial stiffness in healthy postmenopausal women. a double-blind randomised clinical trial. *Thromb. Haemost.* 113, 1135–1144. doi: 10.1160/th14-08-0675

Korakas, E., Dimitriadis, G., Raptis, A., and Lambadiari, V. (2018). Dietary composition and cardiovascular risk: a mediator or a bystander? *Nutrients* 10:1912. doi: 10.3390/nu10121912

Koyama, T., Shibakura, M., Ohsawa, M., Kamiyama, R., and Hirosawa, S. (1998). Anticoagulant effects of 1alpha,25-dihydroxyvitamin D3 on human myelogenous leukemia cells and monocytes. *Blood* 92, 160–167. doi: 10.1182/blood.v92.1.160.413k16_160_167

Kuvin, J. T., Dave, D. M., Sliney, K. A., Mooney, P., Patel, A. R., Kimmelstiel, C. D., et al. (2006). Effects of extended-release niacin on lipoprotein particle size, distribution, and inflammatory markers in patients with coronary artery disease. *Am. J. Cardiol.* 98, 743–745. doi: 10.1016/j.amjcard.2006.04.011

Lin, X., Zhang, I., Li, A., Manson, J. E., Sesso, H. D., Wang, L., et al. (2016). Cocoa flavanol intake and biomarkers for cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials. *J. Nutr.* 146, 2325–2333. doi: 10.3945/jn.116.237644

Liu, J., Sun, L. L., He, L. P., Ling, W. H., Liu, Z. M., and Chen, Y. M. (2014). Soy food consumption, cardiometabolic alterations and carotid intima-media thickness in Chinese adults. *Nutr. Metab. Cardiovasc. Dis.* 24, 1097–1104. doi: 10.1016/j.numecd.2014.04.016

Luo, M., Tian, R., and Lu, N. (2020). Quercetin inhibited endothelial dysfunction and atherosclerosis in apolipoprotein E-deficient mice: critical roles for NADPH oxidase and heme oxygenase-1. *J. Agric. Food Chem.* 68, 10875–10883. doi: 10.1021/acs.jafc.0c03907

McCully, K. S. (1969). Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am. J. Pathol.* 56, 111–128.

Meganathan, P., and Fu, J. Y. (2016). Biological properties of tocotrienols: evidence in human studies. *Int. J. Mol. Sci.* 17:1682. doi: 10.3390/ijms17111682

Mozaffarian, D., and Wu, J. H. Y. (2018). Flavonoids, dairy foods, and cardiovascular and metabolic health: a review of emerging biologic pathways. *Circ. Res.* 122, 369–384. doi: 10.1161/circresaha.117.309008

Mozos, I., Stoian, D., and Luca, C. T. (2017). Crosstalk between vitamins A, B12, D, K, C, and E status and arterial stiffness. *Dis. Markers* 2017:8784971. doi: 10.1155/2017/8784971

Mudd, S. H. (1985). Vascular disease and homocysteine metabolism. *N. Engl. J. Med.* 313, 751–753. doi: 10.1056/nejm198509193131210

Oh, J., Weng, S., Felton, S. K., Bhandare, S., Riek, A., Butler, B., et al. (2009). 1,25(OH)2 vitamin d inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. *Circulation* 120, 687–698. doi: 10.1161/circulationaha.109.856070

Ota, H., Eto, M., Ogawa, S., Iijima, K., Akishita, M., and Ouchi, Y. (2010). SIRT1/eNOS axis as a potential target against vascular senescence, dysfunction and atherosclerosis. *J. Atheroscler Thromb.* 17, 431–435. doi: 10.5551/jat.3525

Parker, R. A., Pearce, B. C., Clark, R. W., Gordon, D. A., and Wright, J. J. (1993). Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme a reductase. *J. Biol. Chem.* 268, 11230–11238. doi: 10.1016/s0021-9258(18)82115-9

Ras, R. T., Geleijnse, J. M., and Trautwein, E. A. (2014). LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: a meta-analysis of randomised controlled studies. *Br. J. Nutr.* 112, 214–219. doi: 10.1017/s0007114514000750

Rasool, A. H., Rahman, A. R., Yuen, K. H., and Wong, A. R. (2008). Arterial compliance and vitamin E blood levels with a self emulsifying preparation of tocotrienol rich vitamin E. *Arch. Pharm. Res.* 31, 1212–1217. doi: 10.1007/s12272-001-1291-5

Ricciarelli, R., Tasinato, A., Clément, S., Ozer, N. K., Boscoboinik, D., and Azzi, A. (1998). alpha-Tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state. *Biochem. J.* 334(Pt 1), 243–249. doi: 10.1042/bj3340243

Rubic, T., Trottmann, M., and Lorenz, R. L. (2004). Stimulation of CD36 and the key effector of reverse cholesterol transport ATP-binding cassette A1 in monocytoid cells by niacin. *Biochem. Pharmacol.* 67, 411–419. doi: 10.1016/j.bcp.2003.09.014

Schunck, W. H., Konkel, A., Fischer, R., and Weylandt, K. H. (2018). Therapeutic potential of omega-3 fatty acid-derived epoxyeicosanoids in cardiovascular and inflammatory diseases. *Pharmacol. Ther.* 183, 177–204. doi: 10.1016/j.pharmthera.2017.10.016

Sekikawa, A., Cui, C., Sugiyama, D., Fabio, A., Harris, W. S., and Zhang, X. (2019). Effect of high-dose marine Omega-3 fatty acids on atherosclerosis: a systematic review and meta-analysis of randomized clinical trials. *Nutrients* 11:2599. doi: 10.3390/nu11112599

Shargorodsky, M., Boaz, M., Pasternak, S., Hanah, R., Matas, Z., Fux, A., et al. (2009). Serum homocysteine, folate, vitamin B12 levels and arterial stiffness in diabetic patients: which of them is really important in atherogenesis? *Diabetes Metab. Res. Rev.* 25, 70–75. doi: 10.1002/dmrr.902

Si, Y., Zhang, Y., Zhao, J., Guo, S., Zhai, L., Yao, S., et al. (2014). Niacin inhibits vascular inflammation via downregulating nuclear transcription factor-κB signaling pathway. *Med. Inflamm.* 2014:263786. doi: 10.1155/2014/263786

Simão, A. F., Precoma, D. B., Andrade, J. P., Correa, F. H., Saraiva, J. F., Oliveira, G. M., et al. (2013). [I Brazilian Guidelines for cardiovascular prevention]. *Arq. Bras. Cardiol.* 101(6 Suppl. 2), 1–63. doi: 10.5935/abc.2013S012

Thies, F., Garry, J. M., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C. P., et al. (2003). Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. *Lancet* 361, 477–485. doi: 10.1016/s0140-6736(03)12468-3

Thompson, A. M., Martin, K. A., and Rzucidlo, E. M. (2014). Resveratrol induces vascular smooth muscle cell differentiation through stimulation of SirT1 and AMPK. *PLoS One* 9:e85495. doi: 10.1371/journal.pone.0085495

Tokede, O. A., Onabanjo, T. A., Yansane, A., Gaziano, J. M., and Djoussé, L. (2015). Soya products and serum lipids: a meta-analysis of randomised controlled trials. *Br. J. Nutr.* 114, 831–843. doi: 10.1017/s0007114515002603

Tomé-Carneiro, J., Gonzálvez, M., Larrosa, M., García-Almagro, F. J., Avilés-Plaza, F., Parra, S., et al. (2012). Consumption of a grape extract supplement containing resveratrol decreases oxidized LDL and ApoB in patients undergoing primary prevention of cardiovascular disease: a triple-blind, 6-month follow-up, placebo-controlled, randomized trial. *Mol. Nutr. Food Res.* 56, 810–821. doi: 10.1002/mnfr.201100673

Tortosa-Caparrós, E., Navas-Carrillo, D., Marín, F., and Orenes-Piñero, E. (2017). Anti-inflammatory effects of omega 3 and omega 6 polyunsaturated fatty acids in cardiovascular disease and metabolic syndrome. *Crit. Rev. Food Sci. Nutr.* 57, 3421–3429. doi: 10.1080/10408398.2015.1126549

Tousoulis, D., Plastiras, A., Siasos, G., Oikonomou, E., Verveniotis, A., Kokkou, E., et al. (2014). Omega-3 PUFAs improved endothelial function and arterial stiffness with a parallel antiinflammatory effect in adults with metabolic syndrome. *Atherosclerosis* 232, 10–16. doi: 10.1016/j.atherosclerosis.2013.10.014

Vásquez-Trespalacios, E. M., and Romero-Palacio, J. (2014). Efficacy of yogurt drink with added plant stanol esters (Benecol®, Colanta) in reducing total and LDL cholesterol in subjects with moderate hypercholesterolemia: a randomized placebo-controlled crossover trial NCT01461798. *Lipids Health Dis.* 13:125. doi: 10.1186/1476-511x-13-125

Wang, X., Chen, Q., Pu, H., Wei, Q., Duan, M., Zhang, C., et al. (2016). Adiponectin improves NF-κB-mediated inflammation and abates atherosclerosis progression in apolipoprotein E-deficient mice. *Lipids Health Dis.* 15:33. doi: 10.1186/s12944-016-0202-y

Waniek, S., di Giuseppe, R., Plachta-Danielzik, S., Ratjen, I., Jacobs, G., Koch, M., et al. (2017). Association of vitamin e levels with metabolic syndrome, and MRI-derived body fat volumes and liver fat content. *Nutrients* 9:1143. doi: 10.3390/nu9101143

Wenzel, U., Fuchs, D., and Daniel, H. (2008). Protective effects of soy-isoflavones in cardiovascular disease. identification of molecular targets. *Hamostaseologie* 28, 85–88.

Wu, D., Koga, T., Martin, K. R., and Meydani, M. (1999). Effect of vitamin E on human aortic endothelial cell production of chemokines and adhesion to monocytes. *Atherosclerosis* 147, 297–307. doi: 10.1016/s0021-9150(99)00199-9

Zhu, Y., Xian, X., Wang, Z., Bi, Y., Chen, Q., Han, X., et al. (2018). Research progress on the relationship between atherosclerosis and inflammation. *Biomolecules* 8:80. doi: 10.3390/biom8030080

Zordoky, B. N., Robertson, I. M., and Dyck, J. R. (2015). Preclinical and clinical evidence for the role of resveratrol in the treatment of cardiovascular diseases. *Biochim. Biophys. Acta* 1852, 1155–1177. doi: 10.1016/j.bbadis.2014.10.016f

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 Bioengineering and Biotechnology

REVIEW
published: 01 July 2021
doi: 10.3389/fbioe.2021.635768

The Review of Anti-aging Mechanism of Polyphenols on *Caenorhabditis elegans*

Limin Liu[1,2], *Peisen Guo*[1], *Peixi Wang*[2], *Shanqing Zheng*[3], *Zhi Qu*[2]* *and Nan Liu*[1,2,4]*

[1] College of Public Health, Zhengzhou University, Zhengzhou, China, [2] Institute of Chronic Disease Risks Assessment, School of Nursing and Health, Henan University, Kaifeng, China, [3] School of Basic Medical Sciences, Henan University, Kaifeng, China, [4] Institute of Environment and Health, South China Hospital, Health Science Center, Shenzhen University, Shenzhen, China

Micronutrients extracted from natural plants or made by biological synthesis are widely used in anti-aging research and applications. Among more than 30 effective anti-aging substances, employing polyphenol organic compounds for modification or delaying of the aging process attracts great interest because of their distinct contribution in the prevention of degenerative diseases, such as cardiovascular disease and cancer. There is a profound potential for polyphenol extracts in the research of aging and the related diseases of the elderly. Previous studies have mainly focused on the properties of polyphenols implicated in free radical scavenging; however, the anti-oxidant effect cannot fully elaborate its biological functions, such as neuroprotection, Aβ protein production, ion channel coupling, and signal transduction pathways. *Caenorhabditis elegans* (*C. elegans*) has been considered as an ideal model organism for exploring the mechanism of anti-aging research and is broadly utilized in screening for natural bioactive substances. In this review, we have described the molecular mechanisms and pathways responsible for the slowdown of aging processes exerted by polyphenols. We also have discussed the possible mechanisms for their anti-oxidant and anti-aging properties in *C. elegans* from the perspective of different classifications of the specific polyphenols, such as flavonols, anthocyanins, flavan-3-ols, hydroxybenzoic acid, hydroxycinnamic acid, and stilbenes.

