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Rising stars in veterinary regenerative medicine 2022

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Rising stars in veterinary regenerative medicine: 2022

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Editorial: Rising stars in veterinary regenerative medicine: 2022

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Editorial on the Research Topic
Rising stars in veterinary regenerative medicine: 2022

It all began at a fast pace, with much hope and little fear: when the regenerative medicine field gained momentum, veterinary medicine was at the forefront of clinical applications for patients with naturally occurring disorders. We have learned that, indeed, there may not be much to fear when it comes to local autologous therapies with adult progenitor cells, which have since then largely proven to be safe. Many case studies were performed with promising results, indicating good efficacy and thereby promoting the tremendous hopes associated with regenerative therapies. Nevertheless, despite a growing research community with rapidly expanding output, progress has slowed down and the field has not yet managed to meet all the high expectations. The more progress the field made, the more questions arose, and we only slowly begin to unravel the complex modes of action of different biologics, their interaction with host cells and tissues, and how to stratify well-responding vs. non-responding patients.

The veterinary profession has an important role to play in the development of regenerative therapies, representing the link between basic science and human clinical applications, owed to the central role of animal experiments in translational research. In addition, animals are an integral part of our society and economy, so they are patients on their own rights in need of advanced treatments. Progress in the field is driven by both the need for companion animal treatments and the value of preclinical data gained from animal models and veterinary patients. Animal models require careful selection and design to ensure they are fit-for-purpose and provide optimal predictive validity while, at the same time, meet ethical animal welfare requirements. In spite of this, regenerative therapies for human patients are often tested in animals that do not mimic the human anatomy and pathophysiology. Small animals, specifically mouse and rat models, are valuable for research into mechanisms of disease and fundamental biology, but findings from these models rarely translate into human clinical applications. Beyond anatomical and physiological differences, the main reason is that experimentally mimicking the high complexity of multifactorial pathologies is extremely challenging. Large animals are well-accepted, well-established and clinically relevant models that commonly suffer from naturally occurring disease/injuries with similar pathophysiology to the human in terms of etiology and risk factors, including, among others, over-exercise, age and genetic factors. Therefore, developing the veterinary regenerative field is important to improve animal health and revert benefit to human medicine, while diminishing the need for experimental animals. However, while the

complexity of orthobiologics requires in-depth research, the veterinary research community faces specific challenges associated to working with non-conventional species. Intensifying the efforts is warranted by the unique landscape offered by the veterinary regenerative field.

Moving the field of regenerative medicine forward requires the cooperation of basic researchers, human and veterinary medical scientists. A big share of the affiliated research is contributed by dedicated young researchers willing to advance the standard of animal care. With this article collection, our goal is to promote promising young scientists in the field of veterinary regenerative medicine. This Research Topic compiles current work of several groups and their young researchers. The collection offers representative insight into the current status of the field, with in-depth studies on long-existing topics, such as the sources and characterization of mesenchymal stromal cells and blood products (Heilen et al.; Melzer et al.; Miguel-Pastor et al.; Andrietti et al.; Phyo et al.) and the clinical application of orthobiologics in osteoarthritis (Mayet et al.). Several articles highlight the importance to understand the immunological properties of orthobiologics (Cequier et al.; Pezzanite et al.; Moellerberndt et al.). In addition, fields which emerged more recently, such as extracellular vesicles and induced pluripotent stem cells, as well as new areas of clinical applications, are discussed in review format (Adamič and Vengust; Barrachina et al.; Jammes et al.; Weeratunga et al.).

We expect that this Research Topic not only holds a great scientific potential highlighting the progress in the veterinary regenerative medicine field, but may also show that human and veterinary medicine share a lot of research interests. The demand for higher standards of care for animals and for better and more ethical translational models is leading to a rapid advancement of the veterinary regenerative field and to the emergence of new frontiers. Thus, an "army" of researchers will be needed to truly unravel and exploit the potential of the veterinary regenerative field. Regenerative medicine is an exciting area of research for young scientists with many different profiles and with a multitude of interests, as the field requires highly multidisciplinary teams, from cell biologists and clinicians to engineers and bioinformaticians. However, developing a career in veterinary regenerative medicine does not come without challenges. For those with clinical interest, it may be difficult to combine research and clinical work as both are highly demanding and time-consuming. Fundraising may also be more difficult than in other research areas as there are very few specific calls for veterinary research, and some may feel discouraged by the competition with human-centered research. Opportunities for dedicated training, dissemination or networking (e.g. conferences, meetings) are sometimes hard to find, as the biggest regenerative medicine events are focused on applications in humans. However, the veterinary community is gaining presence in some of these meetings and is creating new spaces for interaction, which is the key for promoting engagement. Finally, as in other fields, supporting supervisors and institutions are of utmost importance to attract and promote young researchers. Owed to the increasing relevance of veterinary regenerative medicine and the unique challenges that are faced, the rising stars between us should be supported by all possible means.

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The immunomodulation–immunogenicity balance of equine Mesenchymal Stem Cells (MSCs) is differentially affected by the immune cell response depending on inflammatory licensing and major histocompatibility complex (MHC) compatibility

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The immunomodulatory properties of equine mesenchymal stem cells (MSCs) are important for their therapeutic potential and for their facilitating role in their escape from immune recognition, which may also be influenced by donor–recipient major histocompatibility complex (MHC) matching/mismatching and MHC expression level. Factors such as inflammation can modify the balance between regulatory and immunogenic profiles of equine MSCs, but little is known about how the exposure to the immune system can affect these properties in equine MSCs. In this study, we analyzed the gene expression and secretion of molecules related to the immunomodulation and immunogenicity of equine MSCs, either non-manipulated (MSC-naive) or stimulated by pro-inflammatory cytokines (MSC-primed), before and after their exposure to autologous or allogeneic MHC-matched/-mismatched lymphocytes, either activated or resting. Cytokine priming induced the immunomodulatory profile of MSCs at the baseline (MSCs cultured alone), and the exposure to activated lymphocytes further increased the expression of *interleukin 6 (IL6)*, *cyclooxygenase 2*, and *inducible nitric oxide synthase*, and IL6 secretion. Activated lymphocytes were also able to upregulate the regulatory profile of MSC-naive to levels comparable to cytokine priming. On the contrary, resting lymphocytes did not upregulate the immunomodulatory profile of equine MSCs, but interestingly, MSC-primed exposed to MHC-mismatched lymphocytes showed the highest expression and secretion of these mediators, which may be potentially linked to the activation of

lymphocytes upon recognition of foreign MHC molecules. Cytokine priming alone did not upregulate the immunogenic genes, but MSC-primed exposed to activated or resting lymphocytes increased their *MHC-I* and *MHC-II* expression, regardless of the MHC-compatibility. The upregulation of immunogenic markers including *CD40* in the MHC-mismatched co-culture might have activated lymphocytes, which, at the same time, could have promoted the immune regulatory profile aforementioned. In conclusion, activated lymphocytes are able to induce the equine MSC regulatory profile, and their effects seem to be additive to the priming action. Importantly, our results suggest that the lymphocyte response against MHC-mismatched MSC-primed would promote further activation of their immunomodulatory ability, which eventually might help them evade this reaction. Further studies are needed to clarify how these findings might have clinical implications *in vivo*, which will help developing safer and more effective therapies.

KEYWORDS

mesenchymal stem cells, horse, allogeneic therapy, haplotype, co-culture, immune response, gene expression, mediator secretion

Introduction

Mesenchymal stem cells (MSCs) are of great interest to treat several pathologies, including musculoskeletal injuries such as those affecting the horse, which is a species of remarkable value as both patient and translational models (1, 2). The regulatory and immunomodulatory properties of MSCs are currently considered their main therapeutic mechanism and involve both direct cell-to-cell contact and contact-independent paracrine signaling, *via* the expression of adhesion molecules like vascular cell adhesion molecule 1 (*VCAM1*) and the secretion of molecules such as interleukin 6 (IL6) and prostaglandin E2 (PGE_2) (3), respectively (4). Since the immunomodulatory properties of equine MSCs might have profound therapeutic implications in the treatment of many inflammatory-mediated processes in the horse, a growing number of studies have focused on analyzing such immune properties (4). To elucidate possible pathways for immunosuppression exerted by equine MSCs, and how these are influenced by different factors, it is critical to study the expression and secretion of mediators implied in their paracrine mechanisms, including the enzymes producing these molecules, such as cyclooxygenase 2 (*COX2*), indoleamine 2,3-dioxygenase 1 (*IDO1*), or inducible nitric oxide synthase 2 (*iNOS2*).

Furthermore, the MSC immunomodulatory activity is not only important for its therapeutic mechanism but also for its facilitating role in its escape from immune recognition when administered allogenically. Allogeneic application presents several advantages over autologous therapy as it increases the availability of thoroughly characterized cells for therapy, particularly when autologous cells are not suitable because of genetic or metabolic diseases, or in aged patients (5). However, MSCs are no longer considered truly immune-privileged but are considered immune-evasive, so their recognition and elimination by the immune system after their allogeneic administration should be considered (6). Allogeneic MSCs may be rejected due to the expression of foreign antigens on their surface, which may raise both cellular and humoral immune responses against the cells (7) and even lead to immune memory mechanisms that could prevent effective and safe repeated administration of allogeneic MSCs in the horse (8).

The surface expression of major histocompatibility complex (MHC) class I and II antigens on equine MSCs facilitates their immune recognition by lymphocytes, and antibodies can be generated specifically directed against the equine leukocyte antigen (ELA) of the donor, potentially compromising the

Abbreviations: AT, adipose tissue; B2M, beta-2-microglobulin; BM, bone marrow; CB, umbilical cord blood; CT, umbilical cord tissue; COX2, cyclooxygenase 2; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; ELA, equine leukocyte antigen; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IDO1, indoleamine 2,3-dioxygenase 1; IFNγ, interferon gamma; IL6, interleukin 6; iNOS1, inducible nitric oxide synthase 1; iNOS2, inducible nitric oxide synthase 2; iPSCs, induced pluripotent stem cells; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MSC, mesenchymal stem cell; NK, natural killer; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PGE_2, prostaglandin E2; PHA, phytohemagglutinin; RT-qPCR, real-time quantitative polymerase chain reaction; TNFα, tumor necrosis factor alpha; VCAM1, vascular cell adhesion molecule 1.

therapeutic effectiveness of the cells. Therefore, MHC matching between the donor and recipient is receiving increasing attention in the last few years, and ELA haplotypes have been taken into account in equine studies (9, 10), as well as in other species (11, 12). It should be noted that the MHC haplotype is a factor intrinsic to each individual and as such cannot be modified. Furthermore, it has also been reported that the MHC level expression in MSCs in basal conditions is quite dependent on the equine donor (9). The knowledge on these factors is critical to design better therapeutic strategies, including donor selection. As a matter of fact, some researchers are exploring a possible link between low MHC antigen expression and universal blood types to select equine donors whose MSCs would defer immune recognition (7).

Nevertheless, there are other factors that may modify the inherent immune properties of MSCs, such as their exposure to an inflammatory environment. Priming MSCs with pro-inflammatory cytokines like interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) could increase their immunomodulatory properties and may result in enhanced regulatory effects *in vivo* (13). However, priming MSCs may also raise their immunogenicity by inducing the expression of MHCs, thus potentially limiting their allogeneic administration (14). These changes in MSC immune properties upon inflammatory exposure are influenced by the type and duration of priming. For example, while priming with high concentration of IFNγ can increase *MHC-II* gene expression in equine MSCs (9), priming with low doses of IFNγ and TNFα for a short period upregulated several immune regulatory-related genes without significantly increasing the expression of immunogenic markers (15). However, while a significant advance has been made on how different cytokines and ligands may influence the immune properties of equine MSCs, the effects of an immune response environment on MSCs have been less explored.

To develop allogeneic cell therapies is critical to gain knowledge of how factors such as MHC matching/mismatching and inflammation may affect the balance between the immunomodulatory and immunogenic potentials of equine MSCs. Such immune properties can be assessed by evaluating the proliferation of lymphocytes exposed to MSCs in immunosuppression assays or in modified one-way mixed lymphocyte reactions (MLRs) (5). Our group recently reported the effects of equine MSCs on different lymphocyte subpopulations after their *in vitro* co-culture with autologous or allogeneic MHC-matched/-mismatched MSCs, either unstimulated or primed with pro-inflammatory cytokines (16). These *in vitro* assays provide important information on the changes experienced by lymphocytes after contacting with MSCs, contributing to understand the immune response *in vivo*. However, little is known about how MSCs behave when they are exposed to lymphocytes, either if these are already activated during a disease or if they become activated in response to MSCs.

To better understand the fate of MSCs when they enter into contact with the immune system, this study aimed at analyzing the changes in the gene expression and secretion of molecules related to the immune regulatory and immunogenic profiles of equine MSCs after being exposed to activated or resting lymphocytes. Our specific goals were to evaluate the influence of inflammation and compatibility for the MHC in different *in vitro* co-culture settings. For these purposes, equine MSCs in basal conditions (MSC-naive) or pro-inflammatory primed (MSC-primed) were co-cultured with autologous or allogeneic MHC-matched/-mismatched lymphocytes in both immunosuppression assays (activated lymphocytes) and in modified one-way MLRs (resting lymphocytes). Subsequently, MSC gene expression and secretion of different molecules related to their immunomodulatory–immunogenicity balance were assessed.

Our initial hypothesis was that exposure of equine MSCs to lymphocytes would result in increased gene expression and secretion of immunomodulatory molecules accompanied by a slight upregulation of their immunogenic profile. We hypothesized that these changes would be more marked in MSC-primed, particularly after contacting with activated lymphocytes. Regarding the MHC compatibility, it was hypothesized that MSCs exposed to MHC-matched lymphocytes would display a profile similar to that in the autologous setting, whereas the MHC-mismatched co-cultures would result in similar immunomodulation but increased immunogenicity of the equine MSCs.

Materials and methods

Study design

Equine bone marrow (BM)-derived MSCs were obtained from three MHC homozygous donors and were assayed in both basal conditions (MSC-naive) and after pro-inflammatory priming (MSC-primed). MSC-naive and MSC-primed from each donor were co-cultured with peripheral blood lymphocytes (PBLs), either autologous ($n = 3$) or allogeneic from MHC-matched ($n = 8$) and -mismatched ($n = 7$) animals. These PBLs were obtained from the three MSC donors (autologous setting) and from eight horses selected by their MHC haplotype to establish different allogeneic matched and mismatched combinations, as shown in Figure 1A. A total of two types of co-cultures were used: immunosuppressive assays, where PBLs were previously activated, and modified one-way MLR, where resting PBLs were used. After each type of co-culture, PBLs were removed and used in a separate study (16), MSCs were harvested to analyze their gene expression, and supernatants collected to assess their secretion. The gene expression of different molecules related to the immunomodulatory (*VCAM1*, *COX2*, *IDO1*, *iNOS2*, *IL6*) and immunogenic (*MHC-I*, *MHC-II*, *CD40*,

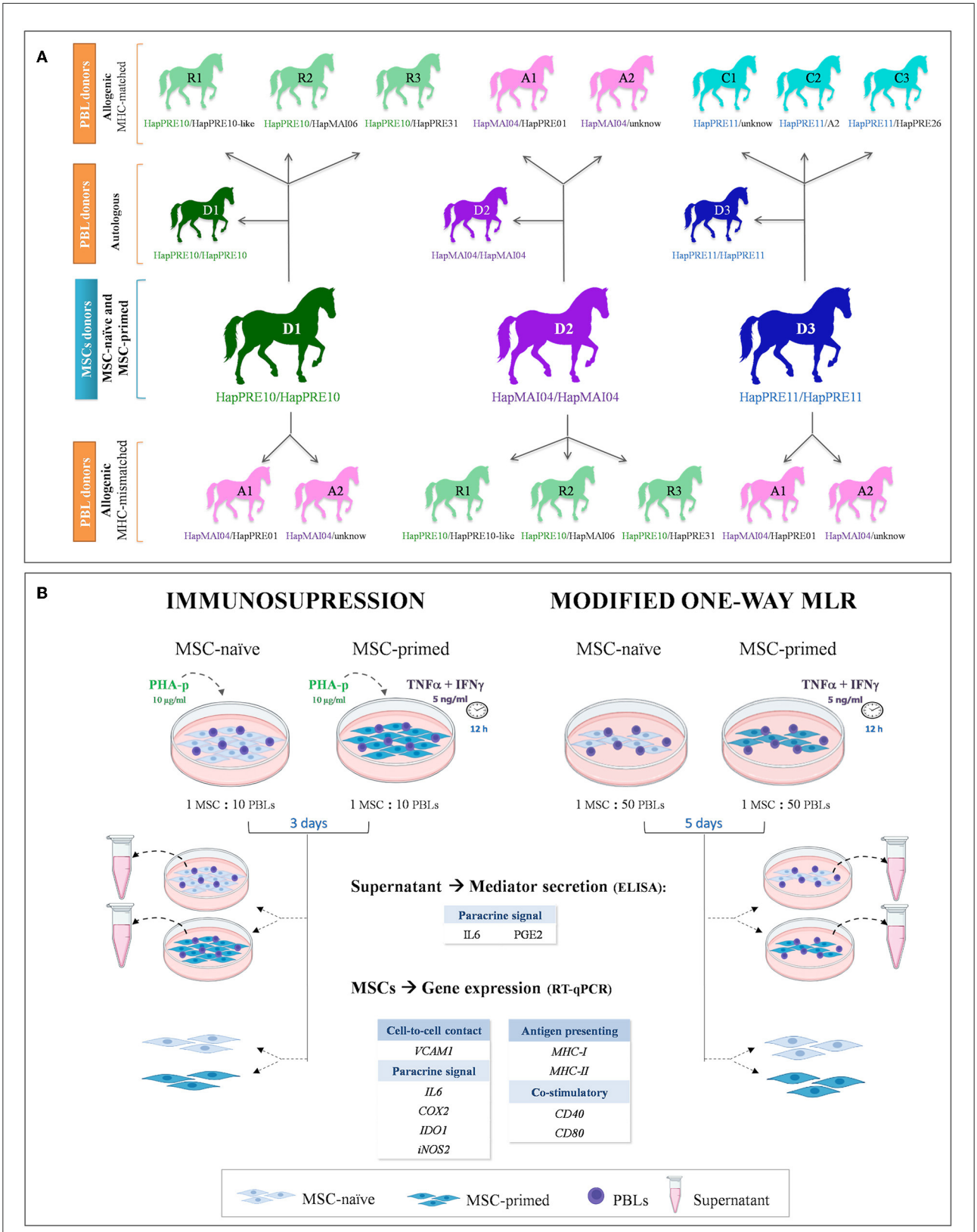

FIGURE 1
Study design. **(A)** Equine donors of mesenchymal stem cells (MSCs) and peripheral blood lymphocytes (PBLs) are presented with their ELA haplotypes and the combinations to establish autologous and allogeneic major histocompatibility complex (MHC)-matched and MHC-mismatched co-cultures. **(B)** Equine MSCs were assayed with unstimulated (MSC-naive) and primed with cytokines (MSC-primed)

(Continued)

FIGURE 1 (Continued)
in both immunosuppression and modified one-way mixed lymphocyte reaction (MLR) assays, using PBLs activated by phytohemagglutinin isoform P (PHA-p) or resting lymphocytes, respectively. After co-culture, supernatant and MSCs were collected to evaluate, respectively, the secretion and gene expression of different mediators involved in the immunomodulatory capacity and immunogenic potential of these cells.

CD80) profiles of MSCs were evaluated by real-time quantitative polymerase chain reaction (RT-qPCR), and secretion of IL6 and PGE_2 was determined by ELISA in the supernatants (Figure 1B).

Animal selection by MHC haplotyping

In total, 11 mixed-breed horses (one stallion, three geldings, seven mares; aged 2–8 years, weight 412–493 kg) in good health status and with no previous pregnancy history were chosen based on their MHC haplotypes. To find and select animals, a screening of 60 Purebred Spanish and mixed-breed horses from a local farm was performed. Haplotypes were determined by microsatellite typing using a validated panel of 10 highly polymorphic intra-MHC regions, as previously described (8, 17). Blood was collected after owner's informed consent, and methodology for DNA extraction, multiplex PCRs, and fragment analysis was performed, as previously reported by our group (8).

Definitive haplotypes were established for homozygous animals, and the remaining animals were assigned with provisional haplotypes based on previously known ones, which are either reported in the literature (10, 18) or described in a preliminary study of our group in Purebred Spanish horses (19). Overall, three groups of animals were selected, with each group including one homozygous horse of the haplotype HapPRE10, HapPRE11, or HapMAI04, and two or three heterozygous animals sharing one haplotype with the donor. Thus, the homozygous horse in each group served as the MSC donor as it was MHC-matched with the heterozygous animals. To establish MHC-mismatched combinations, PBLs from the heterozygous animals in other groups with different haplotypes were used (Figure 1A). Selecting homozygous individuals as MSC donors allows matching them with different heterozygous individuals. This strategy has been proposed to create haplo-banks of human-induced pluripotent stem cells (iPSCs) (20, 21) and has also been used in equine MSC studies (22). Supplementary Table 1 shows the microsatellite alleles of each haplotype identified in the horses involved in this study.

All procedures involving animals were carried out under the Project License PI 15/16 approved by the in-house Advisory Ethics Committee for Animal Research from the University of Zaragoza. The care and use of animals were performed in according with the Spanish Policy for Animal Protection RD53/2013, which is in line with the European Union Directive 2010/63 on the protection of animals used for scientific purposes. All animals were kept on paddocks of the facilities of the Animal Research Service of the University of Zaragoza, with free access to water and grass hay.

Isolation, characterization, and culture of MSCs

Equine bone marrow MSCs were obtained and characterized, as previously described (15) as part of a previous study of our group (16). In brief, bone marrow was harvested from the sternum of D1, D2, and D3 animals under sedation (0.04 mg/kg IV romifidine, Sedivet, Boehringer Ingelheim, and 0.02 mg/kg IV butorphanol, Torbugesic, Pfizer) and local analgesia with lidocaine (Anesvet, Laboratorios Ovejero). Mononuclear cells were separated by density gradient centrifugation and seeded in the culture medium consisting of low-glucose Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 U/mL penicillin, and 10% fetal bovine serum (FBS) (all from Sigma-Aldrich). The cells were expanded until passage three and characterized by their phenotype and tri-lineage differentiation. Characterization data of the MSC lines used in this study were previously published by our group (16). Subsequently, the MSCs were cryopreserved in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 90% FBS medium until the experiments started.

Prior to co-cultures, the cryopreserved MSCs ($n = 3$) were thawed and seeded at 5,000 cells/cm^2 in the basal medium, as described above, at 37°C and 5% CO_2 for 72 h to recover from freezing. At 24 h prior to co-culture, the MSCs were detached with 0.25% trypsin–EDTA (Sigma–Aldrich) and seeded into a 24-well plate at 100,000 cells per well for the immunosuppression assays, and at 20,000 MSCs per well for modified one-way MLR assays.

For the MSC-primed condition, the basal media described above was supplemented with 5 ng/mL of TNFa (R&D Systems) plus 5 ng/mL of IFNg (R&D Systems) and corresponding MSCs were exposed for 12 h to this media, as described earlier (15), before adding PBLs.