Keywords: polyphenols, *Caenorhabditis elegans*, anti-aging, anti-oxidant, insulin/insulin-like signaling pathway

OPEN ACCESS

Edited by:
Miaomiao Yuan,
Sun Yat-Sen University, China

Reviewed by:
Jie Gao,
Guangxi University, China
Rajendran Velmurugan,
Chulalongkorn University, Thailand

***Correspondence:**
Zhi Qu
10210056@henu.edu.cn
orcid.org/0000-0001-9898-4746
Nan Liu
13688869875@163.com;
LNQ555@126.com
orcid.org/0000-0002-8895-3169

Specialty section:
This article was submitted to Synthetic Biology, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *30 November 2020*
Accepted: *07 June 2021*
Published: *01 July 2021*

Citation:
Liu L, Guo P, Wang P, Zheng S, Qu Z and Liu N (2021) The Review of Anti-aging Mechanism of Polyphenols on Caenorhabditis elegans. Front. Bioeng. Biotechnol. 9:635768. doi: 10.3389/fbioe.2021.635768

INTRODUCTION

Aging is considered a universal physiological process that is accompanied by systemic changes in the structural integrity of cells that are caused by alterations in metabolic and signal transduction pathways (Childs et al., 2015). Understanding of the biological mechanisms of aging and longevity has been growing remarkably over the past two decades. At the molecular level, senescence is strongly associated with susceptibility to chronic diseases and disorders, such as chronic fibrosis, severe atherosclerosis, diabetes, osteoarthritis, and ultimately death (Childs et al., 2016; Amor et al., 2020). Among the various anti-aging methods and preventive strategies, the use of micronutrients or biologically active substances is considered a practical and efficient method that targets a variety of intracellular/extracellular pathways (Sahin et al., 2011; Johnson et al., 2013; Li et al., 2017).

Nutrients and bioactive substances have shed new light on the prevention and treatment of chronic diseases and aging. For example, short-term supplementation with appropriate doses of vitamin C or vitamin C plus E has already been confirmed to improve the immunological function

in the elderly and contribute to health and longevity (De la Fuente et al., 2020). Most of the substances that exert bioactive properties originate from natural plants and animals and have been extensively studied for their preventive and therapeutic effects against chronic diseases and aging. Functional nutrition is of great significance to human health; however, the high cost involved in extracting and purifying bioactive compounds from natural sources in the past has limited the rapid growth of the market. Thus, with the development of synthetic biology technology, a few important functional nutrients can be produced at a low cost by biological manufacturing. In the future, biological manufacturing is expected to be replaced by traditional extraction techniques or functional nutritional chemicals. So far, plant polyphenols, such as blueberry polyphenols, black tea and green tea polyphenols, and tocotrienols in vegetable oils, have been proved to delay the aging process in model organisms (Adachi and Ishii, 2000; Wilson et al., 2006; Peng et al., 2009; Salminen et al., 2012; Zarse et al., 2012). The anti-aging effects of these polyphenols are mostly related to their anti-oxidant properties and their ability to scavenge free radicals. It has been reported that resveratrol, a polyphenol compound in red wine, could slow down aging in *Caenorhabditis elegans* due to the reduction of mitochondrial respiration (Wood et al., 2004). The understanding of human aging and longevity might be improved by elucidating the molecular mechanism of aging in *C. elegans* (Park et al., 2020).

ADVANTAGES OF USING *C. ELEGANS* AS A MODEL ORGANISM IN APPLIED ANTI-AGING RESEARCH

Although experiment with a mammalian model is compelling, it is time-consuming and limited by the presence of ethical concerns. *C. elegans* has been proved as a reasonable model organism for biological research on aging because of its advantageous features (Guarente and Kenyon, 2000). Although its anatomical structure is simple, the tissues and organs, such as muscles, nervous system, gastrointestinal tract, and gonads of *C. elegans*, are similar to that of higher animals (Jorgensen and Mango, 2002). In addition, its complete genome sequence is available, and about 50% of human protein-coding sequences have identifiable homologous genes in nematodes (Kim et al., 2018). Similar to humans and other higher mammals, its behavior changes and descending physiological indexes are accompanied by aging. Moreover, there are highly evolutionary conserved mechanisms controlling physiological phenomena, such as development, aging, and disease. Homologous or functionally similar forms of the main enzymes, genes, and transcription factors involved in metabolism have been found in higher animals and *C. elegans* (Chen et al., 2013). For example, the important transcription factor forkhead box O (FOXO), which is involved in longevity, stress resistance, and metabolism, is present in drosophila, nematodes, rodents, and humans (Martins et al., 2016). Therefore, *C. elegans* is broadly utilized in screening for natural bioactive substances (Ye et al., 2020). Numerous transgenes and mutants related to the longevity and aging of *C. elegans* are available (Chen et al., 2015), and many polyphenols have been successfully tested for their effects on general health benefits and longevity on nematodes.

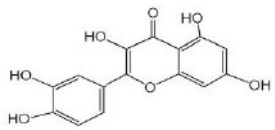
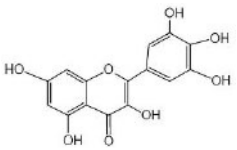
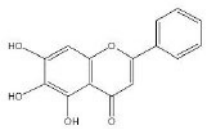
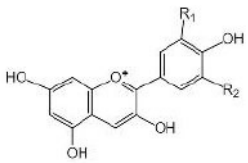
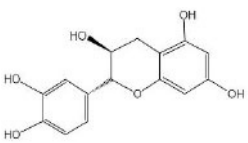
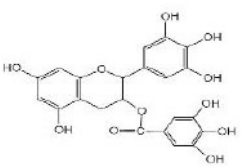
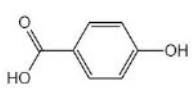
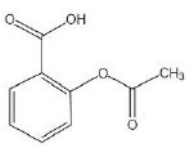
At present, most of the bioactive substances with anti-aging activity were first discovered by using nematodes as model organisms. Since the first use of nematodes by Brenner as a tool in genetics research (Brenner, 1974), the model has been applied to many other research fields, such as development, disease modeling, metabolism, medicine, screening, and others. We also took advantages of this model organism in aging and signal transduction (Zheng et al., 2018; Qu et al., 2020b). Since two American scientists, Friedman and Johnson, discovered in the 1980s that the mutation of a single gene in nematodes can increase lifespan (Johnson and Wood, 1982; Friedman and Johnson, 1988), the genetic control of aging has rapidly developed. It is reported that aging and aging-related diseases are controlled by signaling pathways, such as autophagy-related target of rapamycin (TOR) signaling pathway (McCormick et al., 2011; Laplante and Sabatini, 2012), insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway (Barbieri et al., 2003; Lapierre and Hansen, 2012), mitochondrial-related functional signaling pathway (Sohal and Orr, 2012), and adenosine monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway related to cell energy homeostasis (Salminen and Kaarniranta, 2012; Qu et al., 2020a).

POLYPHENOLS

Polyphenols are the most widely distributed group of phytochemicals (**Table 1**). They are usually classified into flavonoids, phenolic acids, and non-flavonoids. Flavonoids are subdivided into flavonols, flavanones, isoflavones, anthocyanins, and flavan-3-ols according to their chemical structure. Phenolic acids are subdivided into hydroxybenzoic acid and hydroxycinnamic acid. Non-flavonoids are subdivided into lignans, stilbenes, and tannins (Papaevgeniou and Chondrogianni, 2018; Fraga et al., 2019; Majidinia et al., 2019). The category is illustrated in **Figure 1**.

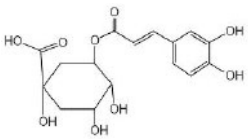
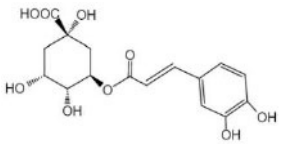
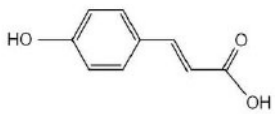
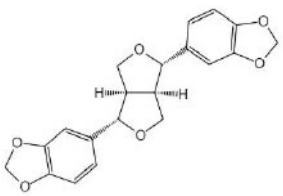
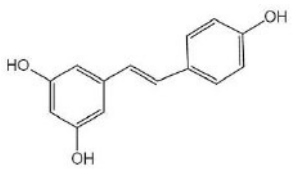
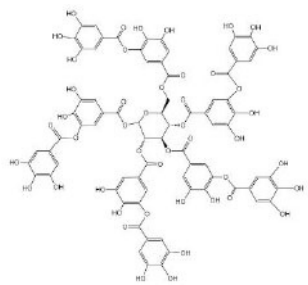
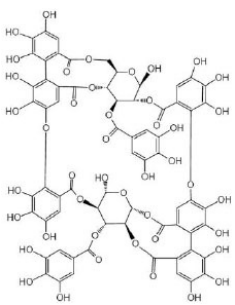
Polyphenols exert beneficial effects on health, owing to their anti-oxidant and anti-inflammatory activities, and they have been commonly employed to treat cancer, autoimmune diseases, type 2 diabetes, cardiovascular disorders, and other diseases. The structural characteristics of the carbocyclic ring of polyphenols and the number of hydroxyl groups on the ring are the main prerequisites for prolonging lifespan (Grunz et al., 2012). In this article, we reviewed literature regarding the anti-aging properties of each specific polyphenol. Different classes of chemicals might activate similar signaling pathways involved in aging processes, and one class of chemicals might be involved in multiple pathways. For example, it was reported that resveratrol can extend *C. elegans* lifespan through the MPK-1/ERK or SIR-2.1/DAF-16 pathway (Yoon et al., 2019). In addition, many kinds of polyphenols can modulate longevity through the IIS pathway, especially through the key transcription factor DAF-16 in the pathway, for example, myricetin (Buchter et al., 2013), blueberry extract (Wang et al., 2018), echinacoside (Wang et al., 2015), and

TABLE 1 | Modulation of the lifespan in *Caenorhabditis elegans* by polyphenols.

Compound	Chemical Structure	Source	Pathway/regulator
Flavonols			
Quercetin		Quercetin was purchased from Sigma-Aldrich (Deisenhofen, Germany) (Kampkotter et al., 2008; Pietsch et al., 2009) Quercetin was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) (Sugawara and Sakamoto, 2020)	IIS/DAF-2/AGE-1, MAPK/UNC-43/SEK-1, DAF-16, HSF-1, SKN-1
Myricetin		Myricetin was purchased from Sigma-Aldrich (Deisenhofen, Germany) (Grunz et al., 2012). Myricetin was obtained from Extrasynthese, Genay, France (Buchter et al., 2013) LC–MS/MS[A] proved that the ethyl acetate fraction of *Eugenia uniflora* is rich in myricetin (Sobeh et al., 2020)	DAF-16
Baicalein		Baicalein (≥98%) was obtained from Sigma-Aldrich (Deisenhofen, Germany) (Havermann et al., 2013, 2016)	SKN-1
Anthocyanins		Anthocyanin contents of the purple wheat and acai extracts were characterized and verified by HPLC–UV/vis[B] (Chen et al., 2013; Peixoto et al., 2016). Anthocyanins extract from bilberry fruit were analyzed by HPLC-DAD[C] and ESI-MS[D] (Gonzalez-Paramas et al., 2020). The purple pitanga extract is rich in anthocyanin, mainly in cyanindin-3-O-glucoside (Tambara et al., 2018) Previous HPLC-MS[E] results proved that the extract of tart cherry (TCE) was rich in anthocyanins, and the concentration of TCE was determined according to the concentration of anthocyanins in a previous experiment (Jayarathne et al., 2020) Mulberry anthocyanins were conducted by HPLC[F] (Yan et al., 2017)	DAF-16, AAK-2, SKN-1, PMK-1
Flavan-3-ols			
Catechin acid		Catechin acid (96%, HPLC) was obtained from Sigma-Aldrich (St Louis, MO, USA) (Wu et al., 2020)	BEC-1, PINK-1
Epigallocatechin-3-gallate (EGCG)		EGCG were obtained from Sigma-Aldrich (St. Louis, MO, USA) (Brown et al., 2006; Zhang et al., 2009; Bartholome et al., 2010; Xiong et al., 2018)	AAK-2, SIR-2.1, DAF-16
Hydroxybenzoic acid			
4-Hydroxybenzoic acid		4-Hydroxybenzoic acid with purity >98% from extract of *Veronica peregrina* was verified by HPLC (Kim et al., 2014)	SIR-2.1, DAF-16
Aspirin		Aspirin was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Huang et al., 2017)	DAF-12, DAF-16

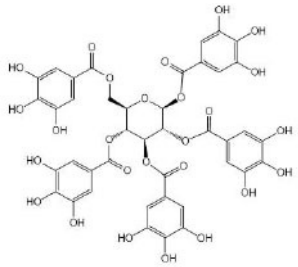
(Continued)

TABLE 1 | Continued

Compound	Chemical Structure	Source	Pathway/regulator
Hydroxycinnamic acid			
Chlorogenic acids (CGA)		NMR[G] and UPLC/ESI-HRMS[H] analyses of green coffee extract confirmed a significant content of CGA (Amigoni et al., 2017) CGA was purchased from Adamas (Basel, Switzerland) (Zheng et al., 2017)	IIS/AKT/DAF-16
5-O-caffeoylquinic acid (5-CQA)		5-CQA is the isomer with the highest content of chlorogenic acid in green coffee extract (Amigoni et al., 2017) 5-CQA were purchased from Aladdin (Shanghai, China) (Zheng et al., 2017)	IIS/AKT/DAF-16
p-Coumaric acid		*p*-Coumaric acid (≥98% pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Yue et al., 2019)	SKN-1, OSR-1
Lignans			
Sesamin		Sesamin was purchased from Wako (Osaka, Japan). γ-Cyclodextrin (γCD) was obtained from Cyclochem (Kobe, Japan) (Yaguchi et al., 2014; Nakatani et al., 2018)	IIS/DAF-2/DAF-16, MAPK/PMK-1/SKN-1, TOR/DAF-15, SIR-2.1, AAK-2, BEC-1
Stilbenes			
Resveratrol		Resveratrol was from purchased from Sigma-Aldrich (St. Louis, MO, USA) (Morselli et al., 2010; Lee et al., 2016; Yoon et al., 2019)	SIR-2.1, DAF-16, AAK-2, MPK-1, BEC-1
Tannins			
Tannic acid		Tannic acid was purchased from Sigma-Aldrich (Taufkirchen, Germany) (Saul et al., 2010, 2011)	SEK-1, *eat-2*
Oenothein B (OEB)		OEB was isolated and purified from eucalyptus leaves, and the isolated and purified OEB was confirmed by HPLC and ^{1}H NMR[I] (Chen et al., 2020)	IIS/AGE-1/DAF-16, SIR-2.1, *eat-2, isp-1*

(Continued)

TABLE 1 | Continued

Compound	Chemical Structure	Source	Pathway/regulator
Pentagalloyl glucose (PGG)		PGG was isolated and purified from eucalyptus leaves and was confirmed by HPLC and MS[J] (Chen et al., 2014)	IIS/AGE-1/DAF-16, SIR-2.1, *eat-2, isp-1*

A: LC-MS/MS, liquid chromatograph-mass spectrometer; B: HPLC–UV/vis, high-performance liquid chromatography–ultraviolet/visible spectroscopy; C: HPLC-DAD, high-performance liquid chromatography-diode array detector; D: ESI-MS, electrospray ionization mass spectrometry; E: HPLC-MS, high-performance liquid chromatography-mass spectrometry; F: HPLC, high-performance liquid chromatography; G: NMR, nuclear magnetic resonance spectroscopy; H: UPLC/ESI-HRMS, hybrid quadrupole-orbitrap mass spectrometry; I: ^1H NMR, ^1H nuclear magnetic resonance spectroscopy; J: MS, mass spectrometer.

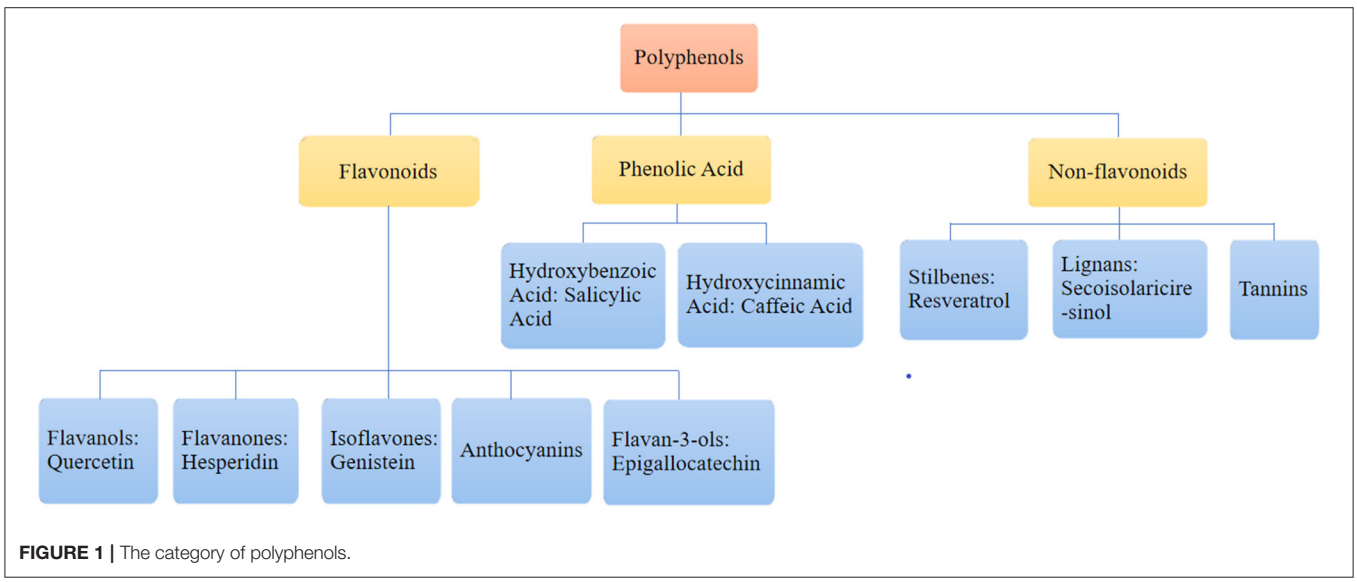


FIGURE 1 | The category of polyphenols.

others. The main reason for this effect might be that the DAF-16 expression increases the ability to scavenge free radicals and resist oxidative stress.

FLAVONOLS

Bioactive phytochemicals, such as flavonols, are abundant in fruits and vegetables, such as onions, peppers, cauliflower, and grapes. The most common flavonol is quercetin, and other common flavonols are kaempferol, myricetin, isorhamnetin, tamarixetin, morin, fisetin, apigenin, and luteolin (Adebamowo et al., 2005; Perez-Vizcaino and Duarte, 2010). **Figure 2** illustrates the model of how flavonols are involved in lifespan regulation.

Quercetin, as a strong anti-oxidant, has been demonstrated to have a positive effect on longevity and stress resistance in various animal models, and its activity and mechanism have also been studied in nematodes (Pietsch et al., 2012; Proshkina et al., 2016). Several studies have confirmed that quercetin accumulates in nematodes and exhibits reactive oxygen species (ROS) scavenging activity, which might be the reason for its beneficial health effects, and this process is regulated by the transcription factor DAF-16 (Kampkotter et al., 2008; Sugawara and Sakamoto, 2020). The *C. elegans* gene, *daf-16*, is homologous to the mammalian gene for the FOXO transcription factor, which plays a key role in controlling several stress response signaling cascades, aging processes, and other important biological functions, and it is also considered as an important downstream factor of the IIS pathway, which is one of the main pathways that regulate the lifespan of nematodes. It starts from the DAF-2 insulin receptor, and it is also the ortholog of the insulin/IGF-1 receptor in *C. elegans*, through AGE-1/PI3K to AKT-1/2 and then to the downstream target DAF-16/FOXO transcription factor, to control the lifespan and metabolism of *C. elegans*. However, the conclusion is contrary. Some reports suggested

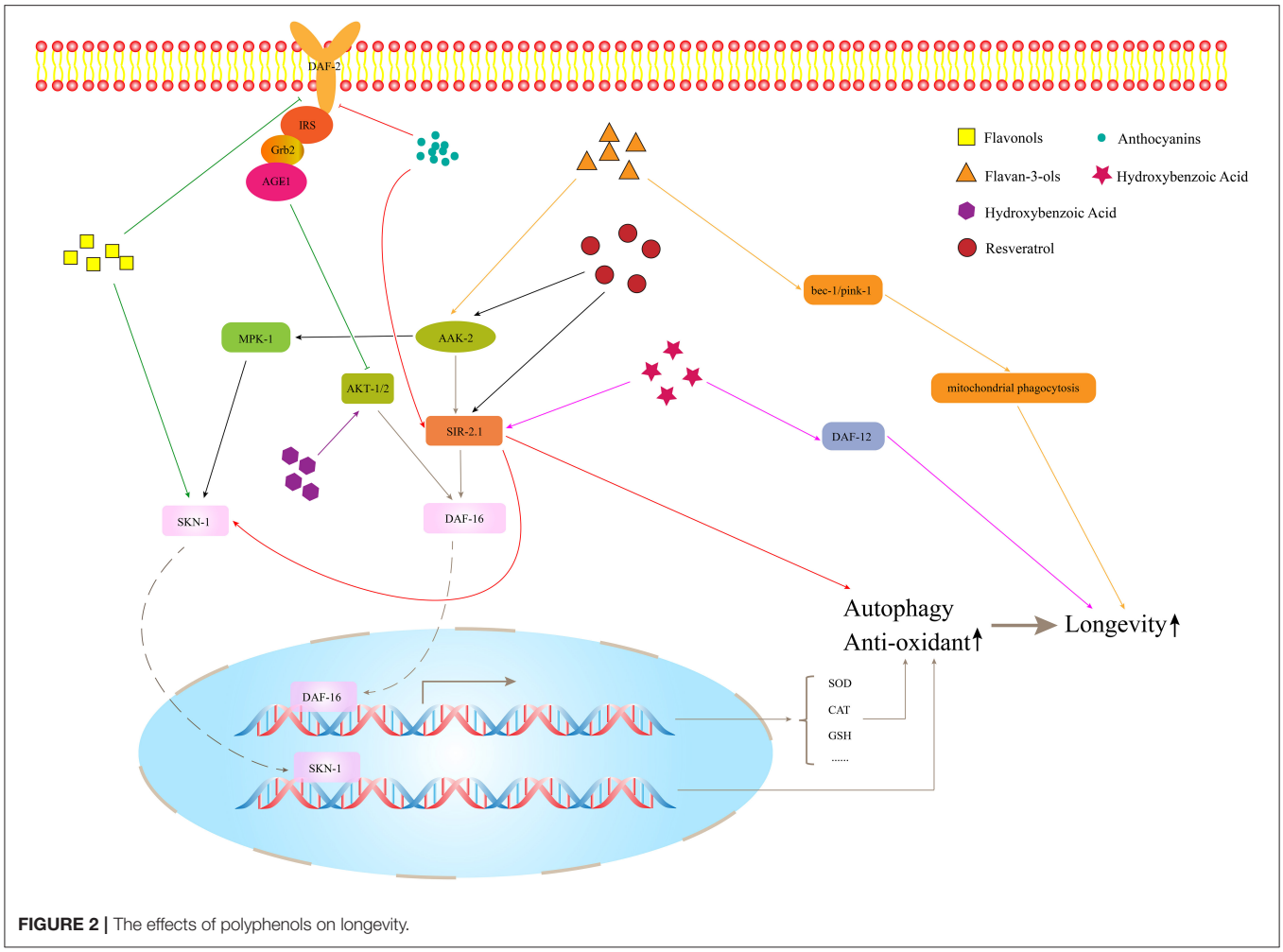


FIGURE 2 | The effects of polyphenols on longevity.