Blood collection and isolation of PBLs

Peripheral blood lymphocytes were isolated using the carbonyl iron granulocyte depletion method, followed by density gradient centrifugation with Lymphoprep^{TM}, as previously described (8, 23). In brief, blood was collected aseptically *via* a jugular venipuncture into sterile 60-mL syringes with 17 IU/mL of lithium heparin (Sigma-Aldrich), and plasma was allowed to separate for 20 min at room

temperature (RT). Plasma was separately collected into conical tubes using extension sets and incubated with carbonyl iron (Sigma-Aldrich) in agitation for 30 min at 37°C. Then, carbonyl iron was placed at the bottom of the tubes by using a magnet, and supernatant was collected and centrifuged at 310× g for 5 min. The cellular pellet was resuspended in PBS and overlayed on Lymphoprep^{TM}. After centrifugation at 690× g for 15 min (without brake), the lymphocyte layer was recovered and washed with PBS. The cells were counted in a hemocytometer chamber using 0.4% trypan blue as dye exclusion. This isolation technique has been reported to provide an enriched lymphocyte population (95–99%) (8, 24).

Co-cultures of MSCs with lymphocytes: Immunosuppression assays and modified one-way MLR

Co-culture of MSCs with activated lymphocytes: Immunosuppression assay

To simulate the environment of an immune response, MSCs were exposed to activated lymphocytes by conducting immunosuppression assays. As described before, corresponding MSCs were previously plated in a 24-well plate at 100,000 cells per well in duplicate and prepared for each condition (MSC-naive and MSC-primed). Lymphocytes from autologous, MHC-matched and -mismatched horses were seeded at 1 × 10^6 PBLs per well (1:10 ratio MSC:PBL), based on previous studies (25, 26) according to the combinations presented in Figure 1. The PBL medium used for co-culture consisted of RPMI 1640 (Thermo Fisher) supplemented with 10% FBS, 0.1 mM 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Sigma-Aldrich). The PBL medium was supplemented with 10 µg/mL of the mitogen phytohemagglutinin isoform P (PHA, Sigma-Aldrich) (27, 28) to activate lymphocyte proliferation.

MSC-naive and MSC-primed were cultured alone in the same conditions to provide baseline measurements for both gene expression and molecule secretion. Appropriate controls were set along with experimental conditions in duplicate. Lymphocytes from all animals, either PHA-activated or unstimulated, were cultured alone as positive and negative controls, respectively, to account for their possible contribution to the secretion of molecules measured by ELISA. All the experimental co-cultures and controls were maintained for 3 days, after which corresponding analyses were performed, as will be detailed in the following text.

Modified one-way MLR

Modified one-way MLRs were performed by co-culturing MSCs with resting (unstimulated) lymphocytes. This setting aims at reflecting what would happen to MSCs if these are recognized by the immune system and raise a response that can simultaneously change the MSC profile. Stimulator MSCs, either naive or primed, were previously plated at 20,000 cells per well on 24-well plates in duplicate for each condition, as described previously. Autologous, MHC-matched and -mismatched responder PBLs were seeded at 1 × 10^6 PBL per well according to the combinations depicted in Figure 1, thus resulting in an MSC/PBL ratio of 1:50 (9, 26).

Positive and negative controls were set by establishing, respectively, matched and mismatched classic MLRs using responder PBLs from each donor. In brief, MHC-matched or -mismatched PBLs were used as stimulators by treating them with 50 µg/mL mitomycin C (Sigma-Aldrich) (37°C 30 min incubation, followed by two washes with PBS at 310 × g 5 min) to inhibit proliferation (10, 29). Stimulator PBLs and responder PBLs were cultured at a ratio of 1:1. The supernatant from the MHC-matched and -mismatched MLRs was used to account for potential contribution of lymphocytes to the secretion of the analyzed molecules. All the co-cultures and controls were maintained for 5 days without media exchange, and corresponding analyses were performed subsequently, as detailed in the following text.

Analysis of expression of genes involved in equine immune response (RT-qPCR)

The expression level of genes coding for immunosuppression- and immunogenicity-related molecules was evaluated in MSC-naive and MSC-primed cultured alone (baseline) and after being co-cultured with autologous, MHC-matched or -mismatched PBLs in both immunosuppression and modified one-way MLR. After the co-cultures, PBLs were removed, and MSCs were washed with PBS and frozen at −80°C until mRNA was extracted. MSCs cultured alone for baseline were processed in the same way.

Isolation of mRNA and complementary DNA (cDNA) synthesis were performed using the Cells-to-cDNA II kit (Ambion) according to the manufacturer's instructions, and RT-qPCRs were performed and monitored with a QuantStudio 3 Real-Time PCR System (Applied Biosystems). All reactions were carried out in a total volume of 10 µL with 2 µL of cDNA as the template and Fast SYBR Green Master Mix (Applied Biosystems). Amplification was performed in

TABLE 1 Primers used for gene expression analysis by RT-qPCR.

Gene	Accession number	Primer sequence (5′–3′)	Amplicon size (bp)
	Housekeeping		
GAPDH	NM_001163856	F: GGCAAGTTCCATGGCACAGT	128
		R: CACAACATATTCAGCACCAGCAT	
B2M	NM_001082502	F: TCGTCCTGCTCGGGCTACT	102
		R: ATTCTCTGCTGGGTGACGTGA	
	Immunomodulation-related molecules		
	Molecules related with cell-to-cell contact mechanism		
VCAM1	NM_001101650	F: TCTATGCTACGCTCTGGCTACG	127
		R: TTGATGGTCTCCCCGATGA	
	Molecules related with paracrine signaling mechanism		
COX2	AB041771	F: GTTTGCATTTTTTGCCCAGC	103
		R: ACTTAAATCCACCCCGTGACC	
IDO1	XM_014736538.2	F: TCATGACTACGTGGACCCAAAA	104
		R: CGCCTTCATAGAGCAGACCTTC	
iNOS2	AY027883	F: CCAACAATGGCAACATCAGGT	85
		R: TGAGCATTCCAGATCCGGA	
IL6	EU438770	F: AACAGCAAGGAGGTACTGGCA	95
		R: CAGGTCTCCTGATTGAACCCA	
	Immunogenic markers: *Antigen presenting-related molecules*		
MHC-I	AB525081	F: CGTGAGCATCATTGTTGGC	92
		R: TCCCTCTTTTTTCACCTGAGG	
MHC-II	NM_001142816	F: AGCGGCGAGTTGAACCTACAGT	172
		R: CGGATCAGACCTGTGGAGATGA	
	Antigen-presenting-related molecules: *Co-Stimulatory molecules*		
CD40	AY514017	F: ACAAATACTGCGACCCCAACC	114
		R: TTTCACAGGCATCGCTGGA	
CD80	XM_005601958.3	F: CAGGAAAGTTGGCTCTGACCA	135
		R: TCTCCATTGTGATCCTGGCTC	

GenBank accession numbers of the sequences used for primers design. Primers (F: forward and R: reverse) and length of the amplicon in base pair (bp). Genes were grouped in agreement with the functions and implications of encoded molecules. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *B2M*, beta-2-microglobulin; *VCAM1*, vascular cell adhesion molecule 1; *COX2*, cyclooxygenase 2; *IDO1*, indoleamine 2 3-dioxygenase 1; *iNOS2*, inducible nitric oxide synthase 2; *IL6*, interleukin 6; *MHC-I*, major complex of histocompatibility I; *MHC-II*, major complex of histocompatibility II; *CD40*, cluster of differentiation 40; *CD80*, cluster of differentiation 80.

triplicate for each sample as follows: 20 s at 95°C for initial activation, followed by 40 cycles consisting of 3 s at 95°C and 30 s at 60°C, and a dissociation curve protocol run after every PCR.

The levels of gene expression were determined by using the comparative ΔΔCt method. As a reference sample, values from MSC-naive cultured alone (baseline) from each donor were used, unless otherwise stated. The normalization factor was calculated as the geometric mean of the quantity of two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta-2-microglobulin (*B2M*). Genes were analyzed, and corresponding primer sequences were previously designed by our group (15) and are presented in Table 1, grouped according to their function.

Assessment of interleukin 6 and prostaglandin E2 secretion

Supernatants collected from MSC-PBL co-cultures were used to evaluate PGE_2 and IL6 production by using commercially available ELISA kits, as previously reported (26, 29–31). The secretion of these molecules was assessed at the baseline (MSC-naive and MSC-primed cultured alone) and after exposure to the different types of PBLs in both immunosuppression and modified one-way MLR assays. The supernatants from unstimulated and PHA-stimulated PBLs seeded alone were used as negative and positive controls, respectively, for the immunosuppression assays. For the modified one-way MLR assays, the supernatants from the classical MLRs with MHC-matched or -mismatched

PBLs as stimulators were used as negative and positive controls, respectively.

After all co-cultures, PBLs were collected, centrifuged at 310 × g for 5 min, and the supernatants recovered. Supernatants were recovered in the same way from controls consisting of PBLs alone and from classic MLRs, as well as from MSC-naive and MSC-primed cultured alone. All the supernatants were centrifuged at 500 × g for 15 min to remove any contaminating cell and subsequently frozen at −20°C for further ELISA. All the procedures were performed as per the manufacturer's instructions and concentrations determined using a standard curve, including a blank.

For PGE_2 analysis (Prostaglandin E2 Parameter Assay Kit, R&D Systems, Ref: KGE004B), control supernatants were diluted 1:10, baseline supernatants were diluted 1:2, and supernatants from co-cultures were diluted 1:50 in the reagent diluent. The standard curve was established from 39 to 5,000 pg/mL. For IL6 analysis (Equine IL-6 DuoSet ELISA, R&D Systems, REF: DY1886), baseline and control supernatants were diluted 1:1 in the reagent diluent, and supernatants from co-cultures were not diluted. The standard curve was set from 62.5 to 16,000 pg/mL. All the samples and points of the standard curve were run in duplicate. All the colorimetric assays were analyzed on a microplate reader SPECTROstar Nano (BMG LABTECH) and read immediately at 450 nm with wavelength correction set to 540 nm. The duplicate readings for each standard, control, and sample were averaged, and the average zero standard optical density was extracted. The standard curve was created generating a four-parameter logistic curve fit, and the concentrations extrapolated were multiplied by the dilution factor. Samples with values beyond the limit of detection were excluded from the analysis.

Statistical analysis

Statistical analysis was performed by GraphPad Prism 9.2 software (San Diego, CA, USA). Results of RT-qPCR and ELISA were separately analyzed for each type of co-culture and not directly compared as the experimental conditions were different. Analytical statistics were performed to check differences in mRNA relative expression or molecule secretion depending on the different study variables. The independent variables were "group" (three categories: autologous, allogenic MHC-matched, and allogenic MHC-mismatched co-cultures) and "cell type" (two categories: MSC-naive and MSC-primed). The existence of outlier samples was evaluated with the Grubbs test (alpha = 0.05). For comparisons between three or more groups, normality and homoscedasticity were evaluated by using the Shapiro–Wilk test and the Levene test, respectively. When data followed a normal distribution and had homogeneous variances, the parametric test ANOVA was used, followed by Tukey's comparisons test as a *post-hoc*. In normally distributed data with unequal variances, Welch's *t*-test was used. In non-normal data, the non-parametric Kruskal–Wallis test was used, followed by Dunn's test as a *post hoc*. The effect of the type of co-culture was analyzed by comparing the results for each type of combination (autologous, MHC-matched, and MHC-mismatched) for each type of MSC (MSC-naive and MSC-primed) using parametric or non-parametric paired tests. The significance level was set at $p < 0.05$ for all analyses.

Results

The expression level of different genes and the secretion of molecules were evaluated in MSCs from three donors under different conditions: MSC-naive and MSC-primed cultured alone (baseline) and after being co-cultured with autologous, and MHC-matched or mismatched PHA-activated PBLs (immunosuppression assays) or resting PBLs (modified one-way MLR assays). The expression level of five genes involved in the immunomodulatory properties of equine MSCs was analyzed (*IL6*, *COX2*, *IDO1*, *iNOS2*, and *VCAM1)*, along with the secretion of IL6 and PGE_2. Also, to account for the effect of the different conditions on MSC immunogenicity, four genes coding for antigen-presenting-related molecules were assessed: *MHC-I*, *MHC-II*, *CD80*, and *CD40*.

For gene expression, data are presented as relative expression (fold change) over corresponding baseline MSC-naive, unless otherwise stated. Baseline values are the same for both types of assays (immunosuppression and modified one-way MLR). For molecule secretion, the supernatants from lymphocytes cultured alone in resembling conditions were used as controls. The IL6 and PGE_2 concentration detected in the lymphocyte controls for the immunosuppression assay (unstimulated, CTL–; PHA-activated, CTL+) and for the modified one-way MLR assays (classic MLRs) was very low and significantly different from that measured in the co-culture supernatants, confirming that MSCs were the major contributors to IL6 and PGE_2 secretion in the co-cultures. The details on these significant differences can be found in Supplementary Figure 1. Note that the scale in Y axes of the graphs presenting results of gene expression and molecule secretion in immunosuppression and modified one-way MLR assays is different to better show the values.

Gene expression and secretion of mediators related to equine MSC immunomodulation

At the baseline (MSCs cultured alone), cytokine priming induced a significant upregulation of all the immunomodulatory genes studied: *IL6* ($p < 0.05$; Figures 2A,B), *COX2* ($p < 0.05$; Figures 3A,B), *IDO1* ($p < 0.01$; Figures 4A,B), and *VCAM1*

($p < 0.01$; Figures 4E,F). The expression of *iNOS2* was not detected in MSC-naive at the baseline, but it was activated in MSC-primed. Therefore, the expression of *iNOS2* is presented as relative expression (fold change) over baseline MSC-primed (cultured alone), instead of MSC-naive. Following the same trend, the stimulation with cytokines induced IL6 and PGE_2 secretion in MSC-primed at the baseline, but significant differences could not be found over MSC-naive as these molecules were only detected in MSC-naive from one donor and at very low concentration (Figures 2C,D, 3C,D).

Effect of activated lymphocytes on the equine MSC immunomodulatory profile

The exposure of MSC-naive to activated PBLs upregulated the expression of different immunomodulatory genes compared with the baseline, regardless of the compatibility scenario (autologous or allogeneic MHC-matched/-mismatched). Specifically, MSC-naive exposed to activated lymphocytes significantly upregulated *IL6* ($p < 0.0001$ in all conditions; Figure 2A), *COX2* ($p < 0.001$ in all conditions; Figure 3A), and *IDO1* (autologous, $p < 0.01$; matched, $p < 0.001$; mismatched, $p < 0.001$; Figure 4A). *iNOS* and *VCAM1* expression also increased compared with the baseline, but these changes were not statistically significant (Figures 4C,E).

MSC-primed exposed to activated lymphocytes showed higher expression than MSC-naive for *IL-6* (matched and mismatched co-cultures, $p < 0.05$; Figure 2A) and *COX2* (matched co-culture, $p < 0.05$; Figure 3A). However, compared with the baseline (MSC-primed alone), the levels of *IL-6* were not further increased by the presence of activated lymphocytes (Figure 2A). On the other hand, *COX2* did experience a significant upregulation compared with the baseline in autologous and MHC-matched co-cultures ($p < 0.05$ for both conditions; Figure 3A). *iNOS2* was also overexpressed after exposure of MSC-primed to activated lymphocytes, but the differences compared with the baseline were not statistically significant (Figure 4C). In contrast to *IL6*, *COX2*, and *iNOS2*, *IDO1* and *VCAM1* were markedly downregulated in MSC-primed exposed to all the types of activated lymphocytes: autologous (*IDO1*, $p < 0.01$; *VCAM1*, $p < 0.05$), matched (*IDO1*, $p < 0.001$; *VCAM1*, $p < 0.01$), and mismatched (*IDO1*, $p < 0.01$; *VCAM1*, $p < 0.01$; Figures 4A,E). Interestingly, there was a clear trend for *VCAM1* downregulation depending on the type of activated lymphocytes, with the mismatched co-culture leading to the greatest reduction ($p < 0.05$ compared with matched, $p < 0.01$ compared with autologous; Figure 4E). Nevertheless, both *IDO1* and *VCAM1* remained higher in MSC-primed than in MSC-naive. This difference was significant for *IDO1* in the mismatched co-cultures ($p < 0.05$), which was also higher than that for the matched co-cultures ($p < 0.05$; Figure 4A) and for *VCAM1* in both allogeneic matched and mismatched co-cultures ($p < 0.05$ for both conditions; Figure 4E). Finally, differences in the expression of *IL6* and *COX2* were not found between MSCs exposed to autologous, matched and mismatched co-cultures, neither for MSC-naive nor for MSC-primed (Figures 2A, 3A).

In agreement with gene expression changes, the secretion of IL6 and PGE_2 increased compared with the baseline when MSCs were exposed to activated lymphocytes, suggesting that this environment activates MSC immunomodulatory potential. MSC-primed tended to produce more IL-6 and PGE_2 than MSC-naive, but significant differences were not observed as in gene expression. The highest concentrations of these molecules were found in the autologous co-cultures, which produced significantly more IL6 (MSC-naive, $p < 0.01$; Figure 2C) and PGE_2 (MSC-naive and MSC-primed, $p < 0.05$) than the baseline and the matched co-culture (PGE_2, MSC-naive and MSC-primed, $p < 0.05$; Figure 3C).

Effect of resting lymphocytes on equine MSC immunomodulatory profile

In contrast to that observed after the exposure to activated lymphocytes, equine MSCs co-cultured with resting lymphocytes showed a downregulation of their immunomodulatory profile. MSC-naive notably downregulated the expression of *IL6* ($p < 0.05$ in all conditions; Figure 2B) and *COX2* (non-significant; Figure 3B), while *IDO1* expression remained low in all the three types of co-cultures (Figure 4B). The expression of *iNOS2* was not detected in MSC-naive exposed to autologous resting lymphocytes and was low in the matched and mismatched co-cultures (Figure 4D). On the contrary, *VCAM1* was upregulated in MSC-naive after being in contact with resting lymphocytes, but these changes were not statistically significant (Figure 4F). In terms of secretion, IL6 was not significantly induced in MSC-naive by resting lymphocytes, and only a slightly higher amount of this molecule was detected after the co-cultures (Figure 2D). In contrast to that observed for *COX2* gene expression, the exposure of MSC-naïve to resting lymphocytes led to an increased PGE_2 secretion compared with the baseline, although, in general, at lower levels than their exposure to activated lymphocytes. Specifically, this increase was statistically significant when MSC-naïve were exposed to resting mismatched lymphocytes ($p < 0.05$; Figure 3D).

Overall, the expression of immunomodulatory genes remained higher in MSC-primed than in MSC-naïve in all the co-cultures with resting lymphocytes (*IL-6*, matched and mismatched, $p < 0.05$, Figure 2B; *COX2*, non-significant, Figure 3B; *IDO1*, mismatched, $p < 0.05$, Figure 4B; *iNOS2*, matched and mismatched, $p < 0.05$, Figure 4D; *VCAM1*, matched and mismatched, $p < 0.05$, Figure 4F). Nevertheless, and similarly to MSC-naive, MSC-primed co-cultured with autologous and matched resting lymphocytes showed a reduced expression of *IL6* ($p < 0.05$ and $p < 0.01$, respectively; Figure 2B), *COX2* (non-significant; Figure 3B), and *IDO1*

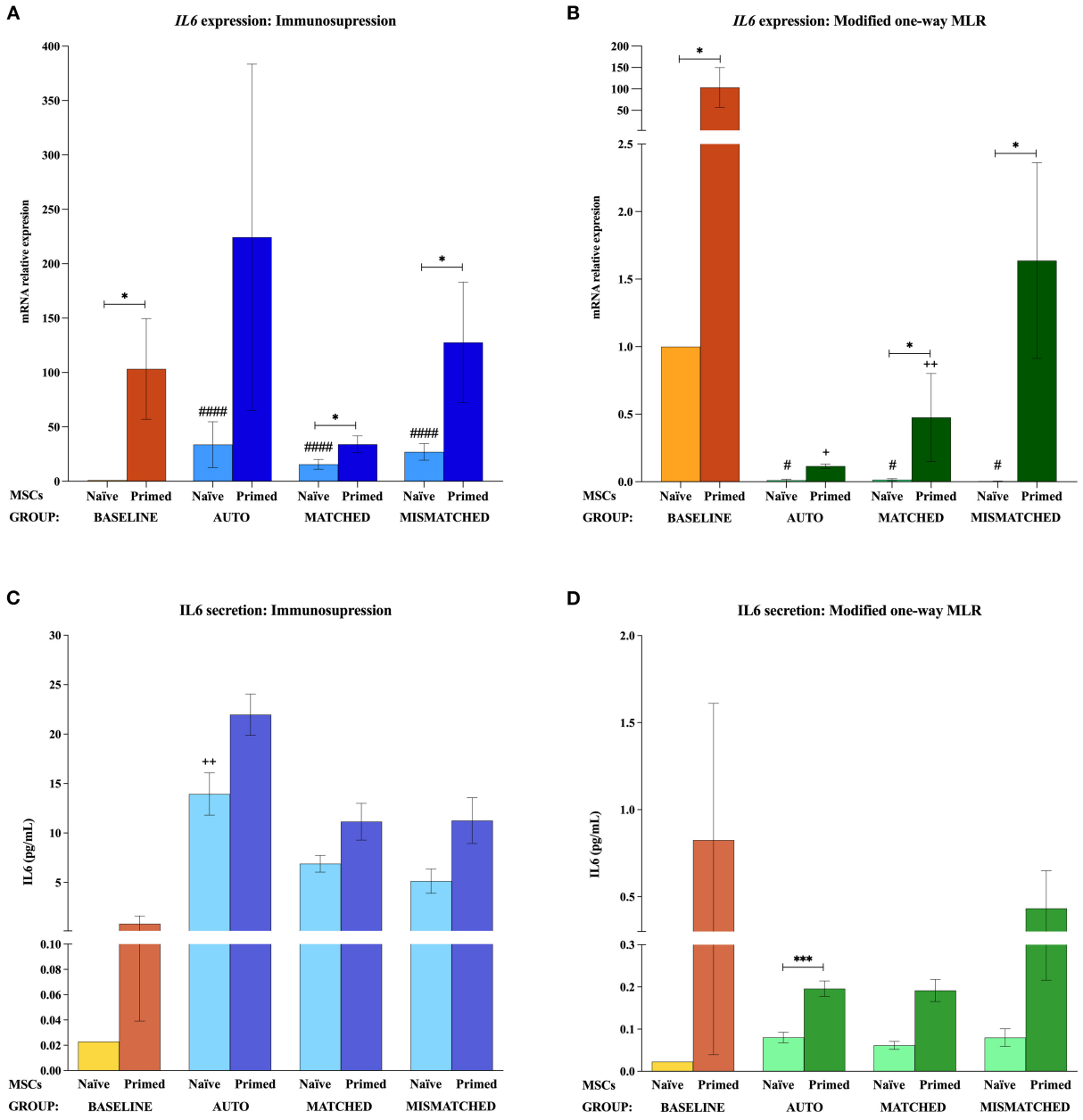

FIGURE 2
Interleukin 6 (IL6) gene expression and secretion by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *IL6* mRNA relative expression and **(C)** IL6 secretion (pg/mL) before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *IL6* mRNA relative expression and **(D)** IL6 secretion before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$), allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Concentration of IL6 in the supernatant from the different conditions is represented as mean ± S.E.M (pg/mL). Significant differences of each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{++}p < 0.01$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$; $^{***}p < 0.001$).