that, although DAF-2 and other components of the IIS pathway mediate the anti-oxidant activity and life-prolonging effects of quercetin on nematodes, these effects seem to be independent of DAF-16 (Pietsch et al., 2009). Hence, the role of DAF-16 in quercetin-induced health effects requires further investigation. Besides DAF-16, SKN-1 and mitogen-activated protein kinase (MAPK) pathways are also involved in the process of scavenging ROS, lifespan extension, and health improvement in *C. elegans*. Quercetin also induces heat resistance by co-activating the expression and/or activity of HSF-1 and DAF-16 (Sugawara and Sakamoto, 2020). HSF orthologous, HSF-1, as a transcriptional regulator of stress-induced gene expression in worms, induces the expression of molecular chaperones. UNC-43 and SEK-1 seem to be parts of the lifespan regulation of quercetin (Pietsch et al., 2009). SEK-1 is an indispensable MAPK in innate immunity, and UNC-43 is a part of the SEK-1 upstream neuron regulation signal pathway. These two regulators belong to the MAPK pathway, a major immune signaling pathway (Troemel et al., 2006). UNC-43 is also a type II Ca^{2+}/calmodulin-dependent kinase (CAMKII) that can also regulate osmotic pressure. Therefore, quercetin can be considered a multitarget nutrient.

In addition, myricetin was first discovered by Spanier for its activation of DAF-16 and its increase in the expression of its downstream gene *sod-3*, but it was found that the activation of DAF-16 was not the cause of the extended lifespan because it was found that DAF-16 activation was not correlated with the myricetin-mediated decrease in mitochondrial ROS and the increase in longevity (Grunz et al., 2012). However, further experiments proved that myricetin does exert its anti-oxidant effect through DAF-16 (Buchter et al., 2013; Sobeh et al., 2020). All these studies found that, in DAF-16 mutants, the ROS-scavenging effect of myricetin was blocked to a great extent, and the beneficial effect on lifespan also disappeared completely. This indicates that, although myricetin is a strong anti-oxidant, its effect on the lifespan of *C. elegans* is heavily dependent on DAF-16 rather than its direct anti-oxidant capacity. At present, the study of myricetin on the life extension of nematodes is limited to its regulation of the IIS pathway. In the future, additional pathways should be further analyzed in this respect, and other mechanisms for myricetin-mediating health effects should be investigated (Buchter et al., 2013).

Baicalein mainly comes from Huangqin, which is one of the commonly used traditional Chinese medicines. It has been

demonstrated that baicalein mediates anti-oxidant effects by activating nuclear factor erythroid 2-related factor 2 (Nrf2) in mammalian cell lines. When it comes to *C. elegans*, SKN-1 is the homologous gene of the mammalian transcription factor Nrf2 (An and Blackwell, 2003). Similar to Nrf2, SKN-1 can also be activated by oxidative stress or exogenous bioactive substances; then, it can be transferred to the nucleus and combined with anti-oxidant response elements (AREs) of various anti-oxidant or protective gene promoter regions. This pathway can induce a variety of anti-oxidant enzymes as the key defense mechanism against oxidative stress. The lifespan of *skn-1* mutant is shortened, and the resistance to oxidative stress is reduced. SKN-1 is the direct target of the IIS pathway and MAPK pathway and has some common downstream targets with DAF-16. It is also required for dietary restriction (DR)-induced longevity because it interacts with amino acid and lipid metabolism during starvation (Dall and Faergeman, 2019). It has been reported that baicalein can modulate the lifespan and stress resistance of nematodes *via* SKN-1, but not DAF-16, a result similar to what was obtained in mammalian cell lines (Havermann et al., 2013, 2016).

ANTHOCYANINS

Anthocyanins are found in a wide variety of colored vegetables, fruits, and cereal, especially in various berry fruits, such as bilberries, blueberries, blackberries, blackcurrants, chokeberries, strawberries, and elderberries (Chen et al., 2013; Wallace and Giusti, 2015; Yan et al., 2017). Many studies have focused on the anti-oxidant capacity of different plant extracts rich with anthocyanins. The vast majority of plant extracts rich in anthocyanins, such as extracts of purple wheat (Chen et al., 2013), acai berry (Peixoto et al., 2016), mulberry (Yan et al., 2017), purple pitanga fruit (Tambara et al., 2018), tart cherry (Jayarathne et al., 2020), and bilberry (Gonzalez-Paramas et al., 2020), which can play their beneficial role by increasing nuclear translocation of DAF-16 and promoting the expression of anti-oxidant genes, such as *sod-3*, and the heat shock gene, *hsp-16.2*, in its downstream. Heat shock proteins (HSPs) are molecular chaperones and play important roles in the protection of molecular damage under environmental stress and have the ability to maintain proteostasis and prolong the longevity of organisms (Swindell, 2009). HSP-16.2 family is expressed under stress conditions and can be considered as stress-sensitive reporters to evaluate lifespan (Strayer et al., 2003). DAF-16 is a key protein for the anti-aging effects. Mechanistically, recent studies have found that anthocyanins could regulate the AAK-2/AMPK signaling pathway to perform its biological function (Jayarathne et al., 2020). *aak-2* is the encoding gene of AMPK in nematodes. AMPK is a regulator of cellular energy homeostasis, which is essential for the metabolic regulation of nematodes during starvation and diapause (Demoinet and Roy, 2018), it can be activated under low energy conditions and can maintain the steady state of energy, linking nutritional availability with longevity (Tullet, 2015). Overexpression of AAK-2 in nematodes prolongs its lifespan, and this effect also requires the downregulation of the IIS pathway and the upregulation and transposition of DAF-16 (Zhao et al., 2017). Additionally, mulberry anthocyanins can also activate transcription factors SKN-1/Nrf2 and PMK-1/MAPK and their downstream targets that are related to oxidative stress (Yan et al., 2017).

FLAVAN-3-OLS

Flavan-3-ols include catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin-3-gallate, epigallocatechin-3-gallate (EGCG), theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3′-digallate, and thearubigins. Flavan-3-ols are mainly found in tea, apples, wine, and cocoa (Lei et al., 2016). **Figure 2** shows the effect of flavan-3-ols on the lifespan of nematode. There are many types of flavan-3-ols, but the current research has focused mostly on tea extracts and the certain classes of flavan-3-ols, such as catechinic acid (CA) and EGCG. Xiong et al. (2014) found that the black tea extracts contain a variety of flavan-3-ols, which can increase the lifespan of *C. elegans* under stress conditions, such as osmotic pressure imbalance, ultraviolet radiation, and heat stress. This effect might be mediated by the SEK-1 signaling and SIR-2.1/DAF-16/SOD-3 pathways, which could increase stress resistance. Worms simultaneously treated with catechins-rich green tea aqueous extract (GTE) and a lethal dose of pro-oxidant, i.e., juglone, showed a decreased expression of *hsp-16.2* and a significantly increased survival rate as compared with worms not receiving GTE. It suggested that GTE could enhance the anti-stress ability of nematodes and reduce oxidative damage *in vivo* (Abbas and Wink, 2014). In addition, it was found that CA, as a natural polyphenol compound, extended the lifespan and declined the age-related behaviors of *C. elegans* by regulating the mitophagy pathway related to genes of *bec-1* and *pink-1*. It was found that it can act in the role of inducing mitochondrial phagocytosis at the early stage, which was also a key period to affect the lifespan (Wu et al., 2020). Mitophagy can prevent the accumulation of dysfunctional mitochondria and prolong lifespan. EGCG is another widely studied flavane. The regulation of EGCG on the lifespan of nematodes is affected by the concentration. The effect on the organism can be described as a hormetic effect; in other words, stimulatory and inhibitory effects would be generated in low and high doses. The health effects of EGCG depend on the hormetic effect. It was found that, when the concentration was $<25\,\mu M$, it could prolong the lifespan of nematodes under the stress and improve their stress ability and the partial decline of age-related physiological behavior, but it was not enough to affect the lifespan of worms under normal conditions (Brown et al., 2006; Zhang et al., 2009). When the concentration is above $800\,\mu M$, it might produce toxic effects (Xiong et al., 2018). At a suitable concentration, EGCG induced ROS in a time-resolved manner, which can temporarily increase ROS level in the early stage and activate AAK-2/AMPK, change the metabolism of NAD^+, and then increase the expression of its downstream target protein SIR-2.1. Previous studies have found that EGCG can increase the nuclear translocation and expression of DAF-16

and activate its downstream antioxidant genes (Zhang et al., 2009; Bartholome et al., 2010). However, the upstream regulation mechanism has not been further studied. Currently, it was found that EGCG acted on SIR-2.1 instead of the IIS pathway to regulate DAF-16 (Xiong et al., 2018). Besides, EGCG can mainly restore the mitochondrial function and increase the biogenesis of early-to-mid adult worms, thus improving the redox steady state of worms. The EGCG-induced longevity of nematodes also depends on the mitochondrial function. The health effect would decrease gradually with age increases (Xiong et al., 2018). Sirtuin of *C. elegans* is the closest homolog to human SIRT1, which is encoded by the gene *sir-2.1*. It is also a conservative transcription regulator. As an NAD^+-dependent histone deacetylase, the overexpression of sirtuin can prolong the lifespan of many species (Smith et al., 2014; Seo et al., 2015). Sirtuin can directly activate DAF-16/FOXO by deacetylation, which affects the lifespan independently of IIS (Kenyon, 2010). It can also induce autophagy by upregulating the autophagy gene and inhibiting the TOR signal together with AMPK (Ruderman et al., 2010). In addition, the anti-oxidant mechanism is activating SKN-1 and regulating lifespan through the pathway, partially overlapping with DR (Jung et al., 2017). DR is one of the most influential environmental interventions for prolonging the lifespan and healthspan of many species.

HYDROXYBENZOIC ACID

Hydroxybenzoic acid is widely distributed in vegetables and fruits and can be synthesized from polyphenols by gut bacteria. It has been confirmed to activate Nrf2 (Juurlink et al., 2014), suggesting that it might have the anti-aging effects on nematodes *via* the Nrf2 signaling pathway. Furthermore, 4-hydroxybenzoic acid could extend the lifespan of nematodes through the activation of DAF-16/FOXO mediated by SIR-2.1/SIR-2, which demonstrated no relationship with DR and IIS pathway. It can also increase the stress resistance under osmotic, heat, and oxidative stress conditions (Kim et al., 2014). By bioinformatics analysis, aspirin was found to alter the expression of genes, which are involved in fat metabolisms, such as *acs-2*, *ech-1.2*, and *cpt-5*, which can extend the longevity of *C. elegans* through the activation of DAF-12 and DAF-16 (Huang et al., 2017). DAF-12 is a nuclear hormone receptor that can be initiated by insulin/IGF-1 and TGF-β and plays an important role in metabolism, longevity, and reproductive development in *C. elegans*. As is known to all, the primary component of aspirin is salicylic acid. As an isomer of hydroxybenzoic acid, it suggests that hydroxybenzoic acid might influence the expression of genes involved in anti-oxidation and fat metabolism.

HYDROXYCINNAMIC ACID

Hydroxycinnamic acid and its derivative, caffeic acid, are abundant in tea leaves, red wine, and coffee. It was reported that extracts of green coffee beans (GCEs), which are composed mainly of chlorogenic acid (CGA) and its derivative, 5-caffeoylquinic acid (5-CQA), have beneficial effects on longevity and reproduction in *C. elegans*. The study also indicated that, compared with CGEs rich in pure 5-CQA, the CGEs rich in 5-CQA have a stronger anti-aging effect, which strongly supports that it might be a better choice to use the mixture of bioactive compounds instead of just one single bioactive molecule (Amigoni et al., 2017). At the same time, it was also found that CGA and its isomers, such as 5-CQA and 4-caffeoylquinic acid (4-CQA), acted on the upstream of AKT in the IIS pathway and then exerted their life-prolonging and anti-aging effects mainly *via* DAF-16 and its downstream stress factors, HSF-1, SKN-1, and HIF-1 (Zheng et al., 2017). Moreover, *p*-coumaric acid, another derivative of hydroxycinnamic acid, can enhance the ability to resist SKN-1-mediated oxidative stress and OSR-1-mediated osmotic stress (OSR-1 can negatively regulate the activity of the MAPK pathway) (Yue et al., 2019).

LIGNANS

Six lignans were isolated from *Arctium lappa* seeds, and all of them were found to have anti-aging properties and to upregulate the expression of *daf-16* and *jnk-1* (Su and Wink, 2015). *jnk-1* is considered as a positive regulator of *daf-16*, which indicates that lignans have the life-promoting activity through the JNK-1/DAF-16 cascade. Sesamin is a major lignan constituent of sesame and possesses various health-promoting effects. That sesamin could not only prolong the life of nematodes but could also reduce the toxicity of Alzheimer's disease (AD) β-amyloid (Aβ) plaque (Keowkase et al., 2018). It was also found that the resistance of nematodes to physical stress and some pathogenic bacteria could not be enhanced by sesamin, but it could protect nematodes from oxidative stress caused by toxins, which is partly due to the indirect hormetic effect of sesamin. Besides, it was found that sesamin could play the anti-aging role *via* the genes constituting the IIS pathway (*daf-2* and *daf-16*) and MAPK pathway (*pmk-1* and *skn-1*) (Yaguchi et al., 2014). PMK-1 is a kinase that plays an important role in immune defense and longevity in the MAPK pathway. In addition, sesamin can also act as a mimic of DR. Sesamin depends on SIR-2.1/SIRT1, AAK-2/AMPK, an autophagic modulator BEC-1, and *daf-15*, which encodes the target of TOR-binding partner raptor, to promote longevity (Yaguchi et al., 2014; Nakatani et al., 2018). The inhibition of the TOR pathway is another well-known intervention method to prolong lifespan. DR might induce autophagy and activate DAF-16 by inhibiting TOR kinase (Cypser et al., 2013). BEC-1 is necessary for the longevity induced by overexpression of *sir-2.1*. SIRT1, TOR, and AMPK are currently known as signaling pathways associated with DR. Unlike other DR analogs, sesamin is likely to be involved in almost all of the known DR-related pathways, which can prolong lifespan.

Another lignan, i.e., pinoresinol has been observed to increase the nuclear translocation of DAF-16, but it has no effect on the longevity of nematodes and has no regulating ability to the stress resistance and oxidation resistance. Although it shows strong

oxidation resistance *in vitro*, its functional effects in organisms need further study at a molecular level (Koch et al., 2015).

STILBENES

The most important representative of stilbene compounds is resveratrol, which is mainly derived from grape skins, grape seeds, red wine (Salehi et al., 2018), blueberries, peanuts, and some traditional Chinese herbal medicines, such as rhubarb (Malaguarnera, 2019), and polygonum cuspidatum (Zhang, 2006). Resveratrol is usually recommended as a dietary supplement to maintain redox balance and to delay aging (Desjardins et al., 2017).

The activation of sirtuins is considered to be an important mechanism of resveratrol-mediated longevity. Research found that resveratrol can activate SIR-2.1 and then prolong the life of nematodes by regulating *bec-1* to induce autophagy (Morselli et al., 2010). Lee et al. (2016) found that resveratrol does not need to exert its health effects through DAF-16 after activating SIR-2.1, which indicates that there might be other regulatory pathways downstream of SIR-2.1, whereas Yoon et al. (2019) found that SIR-2.1 relies on DAF-16 for its function, so the role of DAF-16 in resveratrol-induced longevity needs further study. As research progresses, scientists have learned more about the mechanism of how resveratrol works to delay aging. The effect of resveratrol on life extension might not work entirely in a sirtuin-dependent way. As a DR analog, resveratrol can prolong lifespan through AAK-2, a key factor in the AMPK pathway, and without the association of DAF-16. Similar to SIR-2.1, MPK-1 is also one of the key regulators for lifespan extension (Yoon et al., 2019). However, its contribution to resveratrol-mediated life extension is completely independent of SIR-2.1, and they have different downstream regulatory genes. MPK-1 is also known as human ERK homo that acts by promoting downstream SKN-1 nuclear translocation and is first identified as a longevity factor (Okuyama et al., 2010). Resveratrol can alleviate the damage caused by ROS and prolong the life of nematodes under pressure (Chen et al., 2013). In addition, the two newly synthesized resveratrol derivatives have stronger biological and anti-oxidant activity than resveratrol. Their strong anti-oxidant ability can also regulate DAF-16, SKN-1, SIR-2.1 in the redox activity signal pathway (Fischer et al., 2017).

TANNINS

Tannic acid (TA) belongs to the hydrolyzable tannins, containing five digallic acid residues covalently linked to a central glucose molecule, and it can precipitate protein. As a strong anti-oxidant, the observed increase in heat stress resistance and oxidative stress resistance is not due to its ability to directly remove oxygen free radicals but its ability to act as a stimulus to activate the anti-oxidant system of the body (Saul et al., 2010, 2011). Studies have shown that a low concentration of TA might simulate mild pathogenic stress, strengthen the SEK-1-based pathogen defense system, inhibit the potentially harmful effects of TA, and induce nematodes to prolong their life effectively (Saul et al., 2010). In addition, TA itself does not reduce the food intake of nematodes, but exerts the molecular regulation through *eat-2* in the DR pathway or precipitates and combines nutritional proteins and digestive enzymes (*eat-2* mutant suffered from insufficient food intake due to decreased pharyngeal pumping). It is worth noting that the concentration range of health effects of TA is relatively narrow, so it is also very important to find a suitable concentration to treat *C. elegans*. Unlike TA, ellagic acid (EA) can be used as a chemical repellent to reduce the feeding behavior of nematodes and prolong the lifespan of nematodes by its strong antibacterial ability (Saul et al., 2011). Oenothein B (Chen et al., 2020) and pentagalloyl glucose (Chen et al., 2014) extracted from eucalyptus leaves can prolong healthy life by regulating multiple targets. They can regulate the IIS pathway *via age-1* and *daf-16*, the DR pathway *via eat-2* and *sir-2.1*, and mitochondrial electron transfer chain *via isp-1* to promote healthy life, including reducing age pigment and ROS accumulation and improving exercise flexibility, heat stress tolerance, and lifespan. *isp-1* is one of the genes encoding mitochondrial electron transport chain components, and the deletion of *isp-1* exists in the respiratory chain complex III. Their mechanism of action might be the same because of their similar structure.

CONCLUSIONS

Before studying the signaling pathway implicated in a certain disease in a model organism, some researchers first test this pathway in suitable cell lines. For example, resveratrol was found in the generation of different effects, such as promoting proliferation in mesenchymal stem cells, with possible involvement of the ERK/GSK-3β pathway (Yoon et al., 2015). Nematodes were further utilized to test the MPK-1 (an ERK homolog) signaling (Yoon et al., 2019). When we treat nematodes with plant extracts, we can first analyze each extract component using mass spectrometry, high-performance liquid chromatography, or similar methods; this analysis might help to identify the key components responsible for the biological effects.

At present, researchers rarely treat *C. elegans* with polyphenols, such as flavanones and isoflavones; therefore, it is unknown whether or not these polyphenols have direct effects on *C. elegans*. However, many studies demonstrated that the abovementioned polyphenols can act on homologous genes of *C. elegans* in other species and that these genes are involved in aging and regulation of lifespan. Flavanones, of which the representative molecule is hesperidin, are mainly found in citrus plants and have been demonstrated to be able to reduce oxidative stress caused by a high-fat diet in mice and to slow down the aging process in old-aged rats. Some studies found that one of the targets of flavanones in animals is Nrf2, whereas *C. elegans* has an Nrf2-homolog gene, SKN-1 (Ferreira et al., 2016; Barreca et al., 2017; Habtemariam, 2019; Miler et al., 2020). Isoflavones, such as genistein and daidzein, are generally regarded as phytoestrogens; there is evidence that Nrf2 is also one of the downstream targets of isoflavones and that it can

also regulate fat metabolism in rats with diet-induced obesity, acting through the AMPK pathway (Li and Zhang, 2017; Krizova et al., 2019). In conclusion, further studies should be conducted to test whether these polyphenols have beneficial effects on *C. elegans*.

PROSPECTS

To the best of our knowledge, in the study of total polyphenols in plants, especially medicinal plants, their functions, the bioactive substances, and molecular mechanisms for prolonging lifespan and delaying senescence have not received enough attention. For example, mulberry leaves have been widely used in traditional Chinese medicine and folk dietary therapy for their outstanding effects of detoxifying the liver, improving eyesight, and prolonging life. It is believed that mulberry leaf extracts used in traditional Chinese medicine have anti-oxidant and hepatoprotective effects, and those two activities are related to mitochondria function (Meng et al., 2020). Liver tissue contains a large number of mitochondria, and the fatty acids are activated into ester-acyl-coenzyme A, which is metabolized by β oxidation in mitochondria. Acetyl-coenzyme A and fat synthetases required for fatty acid synthesis come from mitochondria. At present, mulberry leaf extract has been confirmed to have beneficial effects on several diseases, such as cancer, type 2 diabetes, and obesity. In addition, modern medicine experiments have proved that mulberry leaf extract can delay aging in mice (Lim et al., 2013; Turgut et al., 2016). Mulberry leaf extract is an effective and natural free-radical scavenger and anti-oxidant, but the research on mulberry leaf extract and mulberry leaf polyphenol is limited to its anti-oxidant activity *in vitro*, and its specific mechanisms of action have not been elaborated. Moreover, the activities of mulberry leaf polyphenols have not yet been associated with any specific physiological functions. Besides, nematodes being treated by the combination of two extracts from different plants revealed stronger effects than the treatment with only either of the single extract. A recent study shows that nematodes treated with the mixtures of blueberry and apple peel extracts have a longer lifespan than those treated with only one substance (Song et al., 2020a,b). Could mulberry leaf extract exert the effects observed in traditional Chinese medicine by regulating fat metabolism? What are the specific anti-aging mechanisms of mulberry leaves in *C. elegans*? Could the combinations of mulberry leaves, polyphenols, and other polyphenols, or other bioactive substances play their beneficial roles more significantly, and what are their mechanisms? We would like to answer these questions by conducting further research.