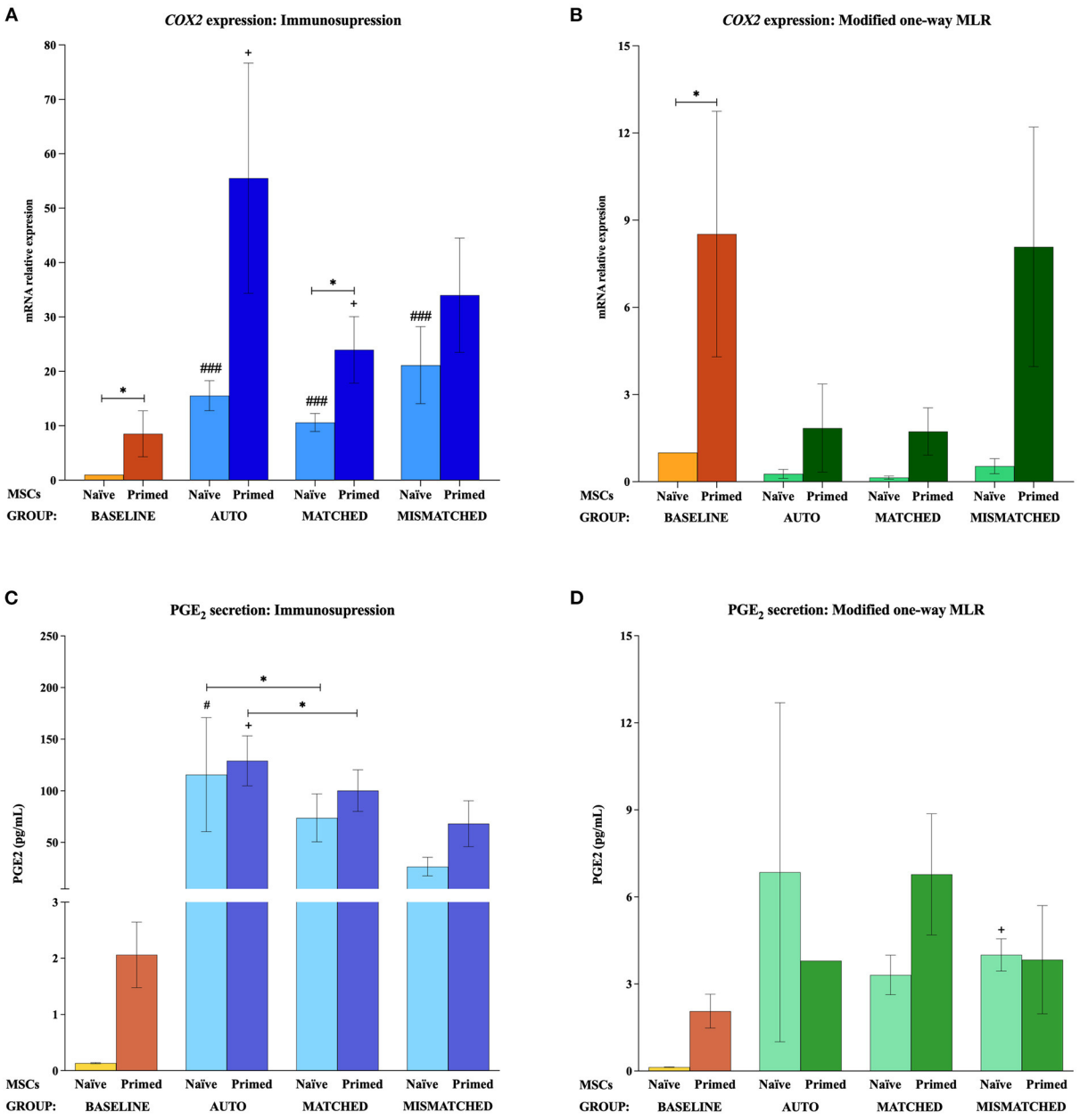

FIGURE 3
Cyclooxygenase 2 (COX2) gene expression and prostaglandin E2 (PGE_2) secretion by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *COX2* mRNA relative expression and **(C)** PGE_2 secretion (pg/mL) before (baseline; orange bars) and after equine MSC-naive (light blue bar) and MSC-primed (dark blue bar) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *COX2* mRNA relative expression and **(D)** PGE_2 secretion before (baseline; orange bars) and after MSC-naive (light green bar) and MSC-primed (dark green bar) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Concentration of PGE_2 in the supernatant from the different conditions is represented as mean ± S.E.M (pg/mL). Significant differences of each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$).

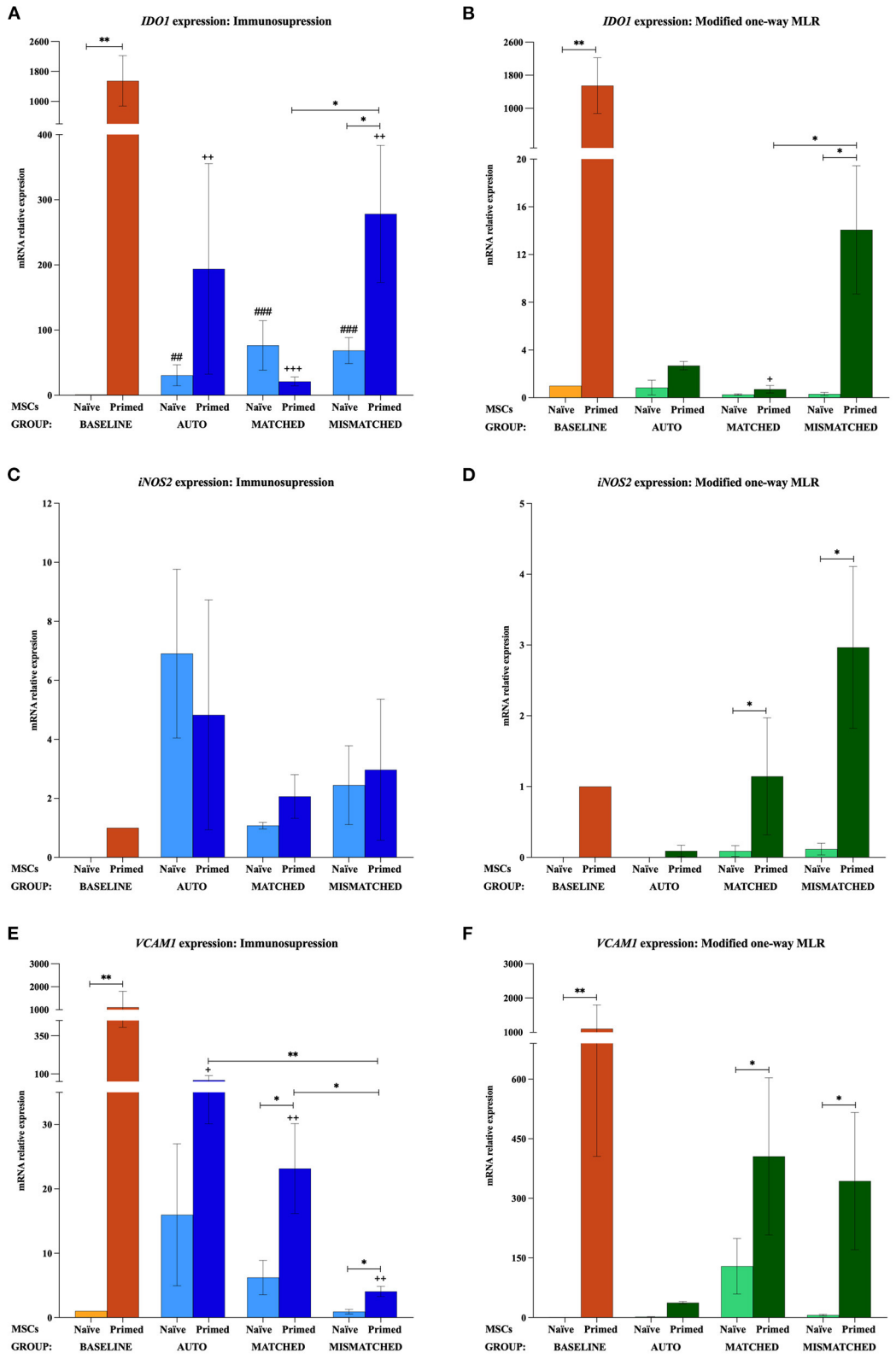

FIGURE 4
Changes in indoleamine 2,3-dioxygenase 1 (*IDO1*), inducible nitric oxide synthase 2 (*iNOS2*), and vascular cell adhesion molecule 1 (*VCAM1*) expression by equine mesenchymal stem cells (MSCs) in the different scenarios **(A)** *IDO1*, **(C)** *iNOS2*, and **(E)** *VCAM1* mRNA relative expression before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to

(Continued)

FIGURE 4 (Continued)
phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *IDO1*, **(D)** *iNOS2*, and **(F)** *VCAM1* mRNA relative expression before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in *IDO1* and *VCAM1* expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Baseline MSC-primed are used as reference sample (dark orange bar, value 1) for *iNOS2* since no expression of this gene was detected in baseline MSC-naive. Significant differences of each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$; $^{**}p < 0.01$).

(matched co-culture, $p < 0.05$; Figure 4B). Accordingly, co-cultured MSC-primed also decreased their secretion of IL6 compared with the baseline but still secreted higher concentrations of this molecule than MSC-naïve ($p < 0.001$ in autologous settings; Figure 2D). Interestingly however, MSC-primed co-cultured with mismatched resting lymphocytes showed the highest expression of *IL6*, *COX2*, *IDO1*, and *iNOS2* among all the co-cultures, even though the differences were not statistically significant, except for *IDO1* (compared with matched co-culture, $p < 0.05$; Figure 4B). Furthermore, the same was observed in terms of IL6 secretion, with the highest concentration of this molecule being produced by MSC-primed exposed to mismatched lymphocytes (Figure 2D), although the difference was not statistically significant. Even though this was also the case for *COX2* gene expression, the same was not replicated at the level of PGE_2 secretion, and the production of this molecule did not follow a clear trend (Figure 3D).

Gene expression of markers related to equine MSC immunogenicity

The analysis of *MHC-I* could only be carried out with the MSCs from two of the donors (D2 and D3) since the MSC-naive and MSC-primed from the other donor (D1) did not express *MHC-I* neither at the baseline (MSCs cultured alone) nor after their exposure to autologous or allogeneic MHC-matched lymphocytes, regardless of these being activated or resting. Interestingly, *MHC-I* gene expression was detected in the MSCs of this donor after these were co-cultured with allogeneic MHC-mismatched lymphocytes, either activated or resting. However, the lack of reference values to establish the relative expression of *MHC-I* prevented to include the data from the D1 donor in the analysis. Even though data from only two donors were used for *MHC-I*, a consistent trend on its expression could be observed, which was very similar to that for *MHC-II* and *CD40*.

The gene expression of *MHC-I*, *MHC-II*, *CD40*, and *CD80* was not increased by the priming at the baseline (MSCs cultured alone). *MHC-I* and *MHC-II* were only upregulated when MSCs were both primed and exposed to lymphocytes, regardless of these being activated or resting, autologous or allogeneic MHC-matched/-mismatched (Figure 5). Activated lymphocytes also induced *CD40* upregulation in MSC-primed but only if mismatched, while resting lymphocytes produced this increase in all the three types of co-cultures. On the contrary, activated lymphocytes tended to downregulate *CD80* expression in MSCs, and resting lymphocytes tended to increase it but only in MSC-naive (Figure 6).

Effect of activated lymphocytes on equine MSC immunogenic profile

When MSC-primed were exposed to activated matched or mismatched lymphocytes, *MHC-I* was significantly overexpressed compared with MSC-naive ($p < 0.05$ for both conditions; Figure 5A), and *MHC-II* increased significantly compared with the baseline ($p < 0.05$ for both conditions; Figure 5C). *MHC-II* overexpression in MSC-primed was also significant compared with MSC-naive in the MHC-matched co-cultures ($p < 0.05$). However, *CD40* was upregulated only in MSC-primed exposed to activated mismatched lymphocytes, and this increase was statistically significant compared with the baseline, the corresponding MSC-naive, and the MSC-primed exposed to autologous and matched activated lymphocytes ($p < 0.05$ for all conditions; Figure 6A). On the other hand, activated lymphocytes led to a reduction of *CD80* expression in MSC-naive in matched and mismatched co-cultures compared with the baseline, the corresponding MSC-primed, and the autologous co-culture ($p < 0.05$ for all conditions; Figure 6C).

Effect of resting lymphocytes on equine MSC immunogenic profile

The exposure to resting lymphocytes upregulated *MHC-I*, *MHC-II*, and *CD40* in MSC-primed compared with MSC-naive, while *CD80* was increased in MSC-naive compared with MSC-primed. Specifically, *MHC-I* increased in MSC-primed in both matched and mismatched co-cultures ($p < 0.05$ for both conditions), this upregulation being significantly higher in the mismatched co-culture than the matched ($p < 0.05$) and the baseline ($p < 0.05$; Figure 5B). In line with this, *MHC-II* and *CD40* increased compared with

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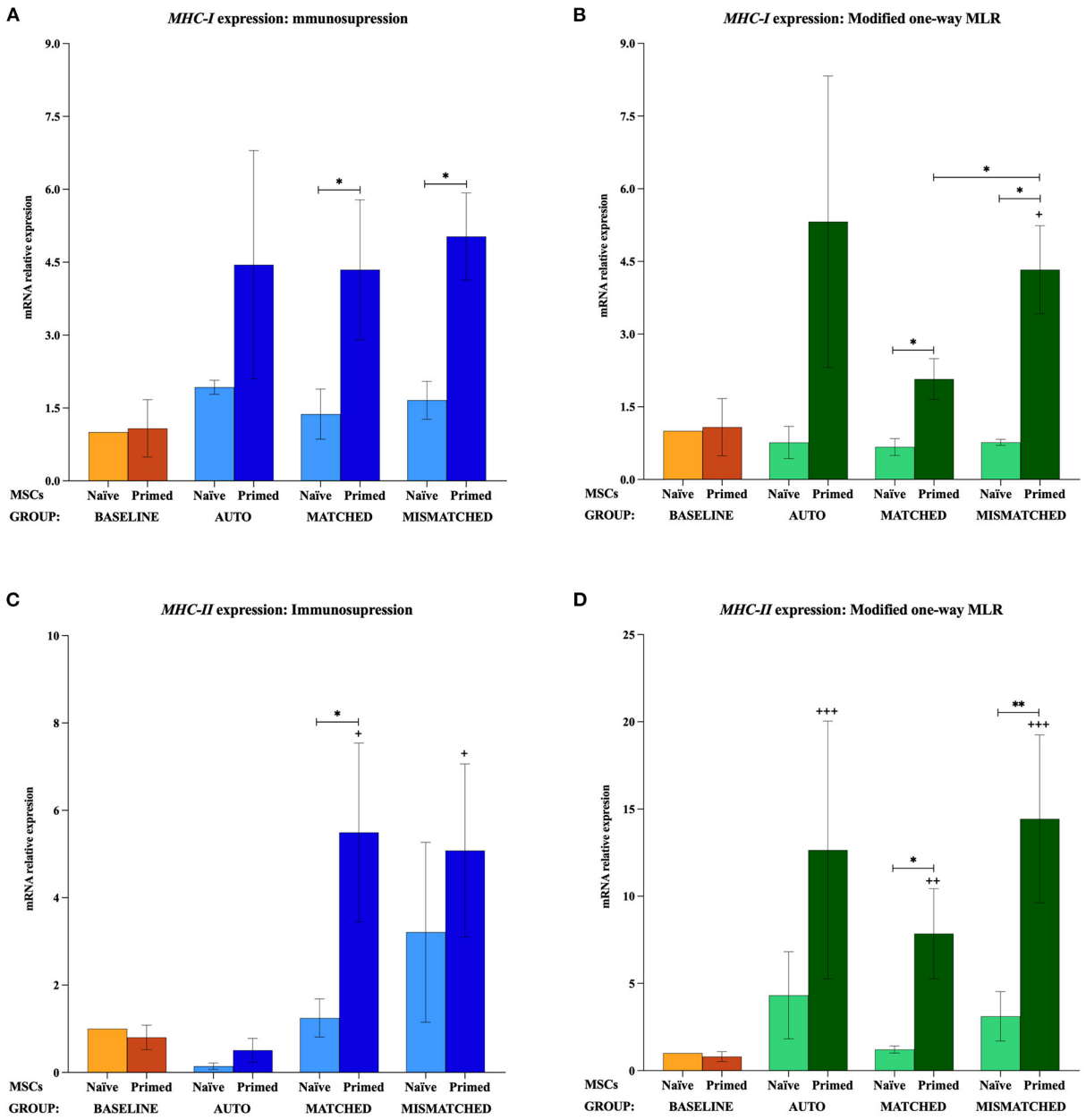

FIGURE 5
Changes in major histocompatibility complex (*MHC*)-*I* and MHC-*II* expression by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *MHC-I* and **(C)** *MHC-II* mRNA relative expression before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *MHC-I* and **(D)** *MHC-II* mRNA relative expression before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the MHC. Data from the D1 donor could not be included in the *MHC-I* analysis because of the lack of reference values since this gene was not expressed at the baseline. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$; $^{**}p < 0.01$).

the baseline in MSC-primed exposed to all the three types of co-cultures (*MHC-II:* $p < 0.001$ autologous, $p < 0.01$ matched, $p < 0.001$ mismatched; *CD40:* $p < 0.05$ autologous, $p < 0.05$ matched, $p < 0.001$ mismatched) (Figures 5D, 6B). Moreover, *MHC-II* was significantly overexpressed compared with MSC-naive in the allogeneic matched and

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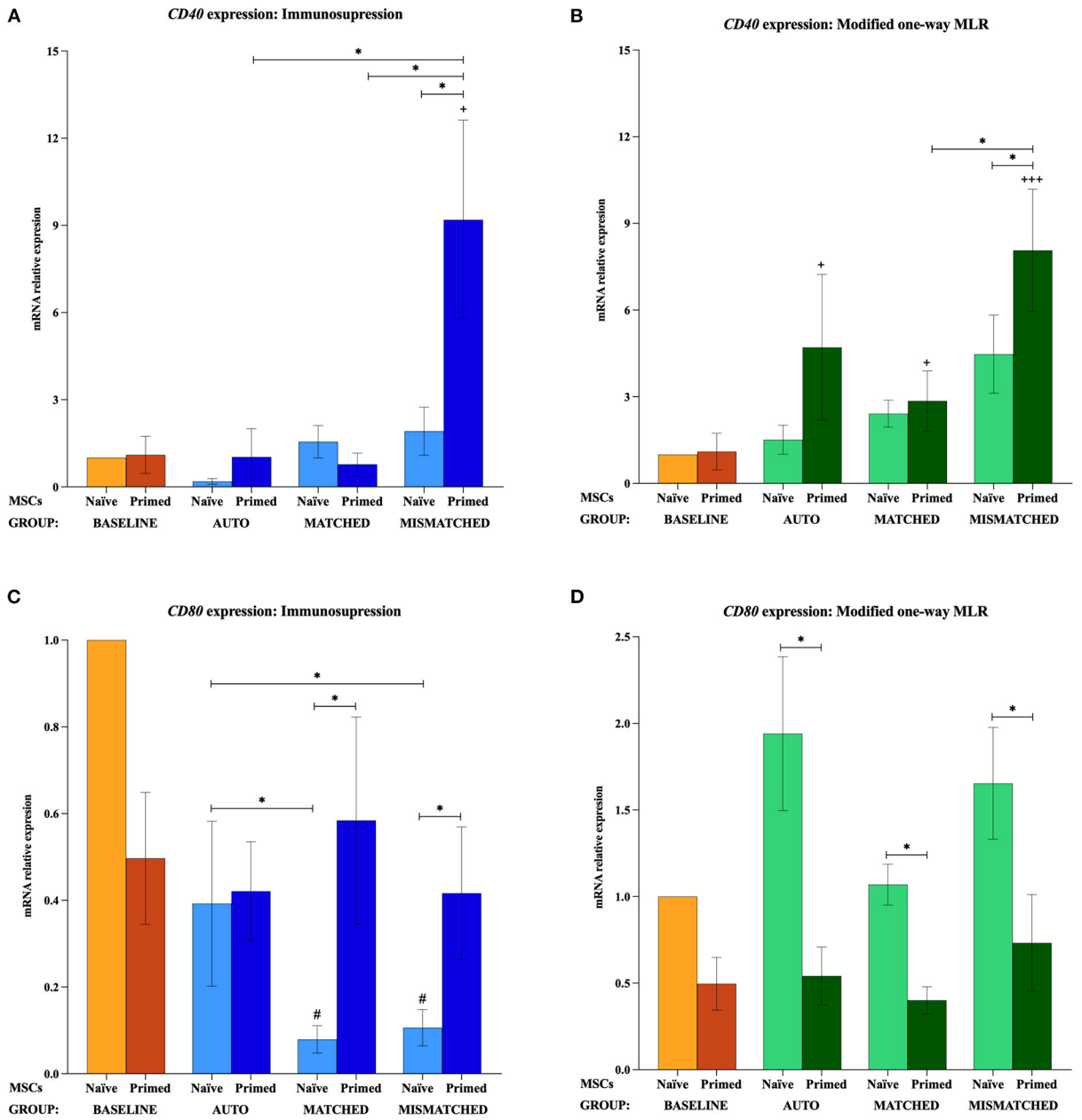

FIGURE 6
Changes in costimulatory molecules *CD40* and *CD80* expression by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *CD40* and **(C)** *CD80* mRNA relative expression before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *CD40* and **(D)** *CD80* mRNA relative expression before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Significant differences in each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#}p < 0.05$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{+++}p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$).

mismatched co-cultures ($p < 0.05$ matched, $p < 0.01$ mismatched). Similarly, the highest *CD40* expression was detected in MSC-primed exposed to mismatched resting lymphocytes, which was significantly increased compared with the corresponding MSC-naive ($p < 0.05$) and the matched co-culture ($p < 0.05$; Figure 6B). In contrast to these

findings, the expression of *CD80* in MSCs exposed to resting lymphocytes was higher in MSC-naive than in MSC-primed in all the three co-cultures ($p < 0.05$ for all conditions; Figure 6D).

Discussion

The role of the immunomodulation and immunogenicity of equine MSCs seems to be key for their therapeutic actions and to evade the immune system in the allogeneic administration. The environment that is encountered by MSCs greatly influences their immune properties, and the changes experienced by these cells in response can either benefit their potency (i.e., increased regulatory capacity) or compromise their effectiveness and safety (i.e., immune targeting and elimination) (6). Therefore, the knowledge on how MSCs respond to different stimuli is key to optimize cell therapies for veterinary and human patients. Although several studies have explored the effects of licensing MSCs with different cytokines and ligands, as well as the changes elicited by these MSCs on different populations of immune cells (9, 32), little is known about how immune cells lead to changes in equine MSCs that may have therapeutic implications. To the best of authors' knowledge, this is the first report on the changes experienced by equine MSCs in their immunomodulatory and immunogenic profiles upon exposure to cytokine priming and/or lymphocytes in different combinations (activated/resting; autologous/MHC-matched/MHC-mismatched). According to our initial hypothesis, co-culture of equine MSCs with activated lymphocytes resulted in an increased gene expression and secretion of immunomodulatory molecules, especially in MSC-primed. However, resting lymphocytes did not elicit remarkable changes, except when MSCs were previously primed and MHC-mismatched. As we hypothesized, a moderate activation of the equine MSC immunogenic profile was also observed, more markedly for MSC-primed but similar between activated and resting lymphocytes. We also hypothesized that MHC-mismatched MSCs would display similar immunomodulation but increased immunogenicity, which we indeed observed mostly for MSC-primed. Interestingly, the increased expression of immunogenic markers seemed to be accompanied by a further activation of the regulatory profile, which might equilibrate the balance between both properties in equine MSCs.