AUTHOR CONTRIBUTIONS

ZQ and NL conceived the idea and wrote the manuscript with input from LL, PG, and SZ. PG and PW prepared the figures. LL, PW, and SZ prepared the tables. All authors edited and approved the final manuscript.

FUNDING

This review was supported by the National Natural Science Foundation of China (Nos. 81872584 and 81472941), the National 863 Young Scientist Program (No. 2015AA020940), the Natural Science Foundation of Guangdong Province (No. 2016A030313138), Key R & D and Promotion Project of Henan Province (No. 192102310259), Key Scientific Research Project of Henan Province (No. 21A330001), the Key Projects of Guangzhou Science and Technology Program (No. 201704020056), Interdisciplinary Research for First-Class Discipline Construction Project of Henan University (No. 2019YLXKJC04), the Scientific Research Project for University of Education Bureau of Guangzhou (No. 201831841), and the Yellow River Scholar Foundation of Henan University.

REFERENCES

Abbas, S., and Wink, M. (2014). Green tea extract induces the resistance of caenorhabditis elegans against oxidative stress. *Antioxidants* 3, 129–143. doi: 10.3390/antiox3010129

Adachi, H., and Ishii, N. (2000). Effects of tocotrienols on life span and protein carbonylation in *Caenorhabditis elegans*. *J. Gerontol. A Biol. Sci. Med. Sci.* 55, B280–285. doi: 10.1093/gerona/55.6.B280

Adebamowo, C. A., Cho, E., Sampson, L., Katan, M. B., Spiegelman, D., Willett, W. C., et al. (2005). Dietary flavonols and flavonol-rich foods intake and the risk of breast cancer. *Int. J. Cancer* 114, 628–633. doi: 10.1002/ijc.20741

Amigoni, L., Stuknyte, M., Ciaramelli, C., Magoni, C., Bruni, I., De Noni, I., et al. (2017). Green coffee extract enhances oxidative stress resistance and delays aging in *Caenorhabditis elegans*. *J. Funct. Foods* 33, 297–306. doi: 10.1016/j.jff.2017.03.056

Amor, C., Feucht, J., Leibold, J., Ho, Y. J., Zhu, C., Alonso-Curbelo, D., et al. (2020). Senolytic CAR T cells reverse senescence-associated pathologies. *Nature* 583, 127–132. doi: 10.1038/s41586-020-2403-9

An, J. H., and Blackwell, T. K. (2003). SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. *Genes Dev.* 17, 1882–1893. doi: 10.1101/gad.1107803

Barbieri, M., Bonafe, M., Franceschi, C., and Paolisso, G. (2003). Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. *Am. J. Physiol. Endocrinol. Metab.* 285, E1064–E1071. doi: 10.1152/ajpendo.00296.2003

Barreca, D., Gattuso, G., Bellocco, E., Calderaro, A., Trombetta, D., Smeriglio, A., et al. (2017). Flavanones: Citrus phytochemical with health-promoting properties. *Biofactors* 43, 495–506. doi: 10.1002/biof.1363

Bartholome, A., Kampkotter, A., Tanner, S., Sies, H., and Klotz, L. O. (2010). Epigallocatechin gallate-induced modulation of FoxO signaling in mammalian cells and C. elegans: FoxO stimulation is masked via PI3K/Akt activation by hydrogen peroxide formed in cell culture. *Arch. Biochem. Biophys.* 501, 58–64. doi: 10.1016/j.abb.2010.05.024

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94. doi: 10.1093/genetics/77.1.71

Brown, M. K., Evans, J. L., and Luo, Y. (2006). Beneficial effects of natural antioxidants EGCG and alpha-lipoic acid on life span and age-dependent behavioral declines in *Caenorhabditis elegans*. *Pharmacol. Biochem. Behav.* 85, 620–628. doi: 10.1016/j.pbb.2006.10.017

Buchter, C., Ackermann, D., Havermann, S., Honnen, S., Chovolou, Y., Fritz, G., et al. (2013). Myricetin-mediated lifespan extension in *Caenorhabditis elegans* is modulated by DAF-16. *Int. J. Mol. Sci.* 14, 11895–11914. doi: 10.3390/ijms140611895

Chen, W., Muller, D., Richling, E., and Wink, M. (2013). Anthocyanin-rich purple wheat prolongs the life span of *Caenorhabditis elegans* probably by activating the DAF-16/FOXO transcription factor. *J. Agric. Food Chem.* 61, 3047–3053. doi: 10.1021/jf3054643

Chen, X., Barclay, J. W., Burgoyne, R. D., and Morgan, A. (2015). Using *C. elegans* to discover therapeutic compounds for ageing-associated neurodegenerative diseases. *Chem. Cent. J.* 9:65. doi: 10.1186/s13065-015-0143-y

Chen, Y., Onken, B., Chen, H., Xiao, S., Liu, X., Driscoll, M., et al. (2014). Mechanism of longevity extension of *Caenorhabditis elegans* induced by pentagalloyl glucose isolated from eucalyptus leaves. *J. Agric. Food Chem.* 62, 3422–3431. doi: 10.1021/jf500210p

Chen, Y., Onken, B., Chen, H., Zhang, X., Driscoll, M., Cao, Y., et al. (2020). Healthy lifespan extension mediated by oenothein B isolated from *Eucalyptus grandis* x *Eucalyptus urophylla* GL9 in *Caenorhabditis elegans*. *Food Funct.* 11, 2439–2450. doi: 10.1039/C9FO02472G

Childs, B. G., Baker, D. J., Wijshake, T., Conover, C. A., Campisi, J., and van Deursen, J. M. (2016). Senescent intimal foam cells are deleterious at all stages of atherosclerosis. *Science* 354, 472–477. doi: 10.1126/science.aaf6659

Childs, B. G., Durik, M., Baker, D. J., and van Deursen, J. M. (2015). Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nat. Med.* 21, 1424–1435. doi: 10.1038/nm.4000

Cypser, J. R., Kitzenberg, D., and Park, S. K. (2013). Dietary restriction in *C. elegans*: recent advances. *Exp. Gerontol.* 48, 1014–1017. doi: 10.1016/j.exger.2013.02.018

Dall, K. B., and Faergeman, N. J. (2019). Metabolic regulation of lifespan from a *C. elegans* perspective. *Genes Nutr.* 14:25. doi: 10.1186/s12263-019-0650-x

De la Fuente, M., Sanchez, C., Vallejo, C., Diaz-Del Cerro, E., Arnalich, F., and Hernanz, A. (2020). Vitamin C and vitamin C plus E improve the immune function in the elderly. *Exp. Gerontol.* 142:111118. doi: 10.1016/j.exger.2020.111118

Demoinet, E., and Roy, R. (2018). Surviving starvation: AMPK protects germ cell integrity by targeting multiple epigenetic effectors. *Bioessays* 40:1700095. doi: 10.1002/bies.201700095

Desjardins, D., Cacho-Valadez, B., Liu, J. L., Wang, Y., Yee, C., Bernard, K., et al. (2017). Antioxidants reveal an inverted U-shaped dose-response relationship between reactive oxygen species levels and the rate of aging in Caenorhabditis elegans. *Aging Cell* 16, 104–112. doi: 10.1111/acel.12528

Ferreira, P. S., Spolidorio, L. C., Manthey, J. A., and Cesar, T. B. (2016). Citrus flavanones prevent systemic inflammation and ameliorate oxidative stress in C57BL/6J mice fed high-fat diet. *Food Funct.* 7, 2675–2681. doi: 10.1039/c5fo01541c

Fischer, N., Buchter, C., Koch, K., Albert, S., Csuk, R., and Watjen, W. (2017). The resveratrol derivatives trans-3,5-dimethoxy-4-fluoro-4'-hydroxystilbene and trans-2,4',5-trihydroxystilbene decrease oxidative stress and prolong lifespan in *Caenorhabditis elegans*. *J. Pharm. Pharmacol.* 69, 73–81. doi: 10.1111/jphp.12657

Fraga, C. G., Croft, K. D., Kennedy, D. O., and Tomas-Barberan, F. A. (2019). The effects of polyphenols and other bioactives on human health. *Food Funct.* 10, 514–528. doi: 10.1039/C8FO01997E

Friedman, D. B., and Johnson, T. E. (1988). A mutation in the age-1 gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. *Genetics* 118, 75–86. doi: 10.1093/genetics/118.1.75

Gonzalez-Paramas, A. M., Brighenti, V., Bertoni, L., Marcelloni, L., Ayuda-Duran, B., Gonzalez-Manzano, S., et al. (2020). Assessment of the *in vivo* antioxidant activity of an anthocyanin-rich bilberry extract using the *Caenorhabditis elegans* model. *Antioxidants* 9:509. doi: 10.3390/antiox9060509

Grunz, G., Haas, K., Soukup, S., Klingenspor, M., Kulling, S. E., Daniel, H., et al. (2012). Structural features and bioavailability of four flavonoids and their implications for lifespan-extending and antioxidant actions in *C. elegans*. *Mech. Ageing Dev.* 133, 1–10. doi: 10.1016/j.mad.2011.11.005

Guarente, L., and Kenyon, C. (2000). Genetic pathways that regulate ageing in model organisms. *Nature* 408, 255–262. doi: 10.1038/35041700

Habtemariam, S. (2019). The Nrf2/HO-1 axis as targets for flavanones: neuroprotection by pinocembrin, naringenin, and eriodictyol. *Oxid. Med. Cell. Longev.* 2019:4724920. doi: 10.1155/2019/4724920

Havermann, S., Humpf, H. U., and Watjen, W. (2016). Baicalein modulates stress-resistance and life span in C. elegans via SKN-1 but not DAF-16. *Fitoterapia* 113, 123–127. doi: 10.1016/j.fitote.2016.06.018

Havermann, S., Rohrig, R., Chovolou, Y., Humpf, H. U., and Watjen, W. (2013). Molecular effects of baicalein in Hct116 cells and *Caenorhabditis elegans*: activation of the Nrf2 signaling pathway and prolongation of lifespan. *J. Agric. Food Chem.* 61, 2158–2164. doi: 10.1021/jf304553g

Huang, X. B., Mu, X. H., Wan, Q. L., He, X. M., Wu, G. S., and Luo, H. R. (2017). Aspirin increases metabolism through germline signalling to extend the lifespan of *Caenorhabditis elegans*. *PLoS ONE* 12:e0184027. doi: 10.1371/journal.pone.0184027

Jayarathne, S., Ramalingam, L., Edwards, H., Vanapalli, S. A., and Moustaid-Moussa, N. (2020). Tart cherry increases lifespan in *Caenorhabditis elegans* by altering metabolic signaling pathways. *Nutrients* 12:1482. doi: 10.3390/nu12051482

Johnson, S. C., Rabinovitch, P. S., and Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-related disease. *Nature* 493, 338–345. doi: 10.1038/nature11861

Johnson, T. E., and Wood, W. B. (1982). Genetic analysis of life-span in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* 79, 6603–6607. doi: 10.1073/pnas.79.21.6603