Prior to engaging into further discussion of our results, it is important to bear in mind the limitations of this study. First, the sample size in the experiments presented is limited due to the implications of working with a large species such as the horse (2), and it is particularly small for the baseline measurements and the autologous co-cultures ($n = 3$). Related to this, even though all horses enrolled in this study had similar characteristics (age, origin, breed, weight), the baseline gene expression of their MSCs and their response to the different conditions considerably varied. Thus, the inter-individual variability among the different donors can also be considered as a limitation and may have prevented to observe further significant differences. To account for this variability, the values of each donor were normalized compared with their corresponding baseline values. In addition, the difficulty in finding MHC-homozygote MSC donors that can be paired with PBL donors should be considered, taking the high variability of ELA haplotypes into account (10, 17). Another limitation is that not all of the gene expression results could be compared to the secretion of the molecule or its surface expression. There are some contradictory reports on iNOS2 and IDO1 activity in the equine MSC supernatant (26). For instance, Cassano et al. (33) described that the increased gene expression of *IDO1* by equine MSCs upon IFNγ stimulation is not enough to be translated into an actual increase in *IDO1* activity. Therefore, we decided to assess the gene expression of these enzymes and focus on IL6 and PGE_2 to conduct ELISA as the role of these mediators has been more consistently reported in equine MSCs (34). The surface expression of the other molecules could not be assessed partly because of the lack of appropriate antibodies for the equine species (35) and partly because of the insufficient number of MSCs to conduct both RT-qPCR and flow cytometry. Therefore, gene expression was prioritized in this study as it has been widely used in the equine MSC literature and can provide relevant information on the changes experienced by these cells (7, 36).

Several authors agree that PGE_2 is the primary mediator responsible for inhibition of lymphocyte proliferation by equine MSCs from different sources, including BM, adipose tissue (AT), umbilical cord blood (CB), and umbilical cord tissue (CT) (4, 33). PGE_2 is produced by COX2, so both the concentration of the soluble molecule and the gene expression of the enzyme are usually assessed in equine studies (26, 27, 30). The secretion of IL6 may not be mainly involved in the inhibition of T-cell proliferation by equine MSCs (30), but studies in other species agree that IL6 may contribute to a more efficient immunosuppression of B lymphocytes (37, 38). iNOS2 is mainly involved in the immune regulatory effects of rodent MSCs, while MSCs from other mammalian species (e.g., monkey, pig, dog, cattle, and human) preferentially use IDO1 (38–41). Equine studies have reported different results regarding iNOS2 participation. Carrade et al. (4) described that NO production by equine MSCs varies from different tissue sources, and subsequent studies have shown that iNOS2 inhibition does not change the inhibitory effect of equine MSCs on lymphocyte proliferation (26, 30, 42). IDO1 activity does not seem to be involved in the capacity of equine MSCs to inhibit allogeneic lymphocyte proliferation (26) but may participate in maintaining this suppressive effect (42). In addition to soluble mediators, cell–cell interactions between MSCs and lymphocytes *via* adhesion molecules may increase the effectiveness of the MSC immunomodulation (43). Indeed, some authors agree that *VCAM1* is only expressed upon direct close contact between

MSCs and lymphocytes, so this molecule may play a key role in the immunosuppressive functions of MSCs (37).

The immunomodulatory actions of MSCs are closely related not only with their therapeutic mechanisms but also with their ability to evade the immune system, in which the expression of immunogenic molecules also plays a role. Expression of MHC-I by MSCs may result in their immune recognition and elimination since cytotoxic T cells attack foreign cells bearing MHC-I receptors that are bound to an alloantigen (44). On the other hand, natural killer (NK) cells can attack cells lacking MHC-I on their surface (45), so the expression of MHC-I, although weak, protects MSCs from NK cell-mediated elimination (46). In addition, expression of MHC-II can also lead to MSC targeting, so its lack confers these cells the ability to escape immune recognition by CD4 helper cells (46). The cell surface expression of MHC-I and II on equine MSCs vary from one donor to another and even among MSC samples (47, 48), so a recent study proposed to classify equine MSCs as MHC class II-high or -low (7). According to this, MSCs from the three donors in this study would be classified as MHC-II low but would be considered MHC-II high after cytokine priming (16). In addition to MHC complexes, other costimulatory molecules are involved in the antigenic presentation and may have an impact on the immune recognition of MSCs. CD40 plays an important role in allograft rejection (49, 50), and its expression in human MSCs may contribute to an effective activation of T cells (51). Furthermore, the coupling ligand of CD80 to the CD28 receptor is the first signaling pathway necessary for T-cell costimulation and enhances T-cell proliferation and cytokine secretion (51). Equine MSCs from peripheral blood and CB-MSCs showed a moderate to strong expression of these costimulatory molecules (35); however, their role in the immune properties of equine MSCs is largely unknown.

All the molecules and/or genes assessed in the current study have been reported to change its expression and/or secretion in response to an inflammatory environment. Specifically, TNFα and IFNγ are considered inductors of immunomodulatory mediators by MSCs from different species and sources, including the horse (38, 42, 50, 52). In this study, the gene expression and secretion of immunomodulatory molecules were induced after the priming in MSCs, whereas the immunogenic markers were not upregulated in this condition; that is, the cytokine exposure alone could activate the immune regulatory profile of equine BM-MSCs without affecting their immunogenic profile. Similarly to our findings, TNFα and IFNγ have been reported to upregulate *VCAM1* in murine MSCs, and this priming rendered MSCs more adhesive to CD8+ T cells, CD4+ T cells, and CD3+ T cells (53). Other reports also observed an upregulation of the expression and secretion of *IL6* after cytokine priming of equine MSCs (15, 42, 54), as well as of the gene expression of *COX2* and secretion of PGE_2 after exposure to TNFα and IFNγ (5, 36, 42). Priming with IFNγ also resulted in significant induction of *iNOS2* and *IDO1* expression in equine MSCs in previous works (36, 42), whereas other studies did not detect its expression after priming (33). Interestingly, MSCs from the three donors in this study showed no expression of *iNOS2* at the baseline unless MSCs were primed, suggesting that some regulatory factors are only expressed upon activation, and this licensing may depend on the type and degree of stimulation.

Even though there are several reports on the effect of cytokine priming on equine MSCs, there is limited information on the changes in the immune profile of these cells after being challenged by lymphocytes. This would more closely resemble the *in vivo* environment that MSCs encounter after administration, in which the immune system may already be activated (inflammation at the injury site, immune-mediated disease) or may be stimulated in response to the MSCs (immunogenic recognition). In our study, when MSC-naive were exposed to activated lymphocytes, the gene expression of *IL6*, *COX2*, and *iNOS2* was upregulated, and the secretion of IL6 and PGE_2 increased to levels similar to or even higher than those after cytokine priming alone. Moreover, in MSC-primed, the baseline expression and secretion of the same mediators were further increased after the co-culture with activated lymphocytes. The consensus on the role of PGE_2 and IL6 in equine MSC immunomodulation is quite broad, so their upregulation upon cytokine priming and/or exposure to activated lymphocytes observed in this study agrees with previous knowledge (30, 36). However, as aforementioned, there is controversy on the participation of *IDO1* and *iNOS2* in the regulatory mechanisms of equine MSCs. Whereas, the dynamics of *iNOS2* gene expression followed the same trend as *IL6* and *COX2* in this study, the expression of *IDO1* decreased in MSC-primed co-cultured with activated lymphocytes. Some studies did not detect IDO1 activity in the supernatants of co-cultures with equine MSCs and PBMCs, either PHA activated or not, suggesting that this pathway may not be functionally active and that equine MSCs failed to produce IDO1 in the presence of stimulated T cells (4, 26). Along with our findings, it may be suggested that the induction of this molecule may depend on the type of stimuli and may require further stimulation to remain active. Similarly to that observed for *IDO1*, *VCAM1* was also downregulated in MSC-primed exposed to activated lymphocytes, which seemed to be influenced by the type of co-culture. While we do not have a clear hypothesis for this observation, it may also be related with a potentially different regulatory ability of equine MSCs in different contexts. Overall, these findings show that activated lymphocytes constitute an environment able to stimulate equine MSCs, so these could be similarly licensed *in vivo*. Furthermore, if MSCs are already primed by cytokines, activated lymphocytes can further contribute to the upregulation of modulatory factors, suggesting that the effect of both stimuli might be additive.

On the contrary, when MSCs were exposed to resting lymphocytes, IL6 secretion and the majority of the immunomodulatory genes were downregulated in both

MSC-naive and MSC-primed. *COX2* gene expression followed the same pattern of downregulation, but PGE_2 secretion differed from this tendency. Even though the deviation observed in the PGE_2 concentration prevents outlining a clear trend, higher levels of PGE_2 were overall observed in MSCs exposed to resting lymphocytes than in MSCs alone. The regulation on eicosanoid pathways is complex and happens at different levels (55), and the data of this study do not allow establishing a definitive explanation for this discrepancy between gene expression and molecule secretion. We have two potential hypotheses for this observation: First, an initial activation of *COX2* could have happened followed by a downregulation, but PGE_2 secreted upon activation could still be present in the supernatant. Second, a regulatory loop could have taken place in which PGE_2 in the medium would have downregulated the expression of *COX2*. In spite of this discrepancy, the general pattern was toward downregulation of the equine MSC modulatory profile, which may be due to the lack of further activation exerted by resting lymphocytes. Thus, the baseline expression of regulatory markers will be reduced after 5 days if MSCs are no longer stimulated. Similarly, a previous report from our group found that the overexpression of these markers induced by cytokine priming diminishes after 7 days in MSCs cultured alone (15). Therefore, it can be hypothesized that resting lymphocytes did not promote or maintain the licensing of equine MSCs, indirectly reflecting their own lack of activation.

Interestingly, the higher regulatory profile in MSC-primed exposed to resting MHC-mismatched lymphocytes may be related to the activation of the latter upon encountering mismatched MSCs as these also presented increased expression of *MHC I*, *MHC-II*, and *CD40* in this condition. The overexpression of these immunogenic markers may facilitate the allo-recognition of MSCs by lymphocytes, which response might activate the regulatory profile of MSCs, and this could facilitate their immune escape. The increased expression of *MHC-I* and *MHC-II* after priming equine MSCs has been previously reported to different extent (9, 15, 36). Similarly, previous studies reported an increase in *MHC-II* expression after equine MSCs were exposed to conditioned media from PBMCs (26). The overexpression of *MHC-I* and *MHC-II* in MSC-primed alone was not observed in this study, but these markers were induced after exposure of MSC-primed to both activated and resting lymphocytes, regardless of the MHC compatibility. It has also been reported that the costimulatory molecule *CD40* could be upregulated on MSCs under inflammatory conditions in different species. In human studies, ∼50% of AT-MSCs expressed *CD40* (50), and cytokine priming enhanced the inhibitory function of MSCs derived from tonsils when expression of *IDO1* and *CD40* increased (49). However, in the conditions of this study, *CD40* was not modified by priming by exposure to activated lymphocytes, separately, and *CD40* was only overexpressed upon simultaneous priming and co-culture with mismatched activated lymphocytes. Previous studies reported that cytokine priming of human MSCs did not increase *CD80* expression as it happens with other immune markers; furthermore, *CD80* expression could be downregulated in MSCs after priming (56, 57). Similarly, in this study, *CD80* expression was reduced upon priming, and its expression after co-culture with activated lymphocytes remained low. Curiously, *CD80* expression was higher in MSC-naive after exposure to resting lymphocytes, differing from the tendencies observed for other genes. Taken together, these findings suggest that different simultaneous stimuli are needed to induce the immunogenic profile of equine MSCs, while a single stimulus would be able to induce their immune regulatory potential.

Even though these observations were not directly correlated with functional implications in this work, it is worth discussing how these changes might translate into immune suppression and immune recognition mechanisms. Previous studies have found that the ability of pro-inflammatory primed equine MSCs to suppress the proliferation of allogeneic activated T cells is enhanced, but these primed cells could also lead more easily to immune activation (32, 42), which would agree with the expression patterns seen in our study. Furthermore, a previous work from our group found that equine MSCs, either naive or primed, were able to change the frequency and proliferation of different subsets of equine activated or resting lymphocytes (16).

In the immunosuppressive assays (activated PBLs) of the current study, the increase in regulatory gene expression and secretion by MSC-primed would agree with our previous results in which the capacity of suppressing CD3+ T cells, CD4+ T cells, CD8+ T cells, and B cells was enhanced in MSC-primed compared with MSC-naive (16). Specifically, PGE_2 can downregulate the proliferation of cytotoxic T cells and B-cell activation (58) and is considered the main factor conferring the ability of equine MSCs to suppress lymphocyte proliferation (26, 30, 42). Even though the role of IL6 in equine MSC immunomodulation needs further elucidation, it has been seen in other species that this molecule is involved in the suppression of B lymphocytes (37, 38). Therefore, the higher *IL6* expression and secretion by MSC-primed might be related with the stronger suppression of B cells in immunosuppression assays and the lack of induction of B cells in modified one-way MLR assays, as observed in a previous study (16). Furthermore, the changes in the profile of equine MSCs may also be implicated in their ability to induce changes in the subpopulation of T reg cells. Human BM-MSCs are known to promote immune suppression by inducing the production of T reg, which would downregulate the proliferation of CD8+ cytotoxic T cells (59). Specifically, PGE_2 secreted by human MSCs induces CD4+ T-cell differentiation into Tregs (39), and it has also been observed in other species that the overexpression of *COX2* prevents the downregulation in the number of CD4+CD25+ Treg cells (58). While this is not well established in horses, in our previous work, the presence of equine MSC-naive and MSC-primed increased the percentage of CD4+ $CD25^{high}$ T cells in a population

of activated lymphocytes (16), which might be related with the overexpression of *COX2* and increased secretion of PGE_2 observed in the present work. Even though a direct relation cannot be established as these are found in separate studies, it is worth mentioning that the conditions in which equine MSCs showed higher *COX2* expression and PGE_2 secretion in the current study were comparable to those that displayed the highest suppressive capacity for CD8+ T cells (16).

By conducting modified one-way MLR assays with resting lymphocytes, we previously described that MSC-primed induced a proliferative response in cytotoxic and helper T cells, and this immunogenic response was more marked when the lymphocytes were MHC-mismatched with the MSCs. Similarly, MHC-mismatched MSC-primed can induce the proliferation of resting CD8+ cytotoxic cells, suggesting their immune recognition, but this condition also activates the T reg cells, which may counter the activation of the first ones (16). Interestingly, in the present study, we observed an induction of the regulatory profile precisely in MSC-primed exposed to resting mismatched lymphocytes, which may be a licensing effect conducted by the activation of the immune cells.

Regarding changes linked to the different MHC matching between equine MSCs and lymphocytes, we overall observed higher expression and/or secretion of regulatory molecules in MSCs in the autologous co-cultures with activated lymphocytes, followed by allogeneic MHC-matched and lastly by MHC-mismatched co-cultures. This observation might relate with a trend previously found for autologous MSCs to further elicit immune suppression of PHA-stimulated PBLs, followed by MHC-matched and mismatched MSCs (16). These are general patterns that cannot be directly compared, and this tendency is not reflected as significant differences among co-cultures for all the mediators assessed; however, it is particularly well-represented by *VCAM1*. Actually, it has been reported that the higher the expression level of *VCAM1*, the greater the MSC inhibitory capacity (53).

The results of this study show that an inflammatory environment can induce a regulatory profile in equine MSCs but can also increase their immunogenic expression. Similar findings have been previously reported, but the novelty of this study is to shed light on the effect of different conditions by directly comparing several scenarios. First, both cytokine priming and activated lymphocytes are able to induce the regulatory profile of equine MSCs separately, but the changes experienced by equine MSCs are different. Furthermore, the action of both stimuli appears to be additive, especially for immunogenic markers. Second, when MSCs have been primed and are specifically exposed to MHC-mismatched lymphocytes, their regulatory profile is further increased. This has been particularly noted when the co-culture was caried out with resting lymphocytes, where MSC-primed also increased their expression of the immunogenic markers *MHC-I*, *MHC-II*, and *CD40*. We hypothesize that such upregulation may facilitate the allo-recognition of foreign MSCs, and thus, the activation of lymphocytes could induce the regulatory profile of MSCs, which, at the same time, would facilitate their immune escape. This potential explanation is in line with the concept of immunomodulation–immunogenicity balance (6, 14), according to which MSCs are able to evade the immune response by equilibrating their capacities to suppress and to activate it. In conclusion, these findings highlight the plasticity of MSCs to respond to stimuli of different nature and degree, and the key role of the balance between their immune regulatory and immunogenic properties. This study also underscores the complexity of the interactions between MSCs and the immune system, giving clues on how these cells may behave once they are administered in the patients, which also can shed light on the mixed results usually obtained in *in vivo* studies. Although the actual clinical impact of these findings remains to be further explored, this information can facilitate the development of *in vivo* studies to further understand the immune properties of equine MSCs, which are key in the path toward safer and more effective cell therapies.

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 Advisory Ethics Committee for Animal Research from the University of Zaragoza (Project License PI 15/16).

Author contributions

LB, AC, EB, and IG were responsible for acquisition of data. AC, LB, IG, FV, and CR were responsible for analysis and interpretation of data. AR, AV, EB, MG-M, and FV provided technical support. AR, FV, and CR provided important conceptual guidance. CR was responsible for obtaining funding. LB and AC drafted the manuscript. FV, AR, AV, EB, MG-M, IG, and CR revised the manuscript for important intellectual content. All authors substantially contributed to the conception, design of the study, approved the final submitted version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and agreed to the published version of the manuscript.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.957153/full#supplementary-material

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Regenerative medicine in lung diseases: A systematic review

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Regenerative medicine has opened the door to the exploration of new therapeutic methods for the treatment of various diseases, especially those associated with local or general disregulation of the immune system. In pulmonary diseases, new therapeutic strategies have emerged that are aimed at restoring functional lung tissue rather than alleviating symptoms. These strategies focus on tissue regeneration using stem cells and/or their derivatives or replacement of dysfunctional tissue using biomedical engineering. Animal health can directly benefit from regenerative therapy strategies and also serve as a translational experimental model for human disease. Several clinical trials have been conducted to evaluate the effects of cellular treatment on inflammatory lung disease in animals. Data reported to date show several beneficial effects in *ex vivo* and *in vivo* models; however, our understanding of the mechanisms that regenerative therapies exert on diseased tissues remains incomplete.

KEYWORDS

lung, regenerative medicine, veterinary medicine, lung diseases, cellular therapies, biomedical engineering

Introduction

Several chronic respiratory diseases in humans and animals remain incurable. Treatments have been relatively successful in relieving some symptoms, but they all ultimately lead to a poorer quality of life and are one of the leading causes of death worldwide (1). Intense and persistent inflammation leads to loss of functional tissue and pulmonary tissue remodeling, which in turn leads to loss of respiratory function. Over time, lung tissue changes are so severe that euthanasia is required in animals or lung transplantation is the only viable option to prolong life in humans (2–9).

The lung has an exceptional ability to respond and regenerate after the tissue injury (7, 9, 10). However, regeneration of lung tissue can often lead to pathological tissue remodeling and subsequent impairment of lung function (10). These changes in the lung can potentially be reversed through regenerative medicine in the form of cellular therapy, extracellular vesicle therapy (ECV), or even tissue engineering (4, 5, 7, 9–12). Several animal studies addressed regenerative therapeutic modalities in the lung in experimental models (13–38) and in clinical trials (39–41) (Table 1).

Although inflammation is the reason for damage to the airways, it is also critical for initiating tissue regeneration and restoration. Inflammatory cells flooding the airways are important for phagocytosis and for stimulating resident progenitor cells through secreted cytokines and growth factors. Some resident cell populations do not appear to exist in a healthy lung, but emerge only in response to lung injury. A more detailed knowledge of this relationship will likely enable new therapeutic options to stimulate lung regeneration and self-repair (43).

Data sources and searches

An online literature search was performed using the PubMed® (U.S. National Library of Medicine and National Institutes of Health) search engine (https://pubmed.ncbi.nlm.nih.gov/),

TABLE 1 List of selected references reporting the use of regenerative treatments for respiratory diseases in animals.

Disease	Species	Therapeutic strategy	Treatment outcomes	References
Acute lung injury	Mice	Bone marrow-derived mesenchymal stem cells	Treatment with intrapulmonary MSC markedly decreases the severity of endotoxin-induced acute lung injury and improved survival in mice	Gupta et al. (13)
	Rabbit	Bone marrow-derived mesenchymal stem cells	Decreased pro-inflammatory cytokines, increased anti-inflammatory cytokines, decreased lung water mass fraction, and ameliorated systemic inflammatory response.	Zhu et al. (15) Chen et al. (16)
	Mice	Human Umbilical cord blood-derived mesenchymal stem cells	Down-modulated inflammatory process and enhanced bacterial clearance.	Kim et al. (17) Sun et al. (19)
	Rats	Human umbilical cord blood-derived mesenchymal stem cells	Reduced systemic inflammation and attenuated ALI	Li et al. (21)
	Sheep	Human bone marrow-derived mesenchymal stem cells	Reduced severity of ALI	Asmussen et al. (22)
	Dogs	Human Umbilical cord blood-derived mesenchymal stem cells	Reduced lung injury.	Hao et al. (33)
	Pigs	Extracellular vesicle therapy	Attenuated influenza virus-induced acute lung injury.	Khatri et al. (34)
	Rats	Extracellular vesicle therapy	Alleviated lung injury and pulmonary fibrosis.	Gao et al. (42)
Acute respiratory distress syndrome	Sheep	Human bone marrow-derived mesenchymal stem cells	Ameliorated inflammation.	Rojas et al. (25) Sadeghian Chaleshtori et al. (37)
	Sheep	Adipose-derived mesenchymal stem cells	Attenuated pulmonary microvascular hyperpermeability.	Ihara et al. (32)
	Sheep	Bone marrow-derived multipotent adult progenitor cells	Recovered arterial oxygenation.	Cardenes et al. (36)
Asthma	Mice	Human bone marrow-derived mesenchymal stem cells	Decreased chronic inflammation	Bonfield et al. (14) Lee et al. (18) Mohammadian et al. (27) Cruz et al. (26)
	Mice	Adipose-derived mesenchymal stem cells	Ameliorated allergic airway inflammation.	Cho et al. (23) Mariñas-Pardo et al. (24) Dai et al. (29) Dai et al. (30)
	Horses	Bone marrow-derived mononuclear cells	Reduced airway inflammation	Barussi et al. (39)
	Mice	Bone marrow, adipose, and lung tissue-derived mesenchymal stromal cells	Reduced airway inflammation and remodeling and improved lung function.	Abreu et al. (28)
	Cats	Adipose-derived mesenchymal stem cells	Delayed effect in reducing airway inflammation, airway hyper-responsiveness and remodeling.	Trzil et al. (40)
	Mice	Human adipose-derived mesenchymal stem cells and their extracellular vesicles	Reduced inflammation and modulated airway remodeling.	de Castro et al. (31)
	Horses	Adipose-derived mesenchymal stem cells	Limited short-term anti-inflammatory effects and long-term stability of clinical signs	Adamič et al. (41)
Lung emphysema	Sheep	Autologous lung-derived mesenchymal stem cell	Ameliorated lung perfusion	Ingenito et al. (20)

Therapeutic strategies and treatment outcomes are also listed.

evaluating reports from January 1, 1990, to October 31, 2022. Reference lists of relevant articles were also reviewed to find additional studies.

Cellular therapy

Regenerative cell therapy is currently the most widely used method for stimulating the regeneration of damaged tissue, in which stem cells (SC) play a leading role. Stem cells are undifferentiated cells capable of self-renewal and transformation into other cell types (44). Traditionally, the therapeutic effect of SC has been associated with their migration to the affected area and their ability to replace damaged tissue (45). However, later discoveries recognized their complex immunomodulatory role through interaction with local cells of the immune system and paracrine signaling (46, 47). Currently, two ways of their potential use for therapeutic purposes are being investigated: (1) induction of endogenous differentiation and

mobilization of resident progenitor cells and (2) *ex vivo* (exogenous) cultivation of SC and their application in patients (4, 11, 48, 49). The former is mainly related to tissue regeneration and repair through activation of resident cells (43, 48), while the latter is mainly associated with paracrine action and immunomodulatory effects (4).

Heterogeneous endogenous stem cells [cells capable of long-term self-renewal and differentiation into other progenitor cells or tissue-specific cells (4)] and progenitor cells [tissue-specific cells capable of differentiation into specific cell types, but are not capable of self-renewal or are capable of self-renewal only in the relatively short term (4)] of the lung, located in different regions of the airway, are capable of self-renewal and of forming one or more mature cell types, allowing local maintenance of epithelial integrity and repair of damage (4, 10, 50). They reside in their unique microenvironmental niches that allow them to maintain their progenitor properties and differentiate into different cell types (10). Several distinct populations of stem and progenitor cells are present in the airways, which can differentiate into different airway cell types (4, 10, 50).

Basal epithelial cells represent a population of stem/progenitor cells from which Club cells (formerly known as Clara cells) and ciliated cells can develop (51, 52). They may also serve as progenitors for multiciliated and goblet cells (10). Submucosal glandular progenitor cells are another group of cells capable of regenerating submucosal glandular tubules, ducts, and surface epithelium (10, 53). Neuroendocrine cells of the lung can also function as progenitor cells that differentiate into Club cells and ciliated cells upon injury (10, 54). Type 2 alveolar cells are critical for surfactant C production and secretion, but are also considered alveolar progenitor cells. They can self-renew and/or differentiate into type 1 alveolar cells, which are responsible for gas exchange (10). Differentiation, proliferation and expansion of type 2 alveolar cells after tissue injury is protracted and takes several months (50).

Resident stem cells, which are thought to share several properties with bone marrow-derived mesenchymal stem cells (BM-MSC), have also been found in the lung (20, 55–57). They are currently referred to as lung mesenchymal stem cells or lung mesenchymal stromal cells (L-MSC). Their potential physiological or pathophysiological functions are not yet known. Similar to BM-MSC, L-MSC secrete immunosuppressive molecules and therefore may influence the course of inflammation, tissue injury and repair (58, 59).

Attempts have also been made to derive the phenotype of structural lung cells for pulmonary vascular regeneration from adipose or bone marrow tissue or from embryonic SC. Despite the ability of SC to differentiate into lung cell types, results of such studies remain controversial because of inadequately derived or described methods (4).

The therapeutic potential of exogenous SC has been repeatedly noted in relation to their immunomodulatory effects. Their complex immunomodulatory role results from their interaction with local immune cells and paracrine signaling, leading to a reduction in proinflammatory stimulus and thus less tissue damage (46, 47, 60–63). Most research on SC therapies has focused on inflammatory airway diseases where conventional treatments have been unsuccessful, and they have been found to have several beneficial effects (36, 64–67). Various cell sources (e.g., BM-MSC, adipose-derived stem cells, embryonic stem cells, umbilical cord blood-derived mesenchymal stem cells), dosages, and delivery methods have been investigated to maximize the potential of their therapeutic use. However, there is not yet sufficient evidence to formulate precise guidelines for clinical use.

Tissue engineering

Pathologic changes in diseased lungs may progress to the point where cell therapy and stimulation of tissue regeneration alone are insufficient and tissue replacement is required to restore lung function. Suitable lung donors are not always available, or lung transplantation is contraindicated (68); therefore, *in vitro*-grown tissue may bridge the time to lung transplantation or serve as a definitive therapeutic modality.

Tissue engineering techniques are still insufficiently developed. The lung is composed of more than 40 different cell types that form a complex three-dimensional (3D) anatomic architecture (69). Generating lungs *in vitro* and mimicking their function is a major challenge that requires a high degree of cell specialization and complex tissue architecture (5, 48, 70, 71). They must provide a variety of organ functions, such as the diversity of airway cell types, the defense mechanisms that protect the upper airways (e.g., secretion of specifically composed mucus and active ciliary apparatus), and the coupling of the alveolar space with the surrounding systemic and pulmonary vasculature to ensure effective tissue perfusion and gas exchange (72).

Most preclinical studies have used biologically derived models or synthetic scaffolds seeded with an appropriate cell source to regenerate functional lung tissue (5, 7). Hybrid scaffolds combining biological materials (extracellular matrix (ECM) components) with synthetic scaffolds currently appear to have the greatest potential. These scaffolds are then seeded with autologous or allogeneic cells to generate functional tissue generation (5). An important advantage of using allogenic cells is the reduction of immunologic complications and tissue rejection (7, 12, 48). In this way, a miniaturized and simplified version of an organ can be produced in the laboratory, called an organoid. This is a 3D structure that replicates the microanatomy of the desired organ. The formation of organoids relies on the self-assembly of cells derived from adult tissues, embryonic stem cells, or induced pluripotent stem cells (70, 71, 73, 74).

Because they represent the overall architecture of the lung, organoids are important models for studying various physiological processes in the airway microenvironment and the effects of various effectors on airway tissue structure, including infectious agents and/or new therapeutic modalities. This is particularly important because the cellular and molecular response to chemical and physical signals *in vivo* and the properties of gene expression can be obscured or lost in more commonly used *in vitro* 2D cell culture systems (73, 74). Lung organoids are broadly divided into proximal lung organoids (containing cells that mimic the conducting airways), distal lung organoids (subsuming the alveoli), or proximal-distal organoids (74).

The creation of a functional epithelial tissue appropriately connected to the vascular component is particularly important for the future development of therapeutically beneficial engineered pulmonary tissues. A more ambitious model of tissue engineering is based on decellularization of the original organ, in which all cells and cellular materials are removed from the entire lung, resulting in an intact three-dimensional scaffold. This represents the innate

ECM, preserving the natural structure of the airways and blood vessels, providing an optimal platform for transplantation of lung cells (48, 74). Lung ECM (collagen and elastic fibers enriched in proteoglycans, glycosaminoglycans, and fibronectin) not only provides a sophisticated scaffold for potential lung organogenesis, but also combines biochemical and mechanical signals that further guide SC behavior during lung re-development and regeneration (74). To generate functional lung tissue *ex vivo*, one would need to define more than 40 different cell types and perhaps hundreds to thousands of different cell subtypes (5).

Nichols et al. (35) transplanted a bioengineered porcine lung, which was generated using autologous cells. The bioengineered lungs successfully formed alveolar tissue and were ventilated, well vascularized, and developed a microbiome similar to that of the natural lung. The authors also noted no evidence of graft rejection (35). However, Yanagiya et al. (38) reported marked bullous changes in the transplanted tissue of bioengineered lungs when they examined unilateral transplantation of porcine lungs generated from autologous cells. They also reported comparable oxygen exchange between the bioengineered lung transplant group and the allograft recipient group, whereas CO_2 exchange was significantly lower in the bioengineered lung transplant group than in the allograft group (38).

Airway anatomy and physiology are highly species-dependent, making it necessary to create species-specific models. In a recent review of mammalian lung organoids, Archer et al. (72) highlighted that the cells lining the bronchiolar or more distal part of the tracheobronchial tree differ considerably between species in terms of their abundance, the cell types present, the ultrastructural features of these cells in adult animals, and the secretory products they produce (72). Mouse models, for example, are not particularly well suited for studying human respiratory diseases. On the other hand, sheep lungs are most commonly used as models for human lungs because of their anatomy and the uniform distribution of differentiated cells at a given age of maturity. These elements make sheep a valuable model for human respiratory physiology and disease (72).

Cell-free therapeutical strategies

Extracellular vesicles are membrane-protected carriers of many substances, including microRNA (miRNA), messenger RNA, proteins, and mitochondria. Extracellular vesicles are broadly classified into exosomes (vesicles of endocytotic origin with a diameter of 30–150 nm, surrounded by a plasma membrane), microvesicles (diameter of 100–1,000 nm, not of endocytotic origin), and apoptotic bodies [diameter of 50 nm−5 μm; they are released by apoptotic cells during membrane budding (blebbing)] (75). The use of ECV offers several important advantages over cell therapy. Due to their smaller size, ECV can penetrate deeper into the airways and potentially be delivered by inhalation techniques (76). In addition, their membrane envelope makes them stable in tissues and body fluids. They also have low immunogenicity and toxicity compared to cell therapies (77, 78). A major obstacle to the therapeutic use of ECV is the lack of standardized methods for isolation and purification of ECV. The lack of standardized methods for isolating exosomes means that exosomes cannot be separated from other ECV of similar size. There is also a lack of standardization of methods for measuring ECV purity (47, 79). In this context, it is advisable to use the generic term "extracellular vesicle" when using ECV therapeutically and to avoid nominal categorization into subtypes. If the name of a single subspecies is used, extraction and selection must be precisely defined.

Confirmation of the functionality of ECV therapy requires that the therapeutic effect occurs without intercellular contact and that this is not achieved by ECV-unrelated soluble paracrine factors (80). Extracellular vesicles are involved in several intercellular signaling pathways, making them critical molecular messengers in various processes responsible for normal homeostasis and disease development. In the regeneration process, they also influence the response of stem/progenitor cells and other cells within their niche (78).

Numerous studies have demonstrated the benefits of systemic administration of ECV in mitigating allergic airway hyperreactivity and resulting inflammation and tissue remodeling (26, 31). Extracellular vesicle treatment has been shown to be beneficial in the treatment of lung injury and pulmonary fibrosis in rats. After intratracheal administration, there was a reduction in apoptosis and necrosis of type 2 alveolar epithelial cells and alleviation of lung injury. Extracellular vesicles decreased reactive oxygen species levels and inflammation in the airways. The authors were able to attribute some of the beneficial effects to a specific miRNA, let-7d-5p (42). Antounians et al. (81) also attributed the therapeutic effects of ECV to miRNA when they investigated its influence on the regenerative capacity of undeveloped fetal lungs in an experimental rodent model. Following ECV treatment, enhanced morphogenesis and alveolarization, restoration of lung tissue homeostasis, and differentiation of epithelial cells and fibroblasts were observed in association with the release of RNA cargo (81). In addition, ECV treatment may limit viral respiratory infections by affecting viral replication and virus-induced apoptosis in lung epithelial cells, which is also thought to depend on the transfer of RNA from ECV to epithelial cells (34).

In addition to the cell-free regenerative medicine options described above, several indirect therapeutic options have been described to stimulate local cells and tissue regeneration in the airways. For example, all-trans-retinoic acid, a derivative of vitamin A (retinol), has been described as a possible candidate to promote alveologenesis (48, 82, 83). It is also suggested that nanoparticles of integrins may influence the regeneration of collapsed alveoli (84). Another area of research is regenerative photobiostimulation, which aims to stimulate resident stem cells with electromagnetic radiation to trigger growth factor production, inhibition of inflammation, and stimulation of angiogenesis (85).

Most probable therapeutic application in animals

Benefits of cell treatments have been reported for the treatment of asthma; experimentally in mouse models (14, 18, 23, 24, 26–31) and animals with natural asthma, such as cats (40) and horses (39, 41). The treatment effects of SC, identified in preclinical studies of asthma treatment, are related to the reduction of airway inflammation through the regulation of inflammatory cytokines. The results of these studies differ in terms of cytokine expression and translation, but all consistently reported a reduction in airway inflammation. The influence of SC on tissue remodeling may play the critical role in the treatment of asthma (18, 24, 26, 31, 86, 87).

Ingenito et al. (20) investigated the effect of autologous L-MSC on experimentally induced lung emphysema in sheep to evaluate their ability to regenerate functional tissue. Animals received endoscopically either cellularized biological scaffolds or scaffolds alone. At four-week follow-up, no immune response to the grafts was detected, but significant improvement in tissue mass (in terms of increased cellularity and extracellular matrix content) and lung perfusion was observed in sheep receiving L-MSC compared with the control group. Detection of labeled L-MSC in the alveolar septum and peribronchial interstitium was also reported. L-MSC therefore have the potential for regeneration of emphysematous lungs (20).

Treatment with SC also significantly affects inflammatory responses and lung tissue regeneration in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (36, 88–91). Aside from symptomatic therapy, no specific treatment for these diseases have been defined that would substantially improve short- and long-term outcomes. Positive effects in terms of reducing pulmonary edema and inflammation and improving gas exchange have been reported after cell treatment in experimentally induced ARDS in sheep (22, 25, 32, 36, 37). Currently, research on the effect of SC on the treatment of ARDS caused by respiratory viruses is particularly relevant due to the COVID-19 pandemic (67, 90, 92). Reductions in oxidative stress and inflammation and resulting lung injury and mortality following treatment with SC have been observed in mice (13, 17, 19, 21), rabbits (15, 16) and dogs (33) with experimentally induced lung injury.

In addition to cell therapy, treatment with ECV has also successfully treated acute airway inflammation caused by viral infection. Khatri et al. (34) investigated the effects of intratracheally administered ECV on influenza virus-induced acute lung injury in pigs. ECV treatment significantly reduced viral secretion (detected in nasal swabs), viral replication in the lungs, and virus-induced inflammatory cytokine formation in the lungs of infected pigs 12 h after viral infection. The authors concluded that intratracheal treatment with ECV attenuates influenza virus-induced ALI in pigs (34).

Conclusions

Further evidence from appropriately designed clinical trials is needed before regenerative therapy is considered an accepted therapeutic modality in respiratory medicine. To date, the use of SC or ECV for the treatment of respiratory disease has consistently been described as relatively safe after local and systemic application. Apart from mild local reactions after administration of cells of allogeneic origin, no severe adverse events have been observed (41, 47, 93–96). The interaction between SC/ECV and the immune system may also provide better insight into the pathophysiology of immune system dysregulation in the respiratory system.

It is also important to focus on a detailed understanding of the functional heterogeneity of each cell type in the respiratory system and the development of protocols for targeted cell differentiation and maturation (70). This is particularly true for tissue engineering, which is less explored compared to SC and ECV due to its anatomical and functional complexity. The creation of a functional epithelial tissue suitably linked to the vascular component is particularly important for the future development of respiratory physiology and medicine.

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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.

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Evaluation of Platelet-Rich Plasma by means of PRGF® -Endoret® protocol in leukemia cats: PDGF-BB and TGF-ß1 valuation

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Introduction: Feline leukemia virus (FeLV) is a chronic disease that leads to the weakening of a cat's immune system. Platelet-rich plasma (PRP) offers therapeutic effects for multiple diseases, the use of PRP and growth factors (GFs) determination could be an alternative treatment to improve the quality of life in these patients. The objectives of this study were to determine and compare the concentration of platelets (PLTs), red blood cells (RBCs) and white blood cells (WBCs) between samples of whole blood (WB), PRP and platelet-poor plasma (PPP) fractions, and to evaluate the concentration of platelet-derived growth factor BB (PDGF-BB) and transforming growth factor β1 (TGF-β1) in both fractions in FeLV cats using a PRGF®-Endoret® protocol previously standardized in this species.

Methods: WB was collected from 11 asymptomatic FeLV-positive cats. PRP and PPP was obtained following PRGF®-Endoret® technology according to centrifugation at 265 g for 10 min. Cellular components, RBCs, WBCs, PLTs, and the PDGF-BB and TGF-β1 concentrations in PRP and PPP fractions were determined.

Results: PLT in the PRP fraction was statistically higher than WB and PPP fraction, with no statistical differences between WB and PPP. PLT concentration increased 1.4 times in PRP fraction compared to WB. Mean platelet volume (MPV) did not differ significantly between the WB, PRP, and PPP fractions. Compared to WB, the absolute numbers of RBCs and WBCs were decreased by 99% and more than 95% in the PRP and PPP fractions, respectively. TGF-ß1 concentrations increased in PRP vs. PPP, with no changes in PDGF-BB.

Discussion: Based on the degree of PLT enrichment and the absence of RBCs and WBCs, this blood product could be classified as a Pure Platelet-Rich Plasma (P-PRP). The presence of GFs in PRP and PPP samples suggests that the PRGF®-Endoret® methodology is suitable for obtaining PRP in FeLV cats, despite future studies are necessary to optimize the technique, standardize the results and assess clinical efficacy.

KEYWORDS

plasma rich in growth factors, PRGF®-Endoret®, feline leukemia, FeLV, cat, PDGF-BB, TGF-ß1, platelet

1. Introduction

Platelet-rich plasma (PRP) is an autologous blood product defined by a platelet (PLT) concentration higher than baseline. Inside, the PLTs have granules that, when activated, release growth factors (GFs), cytokines, and chemokines into the medium. Some of the most important GFs released by PLTs in PRP include platelet-derived growth factor (PDGF, A-B-C), transforming growth factor beta1 (TGF-ß1), vascular endothelial growth factor (VEGF),

fibroblast growth factor (FGF), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1), which are involved in tissue restoration and immunomodulation (1–4).

Taking into account the cellular content and the presence or absence of fibrin, four main groups of PRP products have been identified (5): Pure Platelet-Rich Plasma (P-PRP), Leukocyte and Platelet-Rich Plasma (LR-PRP), Pure Platelet-Rich Fibrin (P-PRF), and Leukocyte and Platelet-Rich Fibrin (L-PRF). P-PRP, as a PRGF®-Endoret® contains a moderate PLT concentration and no leukocytes (WBCs); LR-PRP includes a WBC fraction, but the fibrin matrix is sparse; P-PRF is a blood product with very low concentration of WBCs and is collected owing to the specific separator gel used in the method, PLT activation and fibrin polymerization are triggered using calcium chloride. L-PRF is considered as second-generation PLT concentrate because the natural concentrate is produced without any anticoagulants or gelifying agents, and after centrifugation the PRF clot forms a strong fibrin matrix in which most of the PLTs and WBCs from the harvested blood are concentrated. Continuous revisions of terminology and new classification criteria for PRP bioformulations in regenerative medicine (2) have made it possible to identify up to 15 types of PRP products with differences in formulation, biological properties and mechanisms of action, as well as different clinical indications and clinical results (6, 7). The topical use of PLT concentrates is recent, and its efficiency remains controversial. Several techniques for PLT concentrates are available, and their applications have been confusing because each method leads to a different product with a different biology and potential uses (8). Even though PRP has been widely used in veterinary medicine, no valid standardized classification methods for platelet-derived products have been currently published.

Plasma-Rich Growth Factors (PRGF) represents a novel technology that uses autologous proteins and GFs derived from PLTs as therapeutic formulations for regenerative purposes, cell repair and regeneration. PRGF®-Endoret® technology following a unique centrifugation protocol allows a PRP product to be obtained with moderate PLT enrichment and an extreme reduction of WBCs and RBCs, which decreases the proinflammatory activity of WBCs. The PRGF®-Endoret® protocol for obtaining GFs is widely documented in different species and has been successfully applied in different clinical areas (9–11), such as regenerative medicine for wound healing, ophthalmology, dentistry, osteoarthritis, tendinopathies, or aesthetic medicine in humans (12–19); traumatology and ophthalmology in dogs (20–22) and horses (23); and wound healing (24, 25) or the treatment of complete cartilage defects in rabbits (26). Although different protocols for obtaining PRP in cats have been described (27–32), the use of PRGF®-Endoret® technology in this species has only been reported in a previous study carried out by the same researchers. Due to the cellular characteristics of the autologous blood product obtained following centrifugation, it was classified as a P-PRP product (33). Despite this, there is no clinical evidence of its application, and therefore future research is needed in this field of feline internal medicine (28, 29, 33).

Feline viral leukemia is caused by feline leukemia virus (FeLV), one of the retroviruses with the greatest impact on feline health worldwide. The prevalence is highly variable depending on the geographic location and the animal population analyzed, varying between 3.0 and 28.4% in South America, 0.5 and 24.5% in Asia and Australia/New Zealand, 2.3 and 3.3% in the USA, and 0.7 and 15.6% in Europe, among others (34–40). In Spain, specifically, the reported prevalence of FeLV is 2.6% (41).

Following the course of the infection, four different forms of FeLV infection have been identified: abortive infection ("regressor cats"), regressive infection ("transient viremia" followed by "latent infection"), progressive infection ("persistent viremia"), and focal or atypical infection (42). Progressive infections are categorized by detectable antigenemia, FeLV RNA, and DNA provirus, since the virus persistently sheds into the circulation and tissues have low or no antibodies to FeLV (42). In 30–40% of cats with progressive infection, FeLV virus has tropism for the hematopoietic cells in the bone marrow (BM) associated with proliferative, degenerative, and oncogenic diseases in erythroid, myeloid, and lymphoid cell lineages (42). Both lymphoma (43) and myelodysplastic syndrome are relatively common abnormalities in FeLV-infected cats. Non-neoplastic diseases, such as regenerative anemia, immunosuppression, neutropenia, lymphopenia, and PLT abnormalities like thrombocytopenia or PLT function alterations, can occur in animals persistently infected with FeLV (35, 44–46). Moreover, ∼80% of cats die before 4–5 years of life; prognosis for cats with progressive infections is variable depending upon current immune status, stress, or concurrent disease such that cats can remain without clinical signs for several years after the infection of the BM (47, 48).

Due to the fact that GFs promote wound healing or tissue regeneration in musculoskeletal injuries, such as osteoarthritis in dogs (20–22), horses (23), rabbits (26), and humans (49, 50), the authors considered that GFs could be an advantageous therapeutic option in cats with FeLV. For this reason, the objective of the study was to determine PLT concentrations and two specific types of GFs, such as Platelet-Derived Growth Factor-BB (PDGF-BB) and Transforming Growth Factor-ß1 (TGF-ß1), in both PPP and PRP fractions following the PRGF®-Endoret® protocol previously standardized for its application in cats by these same researchers (33). The authors hypothesize that a P-PRP can be obtained in FeLV cats following PRGF®-Endoret® methodology as in other species or in healthy cats.

2. Material and methods

2.1. Animals

The study protocol was approved by the Animal Welfare Ethics Committee (CEEA) of the CEU Cardenal Herrera University in Valencia (Spain) in accordance with the Spanish Animal Protection Policy (RD53/2013), which complies with the European Union Directive European 2010/63/EU, with the following approval code: 2018/VSC/PEA/0196.

This prospective study included cats brought to CEU-Cardenal Herrera University Veterinary Clinical Hospital between October 2021 and July 2022 as part of a medical clinical study, during which cats were examined for retroviral infections. A total of fourteen non-pedigree FeLV-positive adult cats were included in the study. All animal owners agreed to participate in the study by signing a consent form.

Only animals clinically healthy and with two positive subsequent test results for FeLV by the commercially available combined enzyme-linked immunosorbent assay (ELISA) kit for feline immunodeficiency virus (FIV) antibody and antigen FeLV p27 (IDEXX SNAP® Combo FeLV Ag/FIV Antibody Test) were included in the study. The first samples with positive results were subsequently tested again from 6 to 9 months after, following the same methodology, considering true positives or progressive infection by FeLV those that tested positive twice. Discordant test results were considered negative.

Animals receiving any treatment for the previous or current 6 months of the study and those that developed diseases or tested positive for both FIV and FeLV or only FIV were excluded from the study. Animals with hematological alterations consisting of anemia, leukopenia or true thrombocytopenia were also excluded. Each patient was monitored by a veterinarian throughout the entire procedure.

2.2. Sample processing

To reduce stress, cats were first intramuscularly (IM) sedated with a combination of butorphanol (0.3 mg/kg), dexmedotomidine (12 μg/kg), and alfaxalone (0.8 mg/kg). Following sedation, the cephalic vein was catheterized using a 22 G catheter to collect 0.5 ml of blood, which was immediately transferred to a 0.5 ml tube containing K3-EDTA (BD Vacutainer; Becton, Dickinson) for blood count or whole blood (WB) analysis. Thereafter, 9 ml of blood was collected in sterile conditions from the external jugular vein by means of a vacutainer sodium citrate 3.8% tube (Blood-Collecting Tubes®, BTI Biotechnology Institute, Alava, Spain) for PRGF®-Endoret® preparation. Subsequently, each cat received 9 ml of acetated Ringer's solution IV during the first 20 min to restore the vascular volume and to prevent hemodynamic complications.

2.3. PRGF®-Endoret® processing

Feline samples collected in sodium citrate tubes were immediately centrifugated at room temperature in a PRGF®-Endoret® System IV centrifuge (BTI Biotechnology Institute S.L.) under a single centrifugation protocol of 265 g for 10 min as described by Miguel-Pastor et al. (33).

Following the PRGF®-Endoret® methodology, two fractions were obtained after centrifugation: platelet-poor plasma (PPP) and PRP. Sixty percentage of the upper plasma was considered PPP, and the remaining 40% above the "buffy coat" was considered the PRP fraction. Both fractions were pipetted under maximum sterile conditions with a laminar flow hood and always by the same researcher. Subsequently, samples were transferred to individual fractionation tubes with no additives (PRGF® fractionation tubes, BTI, Institute of Biotechnology, Álava, Spain). In addition, the plasma fractions (PPP and PRP) were activated by adding 5% of the plasma volume of 10% calcium gluconate (activator PRGF®, Institute of Biotechnology, Álava, Spain) to achieve PLT degranulation and release of the GFs, obtaining PRGF. Plasma samples were then aliquoted into eppendorf tubes and immediately frozen at −80°C following PLT activation for subsequent determination of TGF-ß1 and PDGF-BB concentrations (Figure 1).

2.4. Hematological analysis

A complete automated blood count was performed for each feline specimen using the Advia® 2120i (Advia® 2120i Siemens Healthcare Diagnostics Inc.), including red blood cell (RBC; $10e^6/\mu L$), hemoglobin concentration (HB; g/dL), packed cell volume (PCV; %), mean corpuscular volume (MCV; fL), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; g/dL), mean hemoglobin content (CH; pg), hemoglobin concentration distribution width (HDW; d/dL), red blood cell distribution width (RDW; %), reticulocytes absolute count (RET; $10e^9/L$), white blood cell (WBC; $10e^3/\mu L$), neutrophils (NFS; $10e^3/\mu L$), lymphocytes (LYMPH; $10e^3/\mu L$), monocytes (MON; $10e^3/\mu L$), eosinophils (EOS; $10e^3/\mu L$), and basophils (BAS; $10e^3/\mu L$). Platelet (PLT; K/μL) counts and mean platelet volume (MPV; fL) were also determined in WB samples. In the PRP and PPP fractions, RBC, PLT, WBC, LYMPH, NFS, and MON counts, as well as the MPV, were also determined. Verification of absolute PLT numbers, such as the presence of PLT aggregates and differential WBC counts obtained by ADVIA, was performed on Romanowsky-stained blood smears and always by the same pathologist.

2.5. Platelet-Derived Growth Factor-BB and Transforming Growth Factor-ß1 quantification

The concentrations of both GFs in both plasma fractions (PPP and PRP) were determined using an ELISA kit of development with antibodies to human (Human TGF-beta1 DuoSet ELISA de R&D Systems DY240-05 and Human PDGF-BB DuoSet ELISA de R&D Systems DY220, respectively), following the methodology previously published by Miguel-Pastor et al. (33). To the knowledge of the authors, there are no commercial kits for GF determination in cats, so human kits were used for GFs determination as described by other researchers (27, 51).

2.6. Statistical methods

Data were analyzed using SPSS 20.0 for Windows (SPSS® Inc., Chicago, USA). A descriptive study of the mean, standard deviation and confidence intervals was performed for each variable. The normality of the data was verified in each variable with the Shapiro–Wilk test, and the homogeneity of the variance was verified with the Levene test. The means of the variables were studied using a linear mixed model. These models included the treatment group as fixed effects and the cat as a random effect. If the interaction was found statistically significant, analyses using a one-way ANOVA and a Bonferroni test were used. Non-parametric Kruskal–Wallis tests were used to compare variables not adjusted to a normal distribution. $P < 0.05$ was considered significant.

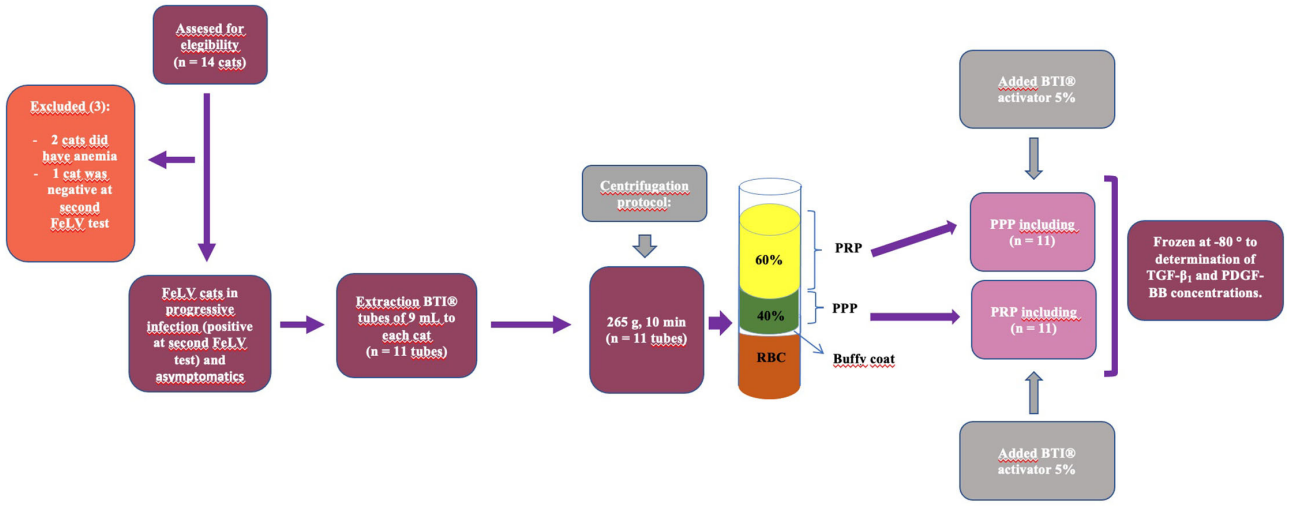

FIGURE 1
Schematization of the study design: blood sample obtention, centrifugation following feline BTI protocol using the PRGF®-Endoret® methodology, PRP and PPP fraction obtention, subsequent activation of both fractions with BTI-activator and freezing at −80°C for subsequent GFs analysis.

3. Results

3.1. Animals and sample acquisition

Of the 14 cats initially included in the study, three of them were excluded: two presented moderate anemia and one tested negative on the second FeLV test. A total of 11 sterilized cats met the inclusion criteria, six males and five females, aged between 1.5 and 6.5 years (Mean: 4.4; SD: 1.6), and weighing between 3.7 and 5.8 kg (Mean: 4.7; SD: 0.7).

The collection and centrifugation of blood were performed with no intercurrence in all cats. Following this procedure, the three fractions (erythrocyte, buffy coat area, and plasma fraction) were obtained.

Table 1 shows the mean values ± SD or median and the 95% CI of all the hematological parameters considered in WB. Comparisons between RBC, PLT, WBC, LYMPH, NFS, and MON counts, as well as MPV between WB and PRP and PPP fractions, and comparisons between PDGF-BB and TGF-ß1 between PRP and PPP fractions are presented in Table 2. Table 3 shows individually the number of PLTs in WB, PRP, and PPP in relation to the volume of recovered plasma. Table 4 shows the recovered volume of PRP and PPP individually, considering the PCV in each animal.

3.2. Platelet concentration and mean platelet volume

The mean number of PLT was statistically higher in PRP (392.1 ± 130.7 PLTs) compared to PPP (260.8 ± 83.9 PLTs) fraction ($p = 0.024$) and WB (307.3 ± 106.2 PLTs) ($p = 0.043$) with no statistical differences between WB and PPP fraction (Tables 2, 3, Figure 2A). MPV did not differ significantly between the WB, PRP, and PPP fractions.

PLT aggregates were present in 4 (36%) WB samples, 2 (18%) PRP samples, and 2 (18%) PPP samples. According to the equivalence between PLT aggregates by ADVIA 2120i and in smears (51), of the eight samples, seven presented 1–7 aggregates with more than 10 PLTs per field, and the remaining 1–3 aggregates with more than 50 PLTs per field.

3.3. RBC concentration

The number of RBCs was statistically higher in WB compared to the PRP and PPP fractions ($p < 0.01$), with no statistical differences between them. The PRGF®-Endoret protocol allowed reducing the number of RBCs by 99% in both PRP and PPP fractions (Figure 2B).

3.4. WBC concentration

The number of WBCs in WB was significantly higher compared to the PRP and PPP fractions ($p < 0.01$). The mean concentration of WBC was reduced by 95% in the PRP fraction and reduced by 97% in the PPP fraction (Figure 2C).

3.5. Lymphocyte concentration

The number of LYMPH was significantly higher in the WB fraction than in the PRP and PPP fractions. LYMPH concentration was significantly decreased by 80% ($p < 0.01$) and 88% in the PRP and PPP fractions ($p < 0.01$), respectively (Figure 2D). The mean number of LYMPH was significantly higher in the WB fraction compared to the PRP and PPP fractions.

3.6. Neutrophil concentration

The number of NFS was significantly higher in the WB fraction compared to the PRP and PPP fractions. The mean NFS

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TABLE 1 Mean ± SD of red blood cell (RBC) count, hemoglobin concentration (HB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean hemoglobin content (CH), hemoglobin concentration distribution width (HDW), red blood cell distribution width (RDW), reticulocytes absolute count (RET), white blood cell (WBC), neutrophil (NFS), lymphocyte (LYMPH), monocyte (MON), eosinophil (EOS), basophil (BAS) and platelet (PLT) counts, and mean platelet volume (MPV) in whole blood samples (WB) in 11 FeLV-positive cats ($n = 11$).

Parameter	*N*	Mean ± SD	Minimun	Maximum	90% CI (lower reference limit)	90% CI (upper reference limit)	Reference range
Erythrogram							
RBC (10^6/μl)	11	7.4 ± 1.1	5.9	9.9	6.7	8.1	5.0–10.0
HB (g/dl)	11	10.6 ± 1.6	8.3	14.0	9.5	11.7	8.0–15.0
PCV (%)	11	30.9 ± 4.6	23.0	40.5	27.9	34.1	24.0–45.0
MCV (fl)	11	41.9 ± 3.8	32.2	46.7	39.4	44.4	39.0–55.0
MCH (pg)	11	14.3 ± 1.5	11.2	16.4	13.3	15.3	12.5–17.5
MCHC (g/dl)	11	34.1 ± 1.6	29.7	35.2	33.1	35.2	30.0–36.0
CH (pg)	11	14.8 ± 1.2	11.8	16.1	13.9	15.6	12.0–16.0
HDW (g/dl)	11	2.4 ± 0.2	2.12	2.88	2.2	2.5	1.6–2.9
RDW (%)	11	16.4 ± 0.9	14.6	18.5	15.8	17.1	14.0–18.0
RET (10^9/μl)	11	54.3 ± 40.6	13.8	134.9	27.1	81.6	15.0–81.0
Leukogram							
WBC (10^3/μl)	11	12.1 ± 5.4	4.31	22.18	8.4	15.7	5.5–19.5
NFS (10^3/μl)	11	8.7 ± 5.0	2.07	17.9	5.4	12.1	2.5–12.5
LYMPH (10^3/μl)	11	2.5 ± 0.5	1.9	3.1	2.1	2.8	1.5–7.0
MON (10^3/μl)	11	0.32 ± 0.19	0.1	0.7	0.2	0.4	0.0–0.9
EOS (10^3/μl)	11	0.9 ± 1.7	0.0	6.1	0.0	2.0	0.0–0.8
BAS (10^3/μl)	11	0.3 ± 0.9	0.0	3.0	0.0	0.9	0.0–0.2
Platelet parameters							
PLT (10^3/μl)	11	307.3 ± 106.2	118.0	432.0	235.9	378.6	200–500
MPV (fl)	11	17.03 ± 5.29	10.0	27.7	13.5	20.6	8.6–18.9

Reference values for the cat determined using ADVIA 2021i Laboratory of the Veterinary Clinical Hospital of the CEU-Cardenal Herrera University.

TABLE 2 Mean ± SD of platelet (PLT) concentrations, mean platelet volume (MPV), erythrocytes (RBCs), leukocytes (WBC), lymphocytes (LYMPH), neutrophils (NFS), and monocytes (MON) concentrations in whole blood (WB) samples and in the PRP and PPP fractions; platelet-derived growth factor BB (PDGF-BB) and transforming growth factor B1 (TGF-β1) concentrations in the PRP and PPP fractions in 11 FeLV cats ($n = 11$).

		Centrifugation protocol (265 g × 10 min)	
	WB	PRP	PPP
PLT ($10e^3$/μL)	307.3 ± 106.2a	392.1 ± 130.7b	260.8 ± 83.9a
MPV (fL)	17.0 ± 5.3a	14.1 ± 3.6a	13.3 ± 3.4a
RBC ($10e^6$/μL)	7.4 ± 1.1a	0.1 ± 0.0b	0.0 ± 0.0b
WBC ($10e^3$/μL)	12.1 ± 5.4a	0.6 ± 0.3b	0.3 ± 0.2b
LYMPH ($10e^3$/μL)	2.5 ± 0.5a	0.5 ± 0.3b	0.3 ± 0.2b
NFS ($10e^3$/μL)	8.7 ± 4.9a	0.1 ± 0.1b	0.0 ± 0.0b
MON ($10e^3$/μL)	0.3 ± 0.2a	0.0 ± 0.0b	0.0 ± 0.0b
PDGF-BB (pg/ml)		248.3 ± 152.3a	198.5 ± 143.2a
TGF-β1 (pg/ml)		17,556.9 ± 7,084.2a	11,487.3 ± 3,427.7b

Different letters (a, b) indicate differences between groups. $P < 0.05$ is considered statistically significant.

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TABLE 3 Number of platelets (PLT) in whole blood (WB) and platelet concentrations considering the total volumes of plasma in the PRP and PPP fractions.

	PLT ($10e^3/\mu L$)			% concentration	
Animal	WB	PRP	PPP	PRP	PPP
1	277	159	123	57.4	44.4
2	421	393	286	93.4	67.9
3	396	295	231	74.5	58.3
4	284	462	364	162.7	128.2
5	118	370	244	313.6	206.8
6	432	670	414	155.1	95.8
7	308	391	301	126.9	97.7
8	375	433	169	115.5	45.1
9	134	273	197	203.7	147.0
10	364	484	295	133.0	81.0
11	271	383	245	141.4	90.4
MEAN	307.3	392.1	260.8	143.4	96.6

TABLE 4 Individual packed cell volume (PCV; %) and recovered volumes of PRP (mL of plasma corresponding to the bottom 40% of the total plasma fraction) and PPP (mL of plasma corresponding to the top 60% of the plasma fraction) for each cat.

Animal	PCV (%) of WB	PRP volume (mL)	PPP volume (mL)
1	33.0	2.4	3.6
2	23.9	2.7	4.1
3	36.9	2.3	3.4
4	29.1	2.5	3.8
5	30.4	2.5	3.8
6	29.7	2.5	3.8
7	40.5	2.1	3.2
8	27.7	2.6	3.9
9	29.6	2.5	3.8
10	32.3	2.4	3.7
11	27.8	2.6	3.9

concentration was significantly decreased by 99% in both PRP and PPP fractions ($p < 0.01$; Figure 2E).

3.7. Monocyte concentration

The number of MON was significantly higher in the WB fraction compared to the PRP and PPP fractions. The mean MON concentration was significantly decreased by 97% in both PRP and PPP fractions ($p < 0.01$; Figure 2F).

3.8. PDGF-BB and TGF-ß1 concentrations

No significant differences were found between the PRP and PPP fractions in PDGF-BB concentrations (Figure 3A), although in samples with PLT aggregates, the values were higher ($p = 0.02$) than those without aggregates. The mean concentration of TGF-ß1 in the PRP fraction was statistically higher than the PPP fraction ($p = 0.02$) were not affected by the presence of aggregates (Figure 3B).

4. Discussion

To the authors' knowledge, this is the first study to separate the PRP and PPP fractions in cats naturally infected with FeLV. The mean PLT enrichment was 1.4 (143%) times higher in the PRP fraction compared to the WB fraction. These results differ little from those observed in a previous study in healthy cats by the same authors in which PLT concentrate was 1.5 (147%) times higher in PRP fraction compared to the WB fraction using the same centrifugation protocol (33). Nevertheless, in other studies in which other types of protocols for obtaining PRP were used, increases in the concentration of PLTs in the PRP fraction were also detected compared to the WB fraction. Thus, Silva et al. showed 183% PLT enrichment in PRP vs. WB using 85 g gravitational force for 6 min in 8.5 mL tubes containing 1.5 mL ACD-A solution (trisodium citrate, citric acid, and dextrose) (27). More recently, Chun et al. reported a 151% PLT enrichment in a 12.5 mL volume of WB in a 30 mL syringe with 2.5 mL citrate and dextrose anticoagulant by double centrifugation at 3,600 rpm for 1 min and then 3,800 rpm for 5 min (29). Likewise, Ferrari and Schwartz (28) evaluated the PLT concentration of PRP within two commercial centrifugation systems. With system 1, 13.5 ml of WB was included in a syringe with 1.5 ml of ACDA and centrifuged at 1,300 rpm for 5 min. Using system 2, 12.5 mL of WB were taken in a syringe with 2.5 mL of ACDA following two consecutive centrifugations at 3,600 rpm for 1 min and at 3,800 rpm for 5 min. While System 1 reduced PLT concentrations by 3%, System 2 increased PLT concentrations by 187% (28). To evaluate the effect of local autologous PRP on healing by secondary intention of skin disorders in cats, a recent study by Angelou et al. (32) included 11 mL of WB in a vacuum tube containing separating gel and anticoagulant (MACD7) and centrifuged at 1,500 rpm for

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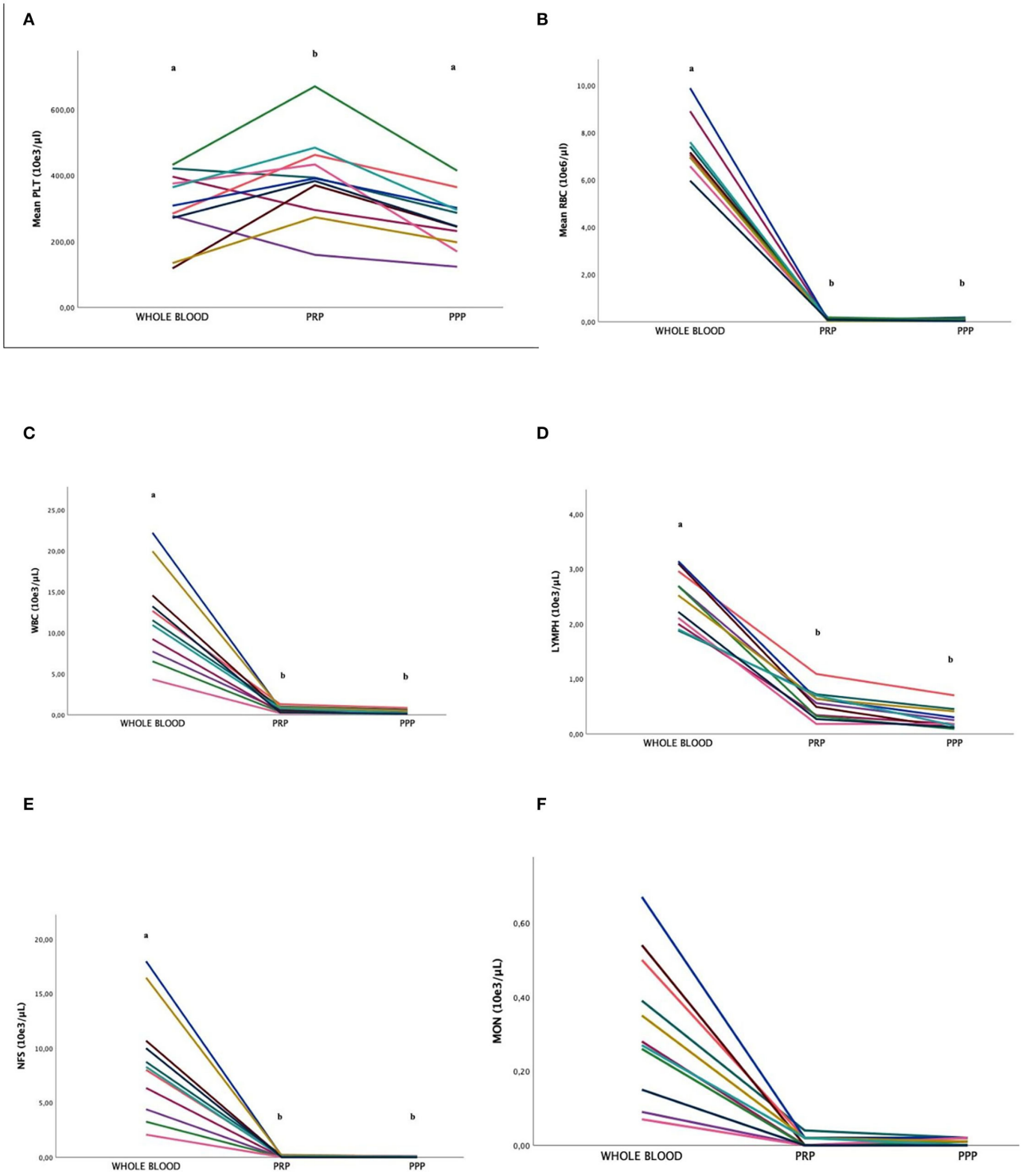

FIGURE 2
Comparison of the platelet [PLT; **(A)**], red blood cell [RBC; **(B)**], white blood cell [WBC; **(C)**], lymphocytes [LYMPH; **(D)**], neutrophils [NFS; **(E)**], and monocyte [MON; **(F)**] concentrations (mean ± SD) in FeLV cats ($n = 11$) between whole blood (WB) and PRP and PPP fractions. Different letters (a, b) indicate differences between groups. $P < 0.05$ statistically different.