Jorgensen, E. M., and Mango, S. E. (2002). The art and design of genetic screens: *Caenorhabditis elegans*. *Nat. Rev. Genet.* 3, 356–369. doi: 10.1038/nrg794

Jung, H. Y., Lee, D., Ryu, H. G., Choi, B. H., Go, Y., Lee, N., et al. (2017). Myricetin improves endurance capacity and mitochondrial density by activating SIRT1 and PGC-1alpha. *Sci. Rep.* 7:6237. doi: 10.1038/s41598-017-05303-2

Juurlink, B. H., Azouz, H. J., Aldalati, A. M., AlTinawi, B. M., and Ganguly, P. (2014). Hydroxybenzoic acid isomers and the cardiovascular system. *Nutr. J.* 13:63. doi: 10.1186/1475-2891-13-63

Kampkotter, A., Timpel, C., Zurawski, R. F., Ruhl, S., Chovolou, Y., Proksch, P., et al. (2008). Increase of stress resistance and lifespan of *Caenorhabditis elegans* by quercetin. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 149, 314–323. doi: 10.1016/j.cbpb.2007.10.004

Kenyon, C. (2010). A pathway that links reproductive status to lifespan in *Caenorhabditis elegans*. *Ann. N. Y. Acad. Sci.* 1204, 156–162. doi: 10.1111/j.1749-6632.2010.05640.x

Keowkase, R., Shoomarom, N., Bunargin, W., Sitthithaworn, W., and Weerapreeyakul, N. (2018). Sesamin and sesamolin reduce amyloid-beta toxicity in a transgenic *Caenorhabditis elegans*. *Biomed. Pharmacother.* 107, 656–664. doi: 10.1016/j.biopha.2018.08.037

Kim, D. K., Jeon, H., and Cha, D. S. (2014). 4-Hydroxybenzoic acid-mediated lifespan extension in *Caenorhabditis elegans*. *J. Funct. Foods* 7, 630–640. doi: 10.1016/j.jff.2013.12.022

Kim, W., Underwood, R. S., Greenwald, I., and Shaye, D. D. (2018). OrthoList 2: a new comparative genomic analysis of human and *Caenorhabditis elegans* genes. *Genetics* 210, 445–461. doi: 10.1534/genetics.118.301307

Koch, K., Buchter, C., Havermann, S., and Watjen, W. (2015). The lignan pinoresinol induces nuclear translocation of DAF-16 in *Caenorhabditis elegans* but has no effect on life span. *Phytother. Res.* 29, 894–901. doi: 10.1002/ptr.5330

Krizova, L., Dadakova, K., Kasparovska, J., and Kasparovsky, T. (2019). Isoflavones. *Molecules* 24:1076. doi: 10.3390/molecules24061076

Lapierre, L. R., and Hansen, M. (2012). Lessons from *C. elegans*: signaling pathways for longevity. *Trends Endocrinol Metab.* 23, 637–644. doi: 10.1016/j.tem.2012.07.007

Laplante, M., and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell* 149, 274–293. doi: 10.1016/j.cell.2012.03.017

Lee, J., Kwon, G., Park, J., Kim, J. K., and Lim, Y. H. (2016). Brief communication: SIR-2.1-dependent lifespan extension of *Caenorhabditis elegans* by oxyresveratrol and resveratrol. *Exp. Biol. Med.* 241, 1757–1763. doi: 10.1177/1535370216650054

Lei, L., Yang, Y., He, H., Chen, E., Du, L., Dong, J., et al. (2016). Flavan-3-ols consumption and cancer risk: a meta-analysis of epidemiologic studies. *Oncotarget* 7, 73573–73592. doi: 10.18632/oncotarget.12017

Li, J., Bonkowski, M. S., Moniot, S., Zhang, D., Hubbard, B. P., Ling, A. J. Y., et al. (2017). AGING A conserved NAD(+) binding pocket that regulates protein-protein interactions during aging. *Science* 355, 1312–1317. doi: 10.1126/science.aad8242

Li, Y., and Zhang, H. (2017). Soybean isoflavones ameliorate ischemic cardiomyopathy by activating Nrf2-mediated antioxidant responses. *Food Funct.* 8, 2935–2944. doi: 10.1039/C7FO00342K

Lim, H. H., Yang, S. J., Kim, Y., Lee, M., and Lim, Y. (2013). Combined treatment of mulberry leaf and fruit extract ameliorates obesity-related inflammation and oxidative stress in high fat diet-induced obese mice. *J. Med. Food* 16, 673–680. doi: 10.1089/jmf.2012.2582

Majidinia, M., Bishayee, A., and Yousefi, B. (2019). Polyphenols: major regulators of key components of DNA damage response in cancer. *DNA Repair* 82:102679. doi: 10.1016/j.dnarep.2019.102679

Malaguarnera, L. (2019). Influence of resveratrol on the immune response. *Nutrients* 11:946. doi: 10.3390/nu11050946

Martins, R., Lithgow, G. J., and Link, W. (2016). Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. *Aging Cell* 15, 196–207. doi: 10.1111/acel.12427

McCormick, M. A., Tsai, S. Y., and Kennedy, B. K. (2011). TOR and ageing: a complex pathway for a complex process. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 366, 17–27. doi: 10.1098/rstb.2010.0198

Meng, Q., Qi, X., Fu, Y., Chen, Q., Cheng, P., Yu, X., et al. (2020). Flavonoids extracted from mulberry (Morus alba L.) leaf improve skeletal muscle mitochondrial function by activating AMPK in type 2 diabetes. *J. Ethnopharmacol.* 248:112326. doi: 10.1016/j.jep.2019.112326

Miler, M., Zivanovic, J., Ajdzanovic, V., Milenkovic, D., Jaric, I., Sosic-Jurjevic, B., et al. (2020). Citrus flavanones upregulate thyrotroph sirt1 and differently affect thyroid Nrf2 expressions in old-aged wistar rats. *J. Agric. Food Chem.* 68, 8242–8254. doi: 10.1021/acs.jafc.0c03079

Morselli, E., Maiuri, M. C., Markaki, M., Megalou, E., Pasparaki, A., Palikaras, K., et al. (2010). Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. *Cell Death Dis.* 1:e10. doi: 10.1038/cddis.2009.8

Nakatani, Y., Yaguchi, Y., Komura, T., Nakadai, M., Terao, K., Kage-Nakadai, E., et al. (2018). Sesamin extends lifespan through pathways related to dietary restriction in *Caenorhabditis elegans*. *Eur. J. Nutr.* 57, 1137–1146. doi: 10.1007/s00394-017-1396-0

Okuyama, T., Inoue, H., Ookuma, S., Satoh, T., Kano, K., Honjoh, S., et al. (2010). The ERK-MAPK pathway regulates longevity through SKN-1 and insulin-like signaling in *Caenorhabditis elegans*. *J. Biol. Chem.* 285, 30274–30281. doi: 10.1074/jbc.M110.146274

Papaevgeniou, N., and Chondrogianni, N. (2018). Anti-aging and anti-aggregation properties of polyphenolic compounds in *C. elegans*. *Curr. Pharm. Des.* 24, 2107–2120. doi: 10.2174/1381612824666180515145652

Park, S., Artan, M., Han, S. H., Park, H. H., Jung, Y., Hwang, A. B., et al. (2020). VRK-1 extends life span by activation of AMPK via phosphorylation. *Sci. Adv.* 6:eaaw7824. doi: 10.1126/sciadv.aaw7824

Peixoto, H., Roxo, M., Krstin, S., Rohrig, T., Richling, E., and Wink, M. (2016). An Anthocyanin-rich extract of acai (Euterpe precatoria Mart.) increases stress resistance and retards aging-related markers in *Caenorhabditis elegans*. *J. Agric. Food Chem.* 64, 1283–1290. doi: 10.1021/acs.jafc.5b05812

Peng, C., Chan, H. Y., Li, Y. M., Huang, Y., and Chen, Z. Y. (2009). Black tea theaflavins extend the lifespan of fruit flies. *Exp. Gerontol.* 44, 773–783. doi: 10.1016/j.exger.2009.09.004

Perez-Vizcaino, F., and Duarte, J. (2010). Flavonols and cardiovascular disease. *Mol. Aspects Med.* 31, 478–494. doi: 10.1016/j.mam.2010.09.002

Pietsch, K., Saul, N., Menzel, R., Sturzenbaum, S. R., and Steinberg, C. E. (2009). Quercetin mediated lifespan extension in *Caenorhabditis elegans* is modulated by age-1, daf-2, sek-1 and unc-43. *Biogerontology* 10, 565–578. doi: 10.1007/s10522-008-9199-6

Pietsch, K., Saul, N., Swain, S. C., Menzel, R., Steinberg, C. E., and Sturzenbaum, S. R. (2012). Meta-analysis of global transcriptomics suggests that conserved genetic pathways are responsible for quercetin and tannic acid mediated longevity in *C. elegans*. *Front. Genet.* 3:48. doi: 10.3389/fgene.2012.00048

Proshkina, E., Lashmanova, E., Dobrovolskaya, E., Zemskaya, N., Kudryavtseva, A., Shaposhnikov, M., et al. (2016). Geroprotective and radioprotective activity of quercetin, (-)-epicatechin, and ibuprofen in *Drosophila melanogaster*. *Front. Pharmacol.* 7:505. doi: 10.3389/fphar.2016.00505

Qu, Z., Ji, S., and Zheng, S. (2020a). BRAF controls the effects of metformin on neuroblast cell divisions in *C. elegans*. *Int. J. Mol. Sci.* 22:178. doi: 10.1101/2020.11.03.367557

Qu, Z., Ji, S., and Zheng, S. (2020b). Glucose and cholesterol induce abnormal cell divisions via DAF-12 and MPK-1 in *C. elegans*. *Aging* 12, 16255–16269. doi: 10.18632/aging.103647

Ruderman, N. B., Xu, X. J., Nelson, L., Cacicedo, J. M., Saha, A. K., Lan, F., et al. (2010). AMPK and SIRT1: a long-standing partnership? *Am. J. Physiol. Endocrinol. Metab.* 298, E751–E760. doi: 10.1152/ajpendo.00745.2009

Sahin, E., Colla, S., Liesa, M., Moslehi, J., Muller, F. L., Guo, M., et al. (2011). Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* 470, 359–365. doi: 10.1038/nature09787

Salehi, B., Mishra, A. P., Nigam, M., Sener, B., Kilic, M., Sharifi-Rad, M., et al. (2018). Resveratrol: a double-edged sword in health benefits. *Biomedicines* 6:91. doi: 10.3390/biomedicines6030091

Salminen, A., and Kaarniranta, K. (2012). AMP-activated protein kinase (AMPK) controls the aging process via an integrated signaling network. *Ageing Res. Rev.* 11, 230–241. doi: 10.1016/j.arr.2011.12.005

Salminen, A., Ojala, J., Kaarniranta, K., and Kauppinen, A. (2012). Mitochondrial dysfunction and oxidative stress activate inflammasomes: impact on the aging process and age-related diseases. *Cell Mol. Life Sci.* 69, 2999–3013. doi: 10.1007/s00018-012-0962-0

Saul, N., Pietsch, K., Menzel, R., Sturzenbaum, S. R., and Steinberg, C. E. (2010). The longevity effect of tannic acid in *Caenorhabditis elegans*: disposable Soma meets hormesis. *J. Gerontol. A Biol. Sci. Med. Sci.* 65, 626–635. doi: 10.1093/gerona/glq051

Saul, N., Pietsch, K., Sturzenbaum, S. R., Menzel, R., and Steinberg, C. E. (2011). Diversity of polyphenol action in *Caenorhabditis elegans*: between toxicity and longevity. *J. Nat. Prod.* 74, 1713–1720. doi: 10.1021/np200011a