10 min. PLT counts in the obtained PRP product increased from 2 to 8.2 times compared to WB in this latter study (32). Even though we cannot specify the exact origin of these differences, factors such as WB volume samples, gravitational force, and centrifugation times in the different protocols used to obtain PRP, should be taken into account since the basal PLT number in the WB fraction were similar.

The mean number of PLTs in the PRP fraction in this study was 392.1 ± 130.7 PLTs, not obtaining homogeneous results in all samples, as can be seen in the standard deviation (145.8 PLTs). This

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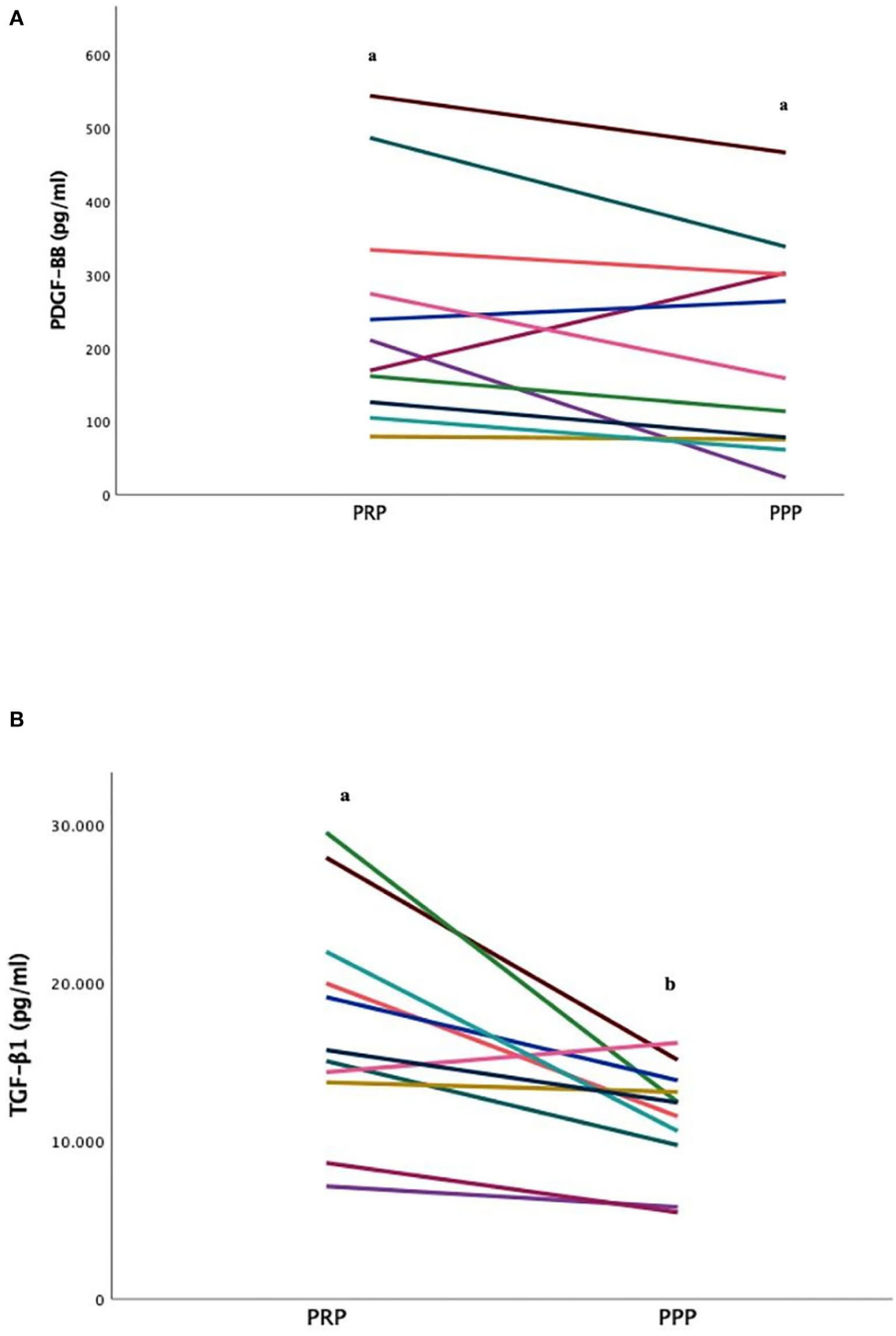

FIGURE 3
Comparison of Platelet-Derived Growth Factor-BB [PDGF-BB; **(A)**] and transforming growth factor ß1 [TGF-ß1; **(B)**] concentrations (mean ± SD) in FeLV cats ($n = 11$) between PRP and PPP fractions. Different letters (a, b) indicate differences between groups. $P < 0.05$ statistically different.

was probably the reason why there were no differences found between WB and PPP. In addition, PLT concentrations in PRP of FeLV cats was lower than that obtained previously in healthy animals (481.4 ± 275.0 PLTs) by these same authors (33). A similar trend of PLTs in PPP (260.8 ± 83.9 PLTs) vs. healthy cats (293.3 ± 161.4 PLT) was observed. This fact is probably related to individual variability since in both cases the PRP fraction was obtained using the same sample volume, relative centrifugal force, temperature, and time of centrifugation. The results of this study show that the PPP fraction contains a considerable proportion PLTs that should have been concentrated in the PRP, as a result optimization of the technique is crucial to obtain a more PLT-concentrated PRP. Following the BTI-Endoret® methodology, the PRP fraction was obtained in this study using a single centrifugation protocol. The authors highlight the need of optimizing the protocol with future studies on blood sample centrifugation protocols by varying the gravitational force or the centrifugation time to obtain PRP with a higher concentration of PLTs following PRGF®-Endoret® characteristics; or even taking into

consideration the need of a second centrifugation to recover more PLTs in the PRP and decrease them in the PPP fractions.

The moderate enrichment in PLT and the absolute reduction in the number of RBCs and WBCs, including LYMPH, NFS, and MON, in both the PRP and PPP fractions enabling a P-PRP in FeLV cats similar to those established in healthy cats by these same researchers (33). However, there was a minimum requirement that a PRP of at least 1.5 should compare to WB according to the PRGF®-Endoret® methodology (9) as previously described in healthy cats (33), dogs (20), and rabbits (52). A larger population of sick cats may elucidate the origin of these differences, since none of the animals included in the study presented thrombocytopenia.

Pseudothrombocytopenia in feline blood samples is a common finding on blood smears in both FeLV positive and healthy animals (53). In fact, from the 11 WB samples analyzed, four presented aggregates, or 36%, as previously reported in these same species (33). Other studies have reported higher rates of pseudothrombocytopenia: 40% (28), 57% (54), 62% (53), and 71% (55, 56). The large size of PLTs, the secretion of granules when exposed to high concentrations of serotonin, and irreversible aggregation in response to adenosine diphosphate (ADP) reduction are factors involved in the formation of PLT clumps in these species (57). However, the sampling quality is the main cause of PLT aggregation in feline WB samples (58, 59). The presence of PLT aggregates in the samples of this study could be related to the collection method, since the WB samples were obtained directly by catheterization of the cephalic vein using a 22 G catheter. It is well-known that the lesion of vascular endothelium produced by venipuncture causes PLT adherence to von Willebrand factor bound to subendothelial collagen with PLT GPIbα receptors, which induces additional PLT recruitment (60, 61). PLT counts by ADVIA were falsely decreased in samples with EDTA-induced pseudothrombocytopenia and was confirmed in the corresponding blood smears. Since pseudothrombocytopenia is a common occurrence in cats, the results of the samples with and without aggregates were included in the same statistical study, which could have induced the variations in PLT numbers in WB.

In this study, the MPV did not vary between the different analyzed samples (WB, PRP, and PPP). The results in relation to this parameter seemed controversial between studies. Indeed, compared to PLT concentrate, MPV increased in WB samples (33) although higher values in PLT concentrates compared to WB have been reported (27). MPV represents the average size of PLTs, increasing during PLT activation (62) and dependent on factors, such as anticoagulant, temperature and storage time of the blood sample (63–65). Although ADVIA is one of the best methods for PLT analysis (53), feline blood can contain PLTs larger than 60 fL (66) and may not be detected by the analyzer. This failure to detect large PLTs may be the reason why the number is falsely lower in some of the samples tested. Also, a low number of large PLT may not sufficiently increase MPV above the upper limit of the reference range.

Studies in cats with naturally occurring FeLV infections are valuable in providing practical clinical guidance and outcome expectations. The diagnosis carried out on these animals consisted of a rapid test (ELISA), detecting the presence of the virus (antigen) in blood, which does not always indicate the presence of the disease. Since these cats lived in an endemic area, the probability of false positives in the test could be excluded. As the test was repeated between 180 and 270 days after the first diagnosis of FeLV, these animals were considered to have the progressive form of the disease. Positive results could not be due to vaccination or maternal transfer of the virus, since the animals were adults and did not receive a vaccination regimen. The normal erytrogram and leukogram in the present study reinforce the hypothesis of the absence of BM involvement, since anemic, leukopenic, or thrombocytopenic animals were excluded. It is common that in persistently infected cats with the progressive form of infection hematopoietic cells invade the bone marrow (42), causing neoplastic hematopoietic pathologies, such as lymphoma (43), myelodysplastic syndrome (48), or non-neoplastic, including non-regenerative or regenerative anemia, immunosuppression, lymphopenia, neutropenia, and thrombocytopenia (35, 44–47). A leukogram characterized by leukocytosis with right shift neutrophilia and lymphopenia in response to stress was identified in two cats. Asymptomatic FeLV-positive animals are known to be less likely to have reduced blood counts than symptomatic ones (47). However, the type of viral strain, its pathogenicity and the type of study with experimentally vs. naturally infected animals may explain these discordant findings (67, 68), though future studies are needed to elucidate it.

Although there is no previous evidence on the effect of viral infections on the quality of PLT concentrate in cats, PDGF-BB and TGF-ß1 concentrations were determined in FeLV-positive animals according to the PRGF®-Endoret® protocol previously described (33). In contrast to Silva's et al. (27) study, lower values of PDGF-BB were obtained in FeLV cats but similar or higher values of TGF-ß1 compared to healthy cats. In addition, in FeLV positive cats, lower values of PDGF-BB but similar values of TGF-ß1 than in healthy cats (33) have been obtained. Several reports in humans have documented elevated TGF-ß1 in blood, lymphoid tissues and cerebrospinal fluid in people infected with human immunodeficiency virus (HIV) (69). This elevation of cytokines was associated with defective T-cell recall, as well as B-lymphocyte proliferative responses and immunoglobulin production related to proapoptotic mechanisms (69). The marked increase in TGF-ß1 with advancing HIV-1 infection suggests an important immunosuppressive role of TGF-ß1 in the pathogenesis of this infection (70, 71). On the other hand, PDGF-BB were associated with severe disease in humans with COVID-19. This pro-inflammatory mediator indicates that innate immune cell responses and anti-viral T-cell responses are responsible for SARS-CoV-2 pathogenesis in COVID-19 patients (72).

The goal of this study was to characterize PRP and to determine GFs in both PRP and PPP fractions in FeLV positive cats. One of the most important limitations of this study was the small sample size in which only positive asymptomatic animals were considered, therefore, it cannot be considered representative of the entire FeLV positive feline population. The lack of a control group or the use of plasma from negative animals, same as the use of samples from positive animals in which the virus was purified by extraction with triton x 100 compared to the immunoassay for the detection of the FeLV p27 antigen used in our study has not allowed us to obtain comparative results.

On the other hand, since the GFs in PLT concentrates are modified by the type of PLT activator and other factors such as time and temperature, it would have been interesting to assess to what extent the time and temperature before proceeding to the

freezing of the samples could have modified the concentrations of TGF-ß1 and PDGF-BB in this study. Taking these limitations into account, further research with regards to GFs obtained by PRGF®-Endoret® methodology is needed. Since PRP obtained in our study showed similar characteristics to PRP products following PRGF®-Endoret® system, asymptomatic animals could benefit from the use of GFs in cutaneous wounds, osteoarthritis, or bone fractures to improve their quality of life. since there is no cure for FeLV. However, further studies are needed to evaluate and define the potential clinical applications of PRP in FeVL-positive cats.

5. Conclusions

Using PRGF®-Endoret® technology, it was possible to obtain and differentiate the PRP and PPP fractions in FeLV positive cats. The moderate PLT enrichment and the absolute reduction of RBCs and WBCs in the samples obtained have allowed the product to be classified as a P-PRP, although the minimum required for the PLT concentrate was not reached. The optimization and standardization of the protocol for the use of PRGF could represent an alternative to alleviate the side effects induced by the virus in feline patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Animal Welfare Ethics Committee (CEEA) of the CEU Cardenal Herrera University in Valencia (Spain). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LM-P performed the experiment. LM-P and KS analyzed data and wrote the manuscript and developed the first draft of the manuscript. DC, MT-T, and PP participated in performing the experiment. JMC, MR, and JS designed the study, supervised all procedures, and coordinated the research. MR and KS performed statistical analysis. JJC analyzed blood samples. JS proofread the manuscript and gave final approval of the version. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

Publisher's note

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Equine osteoarthritis: Strategies to enhance mesenchymal stromal cell-based acellular therapies

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Osteoarthritis (OA) is a degenerative disease that eventually leads to the complete degradation of articular cartilage. Articular cartilage has limited intrinsic capacity for self-repair and, to date, there is no curative treatment for OA. Humans and horses have a similar articular cartilage and OA etiology. Thus, in the context of a One Health approach, progress in the treatment of equine OA can help improve horse health and can also constitute preclinical studies for human medicine. Furthermore, equine OA affects horse welfare and leads to significant financial losses in the equine industry. In the last few years, the immunomodulatory and cartilage regenerative potentials of mesenchymal stromal cells (MSCs) have been demonstrated, but have also raised several concerns. However, most of MSC therapeutic properties are contained in their secretome, particularly in their extracellular vesicles (EVs), a promising avenue for acellular therapy. From tissue origin to *in vitro* culture methods, various aspects must be taken into consideration to optimize MSC secretome potential for OA treatment. Immunomodulatory and regenerative properties of MSCs can also be enhanced by recreating a pro-inflammatory environment to mimic an *in vivo* pathological setting, but more unusual methods also deserve to be investigated. Altogether, these strategies hold substantial potential for the development of MSC secretome-based therapies suitable for OA management. The aim of this mini review is to survey the most recent advances on MSC secretome research with regard to equine OA.

KEYWORDS

osteoarthritis, mesenchymal stromal cells, extracellular vesicles, acellular therapy, horse

1. Introduction

Articular cartilage is the connective tissue that covers the extremities of bones in diarthrodial joints. Its viscoelasticity allows for shock absorption and joint mobility without friction (1). Articular cartilage is composed of specialized cells called chondrocytes, and an abundant extracellular matrix mainly enriched in type II collagen (Col II) and aggrecan. Osteoarthritis (OA) is a degenerative joint disease that, in its later stages, affects the whole joint and leads to decreased joint mobility, pain and impaired quality of life. During OA, articular cartilage homeostasis is disrupted and the overproduction of catabolic enzymes, such as matrix metalloproteinases (MMP) and aggrecanases, leads to cartilage degradation, articular inflammation and, eventually, subchondral bone exposure (1). OA management is challenging because cartilage has a limited capacity for self-repair. To date, there are no curative OA treatments.

As in humans, horses can develop OA due to aging or intense physical activity, directly affecting horse health and welfare, and diminishing performance in sport and race horses (2). OA can thus put an early end to a horse career, leading to economic losses (3). The horse is also an excellent preclinical model for OA, because human and equine articular cartilage share many similarities in terms of anatomy, mechanical functioning, and cellular and molecular composition (2, 4).

Current clinical treatments of equine OA such as anti-inflammatory drugs, dietary supplements or viscosupplementation are only symptomatic and do not prevent the degenerative process of the disease (2). However, among the various emerging regenerative therapies, strategies based on mesenchymal stromal cells (MSCs) appear to hold promise. MSCs possess immunomodulatory and anti-inflammatory effects and regenerative properties that have direct effects or act indirectly through the release of bioactive molecules free or enclosed in extracellular vesicles (EVs) such as exosomes or ectosomes (5).

Here, we explore the relevance and future challenges of MSC-derived EVs (MSC-EVs) as a new orthobiologic approach to manage equine OA.

2. Mesenchymal stromal cell-based therapies

MSCs are defined as multipotent cells able to self-replicate and differentiate into distinct cell types, such as adipocytes, osteoblasts or chondrocytes. Bone marrow (BM) is the most common source of MSCs, even though these cells can be found in several other niches in an organism [adipose tissue (AT), umbilical cord (UC), dental pulp, synovium, etc.] (5). Research over the last few years has suggested that MSCs hold great potential for diverse therapeutic applications through putative immunomodulatory, anti-inflammatory effects or by stimulating tissue regeneration (6–9).

Regarding articular diseases, MSCs have shown the potency to reduce OA-related pain and increase cartilage repair (6, 9). Additionally, OA-afflicted horses treated with intra-articular injections of MSCs show improvement in clinical signs, cartilage appearance and athletic performance (10–12). In the context of autologous chondrocyte transplantation (ACT), equine MSC (eMSC)-derived cartilage organoids overcome the limitations inherent to the use of dedifferentiated chondrocytes and may provide an accurate and reliable drug screening model for OA (13–17).

Although the direct use of MSCs remains promising for equine OA treatments, several challenges have been identified, including their *in vivo* distribution, a low engraftment rate, their immunogenicity and their possible tumorigenicity risk (18–24) (Figure 1).

3. Mesenchymal stromal cell-derived extracellular vesicles as a new orthobiologic therapy

The MSC secretome contains a broad spectrum of compounds including nucleic acids, proteins such as cytokines, growth factors or even lipids. Some of these compounds can be encapsulated in vesicles, called EVs. EVs include apoptotic bodies (>1 μm diameter), ectosomes or microvesicles (100–1,000 nm) and exosomes (30–200 nm). Among the MSC secretome, exosomes contain numerous molecules with proven pro-regenerative and anti-inflammatory properties as reviewed in Hade et al. (25). In addition, numerous studies have demonstrated the cartilage regeneration potential of EVs (26).

Exosomes originate from the endocytic pathway, develop within multivesicular bodies (MVBs) and are delivered to the extracellular environment when MVBs fuse with the plasma membrane (27). Exosomes enter the cells through membrane fusion, endocytosis or interaction with a receptor that is subsequently internalized.

Given the disadvantages attributed to MSC-based cellular therapy, secretome-, EV- and exosome-based strategies are an appealing alternative to explore the therapeutic potential of eMSCs in equine OA management. To date, only a few studies have demonstrated the therapeutic potential of eMSC-EVs in the context of horse OA. Using an *in vitro* cartilage organoid model, our research group has already demonstrated the pro-anabolic potential of eMSC-conditioned media (CM) and the presence of exosomes in eMSC-CM (28). The CM corresponds to the medium in which cell were cultured, hence it contains components that had been secreted by the cells. Noteworthy, because cells cultured *in vitro* do not have exactly the same features than their *in vivo* counterparts, the CM and the secretome of MSC *in vivo* might differ. MSC-EVs can decrease the transcript levels of MMP and pro-inflammatory molecules (29, 30). Furthermore, EVs can be used as biomarkers to evaluate the progression of OA (31). However, to date, *in vivo* cartilage regeneration using EVs in the equine model remains to be demonstrated.

Nevertheless, environment deeply influences MSC secretion and constitutes a variable worth of consideration to improve the capacities attributed to their therapeutic effect (32–34). The MSC therapeutic potential and secretome differ according to the tissue they derive from and can be modulated by several factors as discussed below.

4. Therapeutic potential of mesenchymal stromal cells and their derivatives depends on the source and the culture procedure

MSCs from all sources share similarities, regarding in particular their self-renewal, multipotency and immunomodulation capacities. Nevertheless, the individual, the age, the tissue and the niche MSCs are isolated from lead to slight variations of their properties (35) including their secretory production. For example, AT-eMSCs, peripheral blood (PB)-eMSCs, BM-eMSCs and UC-eMSCs display significant variation in inflammation-related gene expression, although interferon-γ (IFN-γ) stimulation homogenizes the gene expression profile between the studied MSC sources (36). Furthermore, their immunomodulatory properties can be induced through different mechanisms. For example, AT-eMSCs and UC-eMSCs can induce lymphocyte apoptosis, whereas BM-eMSCs, PB-eMSCs and cord blood (CB)-eMSCs induce lymphocyte cell cycle arrest (37). Our research group has demonstrated differences in proliferation and differentiation capacity between BM-eMSCs, CB-eMSCs and UC-eMSCs (13–17). BM-eMSCs are more prone to produce hyaline-like cartilage extracellular matrix (ECM) with low amounts of atypical molecules than are CB-eMSCs and UC-eMSCs. The impact of eMSC origin on antibacterial activity has also been demonstrated (38) and the eMSC secretome also depends on the tissue source they derive from. Indeed, Navarette et al. have reported that the miRNA content of EVs differs between AT- and endometrial eMSCs from the same animal (39). In addition to the inter-tissue origin heterogeneity, eMSCs derived from the same tissue source

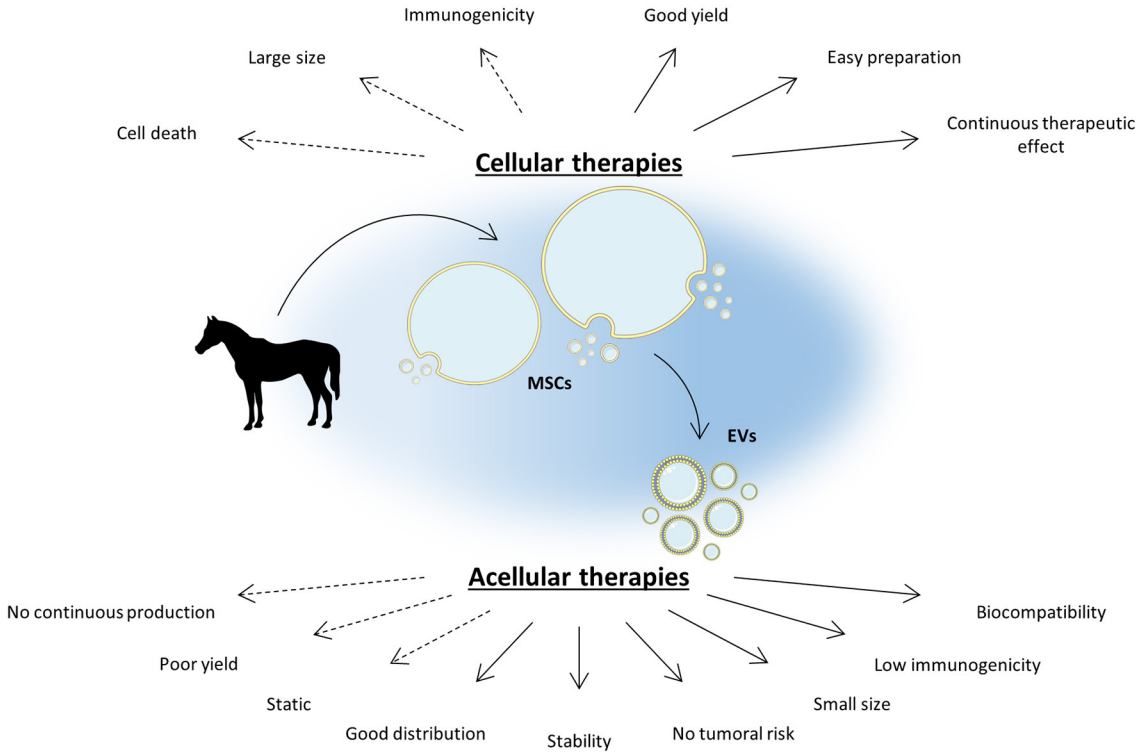

FIGURE 1
MSC-based acellular therapies are an appealing strategy to bypass cellular therapy limitations. Cellular therapies based on mesenchymal stromal cells (MSCs) demonstrate a promising potential in the management of equine osteoarthritis (OA) by reducing joint inflammation and enhancing cartilage regeneration. However, MSC intra-articular injection can be immunogenic and brings several concerns inherent to their nature. As the main part of MSC therapeutic potential lies in their secretome, notably in extracellular vesicles (EVs), acellular therapies appear to be a promising alternative to increase the safety of MSC-based therapy while improving its efficacy. EVs are highly biocompatible and can easily diffuse into tissues thanks to their small size. To date, acellular therapies still exhibit a few limitations but several strategies are in progress to overcome them. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

can show differences in gene expression and functional heterogeneity (40). Ultimately, the differentiation status of MSCs modulates their properties. As an example, BM-eMSCs engaged in a chondrogenic differentiation process exert a weaker inflammatory response to IL-1β than naive BM-eMSCs (41). eMSC heterogeneity suggests that these cells are highly influenced by their environment. Considering the impact of MSC origin on their properties, tissue source should be wisely selected before exploiting their secretome. Hence, in the study of the therapeutic potential of eMSCs, medium composition and culture conditions must be carefully selected.

Culture medium supplementation with fetal bovine serum (FBS) is widely used to support *in vitro* MSC proliferation. One of the challenges in the use of FBS resides in its non-standardized and variable composition between batches (42). This xenogeneic supplement can interfere with MSC metabolism, phenotype and, by consequence, the properties of their secretome. Additionally, FBS is a limiting factor in *in vivo* applications because it can trigger an immune response. For instance, eMSCs cultured with FBS have exacerbated immunogenicity compared with eMSCs cultured with allogenic or autogenic equine BM supernatant-supplemented culture medium (43). The use of autologous equine serum can be considered for the culturing eMSCs because horses can tolerate the removal of 25% of their blood volume (44). Replacing FBS with equine platelet lysate as a medium supplement has also been tested, and resulted in similar growth and phenotypical BM-MSC characteristics (45) as well in moderately increased immunomodulatory marker expression (46). In contrast, Pezzanite et al. demonstrated the superiority of FBS over equine serum supplementation to generate functional eMSCs (47). Serum-free medium is another alternative to FBS supplementation during MSC expansion. This option is being investigated, especially during CM and EV harvest, to avoid the co-isolation of xenogeneic contaminants that can reduce the therapeutic efficacy of EVs. Serum-free-cultured eMSCs decrease the pro-inflammatory mediator secretion of activated T-cells, but to a lesser extent than eMSCs cultured with FBS (48). In the last few decades, efforts have been made to culture the cells *in vitro* in contexts similar to those *in vivo*, particularly using three-dimensional (3D) cultures instead of monolayers (2D). MSCs cultured in 3D undergo morphological and metabolic changes, and their proliferation and survival rate are increased (49). Compared with a monolayer culture, CM from MSCs cultured in spheroids suppress macrophage pro-inflammatory cytokine secretion and enhance the production of the anti-inflammatory cytokine interleukin (IL)-10 (50). Additionally, MSCs grown in dynamic 3D cultures—spinner flasks and a rotating bioreactor—show enhanced therapeutic properties, but mRNA profiles differ according to the method used (51). To date, these culture methods have not yet been tested in the equine model.

Long-term *in vitro* expansion affects MSCs (52). A large proportion of MSCs become senescent and display altered differentiation and immunosuppressive potential (53, 54).

Therefore, early passages should be preferred to harness eMSC therapeutic properties. Noteworthily, cryopreservation does not appear to interfere with eMSC differentiation potential and therapeutic potential (55, 56), but the isolation protocol can affect the characteristics of these cells (57). Nevertheless, the MSC secretome, particularly EVs, can exhibit diminished immunosuppressive properties after freeze-thawing (58).

The surrounding environment inevitably affects eMSCs. Controlling it remains a real challenge that must be addressed to increase the reproducibility of the therapeutic effects of MSCs or MSC-derived products such as EVs. On the other hand, the ability of MSCs to adapt to their environment also represents a tremendous opportunity to improve their therapeutic potential (Figure 2).

5. Future directions to enhance the therapeutic potential of MSC and their derivatives

It has already been proven that the unstimulated eMSC secretome can enhance the equine articular chondrocyte phenotype and increase their migratory capacity (28, 30). Nevertheless, therapeutic capacities of naive MSCs developing in a healthy environment have not been optimized. To maximize the MSC therapeutic properties, stimulation by extrinsic factors can mimic a pathological situation, leading to a boost in the MSC immunomodulatory and therapeutic capacities (8). In OA, this is illustrated *in vivo* by a pro-inflammatory environment induced by a cartilage lesion, triggering an MSC reaction to external aggression. These diverse procedures are collectively called preconditioning, licensing or priming and are probably the key to improvements in the regenerative potential of the MSC secretome (Figure 2).

Given that MSCs evolve *in vivo* in hypoxic conditions (2%−9% oxygen) (59), growing them under a 21% oxygen atmosphere can alter their phenotype and their secretome. Low oxygen tension regulates hypoxia-inducible factor (HIF-1α) activity that triggers the transcription of diverse genes involved in eMSC stemness-associated features, differentiation and self-renewal (60). Because EV cargo reflects the nature and composition of their cell source, these factors are likely to be found in the eMSC secretome and modify their properties (61). Recently, Zhang et al. (62) showed that the secretome of hypoxia-preconditioned MSCs enhanced rat chondrocyte proliferation and migration and inhibited apoptosis compared with rat chondrocytes cultured with the secretome from MSCs grown in normoxia. To our knowledge, none of the hypoxia preconditioning advantages described previously have yet been demonstrated in equines.

When tissue is damaged, inflammatory factors and chemokines are released by immune cells recruited to the inflammation site, triggering eMSC activation. Reproducing this process *in vitro* is one way to enhance eMSC-EV therapeutic capacities. Interferon γ (IFN-γ) is known as the gold standard cytokine priming for MSCs. Many studies confirm its abilities to enhance eMSC secretome-mediated chondroprotection and downregulate inflammatory genes in equine chondrocytes (63, 64). IFN-γ can also increase the immunosuppressive properties of murine BM-MSCs, but priming does not enhance the capacities of EVs (58). Depending on their source, eMSCs vary in their response to IFN-γ, but this cytokine lowers inter-tissue differences in unstimulated eMSC immunomodulatory gene expression (36). Therefore, tissue of origin may not be a crucial parameter when IFN-γ is used to license eMSCs. Moreover, eMSC surface expression of major histocompatibility complex (MHC)-II in horses is increased by IFN-γ and decreased by IL-1β. However, tumor necrosis factor-α (TNF-α) has no impact on the expression of MHC-II, demonstrating the importance of the nature of the cytokine used for eMSC stimulation on their antigenicity and immunomodulation (65).

However, a single molecule is not an accurate replication of the whole inflammatory environment. Pro-inflammatory cytokines can exert distinct actions. For example, preconditioning human MSCs with TNF-α enhances the chondrogenic differentiation potential of the cells, whereas IL-1β does not enhance the chondrogenic potential of MSCs (66). Thus, a combination of several of these factors may be more accurate. Stimulation of eMSCs with IFN-γ and TNF-α resulted in the overexpression of immunomodulation-related genes (67). Alone or in combination, these cytokines also significantly increased the expression of prostaglandin-endoperoxide synthase 2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) in eMSCs (68). PGE2 (37) and IDO both mediate most of the inhibition of equine lymphocyte proliferation, although the involvement of IDO in the horse model is a subject of debate (69). Recently, injection of the secretome from TNFα and IFNγ-stimulated eMSCs in LPS-induced osteoarthritic equine joints (70) led to reduced inflammatory symptoms and higher ECM marker expression in joints treated with concentrated CM. Nonetheless, no differences were noted between MSC-secretome and MSC groups. To our knowledge, this is the only MSC-CM *in vivo* experiment that has been carried out in the equine model.

However, combining cytokines only considers a small part of *in vivo* molecular content and interactions. Cytokine priming can have a deleterious effect on eMSC viability and trilineage differentiation, which is not observed when they are primed with inflammatory synovial fluid (SF) (71). Because immunomodulatory cytokines are also released by activated T-cells, CM from PB mononuclear cell-activated eMSCs can diminish T-cell proliferation in a significative manner compared with naive eMSCs (69). Platelet-rich plasma (PRP) and bone marrow concentrate (BMC) can stimulate the migration of eMSCs (72). These biological fluids contain various healing-related factors and, because migration is linked to immunomodulation (73), they may represent a strategy for eMSC preconditioning.

Hypoxia and cytokine priming are the most investigated strategies for improving eMSC therapeutic potential. Nevertheless, some less well-known methods may be promising. eMSCs are naturally exposed to mechanical forces such as fluid shear stress, hydrostatic compression or mechanical loading that affect MSC proliferation, differentiation and migration (74). Moreover, the human MSC secretome's ability to modulate angiogenesis is influenced by the mechanical environment of MSCs in both 2D and 3D culture systems (75). To date, mechanostimulation efficiency has not been demonstrated in the equine model. Extracorporeal shock wave therapy (ESWT) is a type of mechanical sensing using acoustic waves already employed in the therapies for tendon and ligament affections, but only as an auxiliary treatment in equine OA management. ESWT can increase metabolic activity and differentiation of eMSCs, but no effects on immunomodulatory potential have been observed (76). Furthermore, CM from human

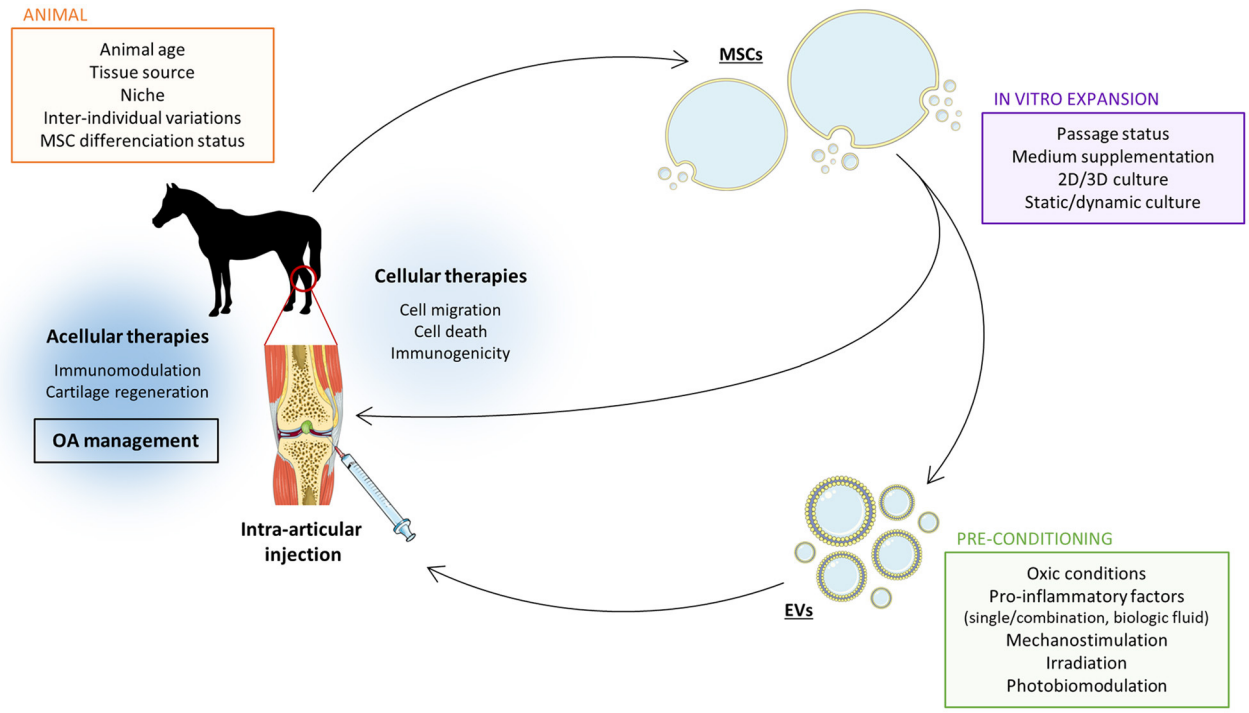

FIGURE 2
Several parameters must be considered to improve the therapeutic potential of MSC-derived EVs in the treatment of equine OA. Mesenchymal stromal cells (MSC)-derived extracellular vesicles (EVs) have the capacity to improve equine osteoarthritis (OA) management *via* immunomodulation and stimulation of cartilage matrix synthesis. However, this therapeutic potential can be enhanced by adjusting the steps in the EV production protocol. The source of MSCs affects EV properties and their therapeutic capacities. The MSC culture methods can be used as a tool to optimize the therapeutic effect of MSC-derived EVs. In the last years, it has been demonstrated that EV properties can be refined by adjusting oxic conditions, adding pro-inflammatory cytokines or other physical parameters such as mechanical stress or light exposition. Setting the balance between all these factors is crucial to achieve the most effective therapeutic effect of MCS-derived EVs for equine OA. Moreover, acellular therapy has the advantage to avoid most of direct MSC intra-articular injections concerns such as immunogenicity. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

MSCs exposed to pulsed electromagnetic fields can also enhance articular chondrocyte migration and reduce the inflammatory state and apoptosis of these cells (77), but no proof is available regarding equine cells. Lastly, 1064 nm irradiation enhances IL-10 and VEGF expression in naive eMSCs (78). Photobiomodulation may be another way of maximizing stimulation and therapeutic potential of eMSCs in the treatment of equine OA.

6. Conclusion

The relevance of the equine model in OA therapy research contributes to the emergence of new studies and better understanding of the therapeutic potential of eMSCs. Despite recent advances in MSC-based therapies, several hurdles still need be overcome to propose a MSC therapy to treat equine OA. Notwithstanding the difficulties of quantification and large-scale production due to the novelty of the approach, eMSC-EVs may be an appropriate adjunct to improve MSC-based equine OA management. This strategy can benefit from the immunomodulatory, anti-inflammatory and regenerative properties of MSCs without inducing side effects such as immunogenicity or tumoral transformation (Figure 1).

Nonetheless, there still are numerous questions before considering therapies based on MSC-EVs for equine joint disorders. Issues involving eMSC origin, culture and preconditioning conditions, method of EV isolation, enrichment, storage and dosing need to be addressed, as well as the safety of allogenic or autologous EVs (Figure 2). Another critical issue that needs to be examined is the *in vivo* targeting of cartilage. Currently, a promising strategy is the use of a cationic molecule that can coat EVs and reverse their negative surface charge to infiltrate the negatively charged cartilage more easily (79).

To address all these considerations, *in vitro* organoid models of equine chondrocytes or eMSCs can be useful to optimize EV preparation and to identify the ideal treatment for use in controlled clinical trials on horses affected by OA.

Finally, progress in equine OA treatment using the therapeutic potential of MSC-EVs is critical for horse welfare and the equine industry, and may even eventually be transposable to humans as part of a One Health approach.

Author contributions

Conceptualization, literature search, and writing: MJ, RC, and FC. Review and editing: MJ, RC, FC, and PG. Supervision and approval: PG. All authors contributed to this review and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Distinct differences in immunological properties of equine orthobiologics revealed by functional and transcriptomic analysis using an activated macrophage readout system

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Introduction: Multiple biological therapies for orthopedic injuries are marketed to veterinarians, despite a lack of rigorous comparative biological activity data to guide informed decisions in selecting a most effective compound. Therefore, the goal of this study was to use relevant bioassay systems to directly compare the anti-inflammatory and immunomodulatory activity of three commonly used orthobiological therapies (OTs): mesenchymal stromal cells (MSC), autologous conditioned serum (ACS), and platelet rich plasma (PRP).

Methods: Equine monocyte-derived macrophages were used as the readout system to compare therapies, including cytokine production and transcriptomic responses. Macrophages were stimulated with IL-1ß and treated 24 h with OTs, washed and cultured an additional 24 h to generate supernatants. Secreted cytokines were measured by multiplex immunoassay and ELISA. To assess global transcriptomic responses to treatments, RNA was extracted from macrophages and subjected to full RNA sequencing, using an Illumina-based platform. Data analysis included comparison of differentially expressed genes and pathway analysis in treated vs. untreated macrophages.

Results: All treatments reduced production of IL-1ß by macrophages. Secretion of IL-10 was highest in MSC-CM treated macrophages, while PRP lysate and ACS resulted in greater downregulation of IL-6 and IP-10. Transcriptomic analysis revealed that ACS triggered multiple inflammatory response pathways in macrophages based on GSEA, while MSC generated significant downregulation of inflammatory pathways, and PRP lysate induced a mixed immune response profile. Key downregulated genes in MSC-treated cultures included type 1 and type 2 interferon response, TNF-α and IL-6. PRP lysate cultures demonstrated downregulation of inflammation-related genes IL-1RA, SLAMF9, ENSECAG00000022247 but concurrent upregulation of TNF-α, IL-2 signaling, and Myc targets. ACS induced upregulation of inflammatory IL-2 signaling, TNFα and KRAS signaling and hypoxia, but downregulation of MTOR signaling and type 1 interferon signaling.

Discussion: These findings, representing the first comprehensive look at immune response pathways for popular equine OTs, reveal distinct differences between therapies. These studies address a critical gap in our understanding of the relative immunomodulatory properties of regenerative therapies commonly used in equine

practice to treat musculoskeletal disease and will serve as a platform from which further *in vivo* comparisons may build.

KEYWORDS

biologic, intra-articular, mesenchymal stromal cell, autologous conditioned serum, equine, platelet rich plasma

Introduction

Osteoarthritis (OA) represents one of the most common conditions treated by equine practitioners and is estimated to affect 80% of horses over 15 years of age and up to 2/3 of Thoroughbred racehorses (1–3). Despite this high prevalence, no approved pharmacological intervention, biological therapy, or procedure prevents or reverses progressive destruction of the degenerative joint. Orthobiologic therapies (OTs) are increasingly popular but their true efficacy remains controversial due to lack of rigor in clinical study design and the lack of demonstrated consistency in product formulation. Progressive joint degeneration is increasingly thought to be a multifactorial disease in which the innate immune system, particularly macrophages, plays an important role in regulating and perpetuating low-grade inflammation, resulting in continued articular cartilage breakdown for years following initial joint trauma. Synovial macrophages are the most numerous immune cells in the joint and among the most immunologically active cells, responding to signals released from cartilage degradation products, among other environmental triggers (4). Macrophages display high phenotypic and functional heterogeneity ranging from classical pro-inflammatory (M1) macrophages to reparative (M2) macrophages (5, 6). Alterations in synovial macrophage functional activity have been implicated in the pathogenesis of OA, propagating cartilage destruction and synovitis, with a higher ratio of M1/M2 associated with greater severity in human knee OA (7–14). Therefore, efforts to reduce inflammation associated with progression of OA would include resident synovial macrophages as well as infiltrating inflammatory monocytes as primary targets for immune modulation.

Orthobiologic therapies (OTs) frequently used in veterinary practice to treat OA include autologous conditioned serum (ACS) (15, 16), platelet rich plasma (PRP) (17–21), and mesenchymal stromal cells (MSC) (22, 23). However, relatively little work has been done to evaluate and compare the biological activities of these compounds more fully (24–27). There is also a paucity of evidence to support optimal processing and storage conditions, currently recommended doses, and evidence-based protocols for application of OTs clinically (15, 16, 28, 29). The decision on which OT to use in specific disease conditions (e.g., soft tissue vs. cartilage injury) is often based on incomplete information on the specific pathological physiology and thus may lead to inappropriate choices regarding the most effective OT.

Therefore, the purpose of the current study was to directly evaluate and compare the immune modulatory properties of three commonly used OTs using multiple functional and transcriptomic readouts. This in-depth analysis provides a more complete understanding of the different OT mechanisms of action and how they may resemble and differ from one another. Specifically, the first aim was to determine the macrophage cytokine response to OTs using relevant cell culture assays with equine macrophages. The second aim was to use transcriptomic analysis of OT-treated macrophages to identify unique and potentially disease-modifying pathways and how they may differ between the three OTs. We hypothesized that all three OTs would suppress IL-1ß induced macrophage activation and would also activate unique and distinctive gene expression pathways in macrophages. This approach was based on the fact that IL-1ß is one of the key cytokines associated with cartilage degradation in OA, and a cytokine known to strongly activate synovial macrophages (30). The long-term goal of this study is to add to our understanding of the mechanisms by which OTs function to guide more evidence-based treatment decisions with different OT products.

Methods

Study overview

Three healthy Quarter Horses (aged 2, 5, and 5 years; one gelding and two mares) were used as donors of blood and bone marrow aspirate to prepare orthobiologic therapies. Two additional Quarter Horse geldings, aged 3 and 5 years, were used as blood donors to generate monocyte-derived macrophage cultures. All procedures were approved by the University's Institutional Animal Care and Use Committee (IACUC protocol #927) and were performed in accordance with CONSORT guidelines according to national guidelines under which the university operates and NIH guidelines for the Care and Use of Laboratory Animals (8th edition). Study overview is summarized in Figure 1.

Orthobiologic therapy preparation

To isolate MSC, the sternum of donor horses ($n = 3$) was clipped and aseptically prepared. Bone marrow aspirate (5–15 ml) was obtained from the sternebrae using a jamshidi into a syringe containing 1 ml heparin (5,000 U/ml). Bone marrow aspirates were centrifuged by Ficoll density separation (Ficoll-Paque^{TM} Plus; GE Healthcare BioSciences) at 400 g for 18 mins to pellet red cells as previously described (31, 32). The mononuclear cell population was plated at 10,000 cells/cm^2 and expanded in culture (37°C, 5% CO_2, 95% humidity) to 80% confluence for approximately seven days in complete growth medium [Dulbecco's Modified Eagle's Medium (DMEM) with 1,000 mg/L glucose, 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), 1M HEPES]. Cells were detached from flasks by trypsinization, then frozen at 5×10^6 cells/ml in freeze media [90% FBS, 10% dimethyl sulfoxide (DMSO)] in liquid nitrogen vapor phase until further use. To generate MSC-CM for use in co-culture assays, cells were thawed quickly in a 37°C water bath and cultured 48 h in complete growth medium under standard incubation conditions (37°C with 5% CO_2). MSC were subsequently plated at 100,000 cells/well on 24-well plates for 24 h and supernatants were collected and frozen at −80°C for use in immunoassays.