Seo, H. W., Cheon, S. M., Lee, M. H., Kim, H. J., Jeon, H., and Cha, D. S. (2015). Catalpol modulates lifespan via DAF-16/FOXO and SKN-1/Nrf2 activation in *Caenorhabditis elegans*. *Evid. Based Complement. Alternat. Med.* 2015:524878. doi: 10.1155/2015/524878

Smith, M. R., Syed, A., Lukacsovich, T., Purcell, J., Barbaro, B. A., Worthge, S. A., et al. (2014). A potent and selective Sirtuin 1 inhibitor alleviates pathology in multiple animal and cell models of Huntington's disease. *Hum. Mol. Genet.* 23, 2995–3007. doi: 10.1093/hmg/ddu010

Sobeh, M., Hamza, M. S., Ashour, M. L., Elkhatieb, M., El Raey, M. A., Abdel-Naim, A. B., et al. (2020). A polyphenol-rich fraction from eugenia uniflora exhibits antioxidant and hepatoprotective activities *in vivo*. *Pharmaceuticals* 13:84. doi: 10.3390/ph13050084

Sohal, R. S., and Orr, W. C. (2012). The redox stress hypothesis of aging. *Free Radic. Biol. Med.* 52, 539–555. doi: 10.1016/j.freeradbiomed.2011.10.445

Song, B., Wang, H., Xia, W., Zheng, B., Li, T., and Liu, R. H. (2020a). Combination of apple peel and blueberry extracts synergistically induced lifespan extension via DAF-16 in *Caenorhabditis elegans*. *Food Funct.* 11, 6170–6185. doi: 10.1039/D0FO00718H

Song, B. B., Zheng, B. S., Li, T., and Liu, R. H. (2020b). SKN-1 is involved in combination of apple peels and blueberry extracts synergistically protecting against oxidative stress in *Caenorhabditis elegans*. *Food Funct.* 11, 5409–5419. doi: 10.1039/D0FO00891E

Strayer, A., Wu, Z., Christen, Y., Link, C. D., and Luo, Y. (2003). Expression of the small heat-shock protein Hsp16-2 in *Caenorhabditis elegans* is suppressed by Ginkgo biloba extract EGb 761. *FASEB J.* 17, 2305–2307. doi: 10.1096/fj.03-0376fje

Su, S., and Wink, M. (2015). Natural lignans from *Arctium lappa* as antiaging agents in *Caenorhabditis elegans*. *Phytochemistry* 117, 340–350. doi: 10.1016/j.phytochem.2015.06.021

Sugawara, T., and Sakamoto, K. (2020). Quercetin enhances motility in aged and heat-stressed *Caenorhabditis elegans* nematodes by modulating both HSF-1 activity, and insulin-like and p38-MAPK signalling. *PLoS ONE* 15:e0238528. doi: 10.1371/journal.pone.0238528

Swindell, W. R. (2009). Heat shock proteins in long-lived worms and mice with insulin/insulin-like signaling mutations. *Aging* 1, 573–577. doi: 10.18632/aging.100058

Tambara, A. L., de Los Santos Moraes, L., Dal Forno, A. H., Boldori, J. R., Goncalves Soares, A. T., de Freitas Rodrigues, C., et al. (2018). Purple pitanga fruit (*Eugenia uniflora* L.) protects against oxidative stress and increase the lifespan in *Caenorhabditis elegans* via the DAF-16/FOXO pathway. *Food Chem. Toxicol.* 120, 639–650. doi: 10.1016/j.fct.2018.07.057

Troemel, E. R., Chu, S. W., Reinke, V., Lee, S. S., Ausubel, F. M., and Kim, D. H. (2006). p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. *PLoS Genet.* 2:e183. doi: 10.1371/journal.pgen.0020183

Tullet, J. M. (2015). DAF-16 target identification in *C. elegans*: past, present and future. *Biogerontology* 16, 221–234. doi: 10.1007/s10522-014-9527-y

Turgut, N. H., Mert, D. G., Kara, H., Egilmez, H. R., Arslanbas, E., Tepe, B., et al. (2016). Effect of black mulberry (Morus nigra) extract treatment on cognitive impairment and oxidative stress status of D-galactose-induced aging mice. *Pharm. Biol.* 54, 1052–1064. doi: 10.3109/13880209.2015.1101476

Wallace, T. C., and Giusti, M. M. (2015). Anthocyanins. *Adv. Nutr.* 6, 620–622. doi: 10.3945/an.115.009233

Wang, H., Liu, J., Li, T., and Liu, R. H. (2018). Blueberry extract promotes longevity and stress tolerance via DAF-16 in *Caenorhabditis elegans*. *Food Funct.* 9, 5273–5282. doi: 10.1039/C8FO01680A

Wang, X., Zhang, J. L., Lu, L. L., and Zhou, L. J. (2015). The longevity effect of echinacoside in *Caenorhabditis elegans* mediated through daf-16. *Biosci. Biotechnol. Biochem.* 79, 1676–1683. doi: 10.1080/09168451.2015.1046364

Wilson, M. A., Shukitt-Hale, B., Kalt, W., Ingram, D. K., Joseph, J. A., and Wolkow, C. A. (2006). Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. *Aging Cell* 5, 59–68. doi: 10.1111/j.1474-9726.2006.00192.x

Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M., et al. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* 430, 686–689. doi: 10.1038/nature02789

Wu, X., Al-Amin, M., Zhao, C., An, F., Wang, Y., Huang, Q., et al. (2020). Catechinic acid, a natural polyphenol compound, extends the lifespan of *Caenorhabditis elegans* via mitophagy pathways. *Food Funct.* 11, 5621–5634. doi: 10.1039/D0FO00694G

Xiong, L. G., Chen, Y. J., Tong, J. W., Gong, Y. S., Huang, J. A., and Liu, Z. H. (2018). Epigallocatechin-3-gallate promotes healthy lifespan through mitohormesis during early-to-mid adulthood in *Caenorhabditis elegans*. *Redox Biol.* 14, 305–315. doi: 10.1016/j.redox.2017.09.019

Xiong, L. G., Huang, J. A., Li, J., Yu, P. H., Xiong, Z., Zhang, J. W., et al. (2014). Black tea increased survival of *Caenorhabditis elegans* under stress. *J. Agric. Food Chem.* 62, 11163–11169. doi: 10.1021/jf503120j

Yaguchi, Y., Komura, T., Kashima, N., Tamura, M., Kage-Nakadai, E., Saeki, S., et al. (2014). Influence of oral supplementation with sesamin on longevity of *Caenorhabditis elegans* and the host defense. *Eur. J. Nutr.* 53, 1659–1668. doi: 10.1007/s00394-014-0671-6

Yan, F. J., Chen, Y. S., Azat, R., and Zheng, X. D. (2017). Mulberry anthocyanin extract ameliorates oxidative damage in HepG2 cells and prolongs the lifespan of *Caenorhabditis elegans* through MAPK and Nrf2 pathways. *Oxid. Med. Cell. Longev.* 2017:7956158. doi: 10.1155/2017/7956158

Ye, Y., Gu, Q., and Sun, X. (2020). Potential of *Caenorhabditis elegans* as an antiaging evaluation model for dietary phytochemicals: a review. *Compr. Rev. Food Sci. Food Saf.* 19, 3084–3105. doi: 10.1111/1541-4337.12654

Yoon, D. S., Cha, D. S., Choi, Y., Lee, J. W., and Lee, M. H. (2019). MPK-1/ERK is required for the full activity of resveratrol in extended lifespan and reproduction. *Aging Cell* 18:e12867. doi: 10.1111/acel.12867

Yoon, D. S., Choi, Y., Choi, S. M., Park, K. H., and Lee, J. W. (2015). Different effects of resveratrol on early and late passage mesenchymal stem cells through beta-catenin regulation. *Biochem. Biophys. Res. Commun.* 467, 1026–1032. doi: 10.1016/j.bbrc.2015.10.017

Yue, Y., Shen, P., Xu, Y., and Park, Y. (2019). p-Coumaric acid improves oxidative and osmosis stress responses in *Caenorhabditis elegans*. *J. Sci. Food Agric.* 99, 1190–1197. doi: 10.1002/jsfa.9288

Zarse, K., Jabin, S., and Ristow, M. (2012). L-Theanine extends lifespan of adult *Caenorhabditis elegans*. *Eur. J. Nutr.* 51, 765–768. doi: 10.1007/s00394-012-0341-5

Zhang, J. (2006). Resveratrol inhibits insulin responses in a SirT1-independent pathway. *Biochem. J.* 397, 519–527. doi: 10.1042/BJ20050977

Zhang, L., Jie, G., Zhang, J., and Zhao, B. (2009). Significant longevity-extending effects of EGCG on *Caenorhabditis elegans* under stress. *Free Radic. Biol. Med.* 46, 414–421. doi: 10.1016/j.freeradbiomed.2008.10.041

Zhao, L., Zhao, Y., Liu, R. H., Zheng, X. N., Zhang, M., Guo, H. Y., et al. (2017). The transcription factor DAF-16 is essential for increased longevity in *C. elegans* exposed to *Bifidobacterium longum* BB68. *Sci. Rep.* 7:7408. doi: 10.1038/s41598-017-07974-3

Zheng, S., Qu, Z., Zanetti, M., Lam, B., and Chin-Sang, I. (2018). *C. elegans* PTEN and AMPK block neuroblast divisions by inhibiting a BMP-insulin-PP2A-MAPK pathway. *Development* 145:dev166876. doi: 10.1242/dev.166876

Zheng, S. Q., Huang, X. B., Xing, T. K., Ding, A. J., Wu, G. S., and Luo, H. R. (2017). Chlorogenic acid extends the lifespan of *Caenorhabditis elegans* via insulin/IGF-1 signaling pathway. *J. Gerontol. A Biol. Sci. Med. Sci.* 72, 464–472. doi: 10.1093/gerona/glw105

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.

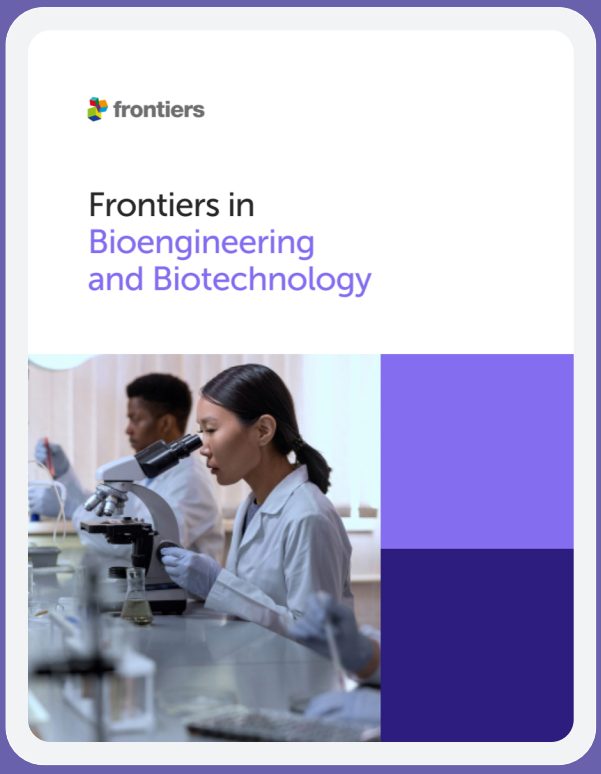
Frontiers in
Bioengineering and Biotechnology

Accelerates the development of therapies, devices, and technologies to improve our lives

A multidisciplinary journal that accelerates the development of biological therapies, devices, processes and technologies to improve our lives by bridging the gap between discoveries and their application.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact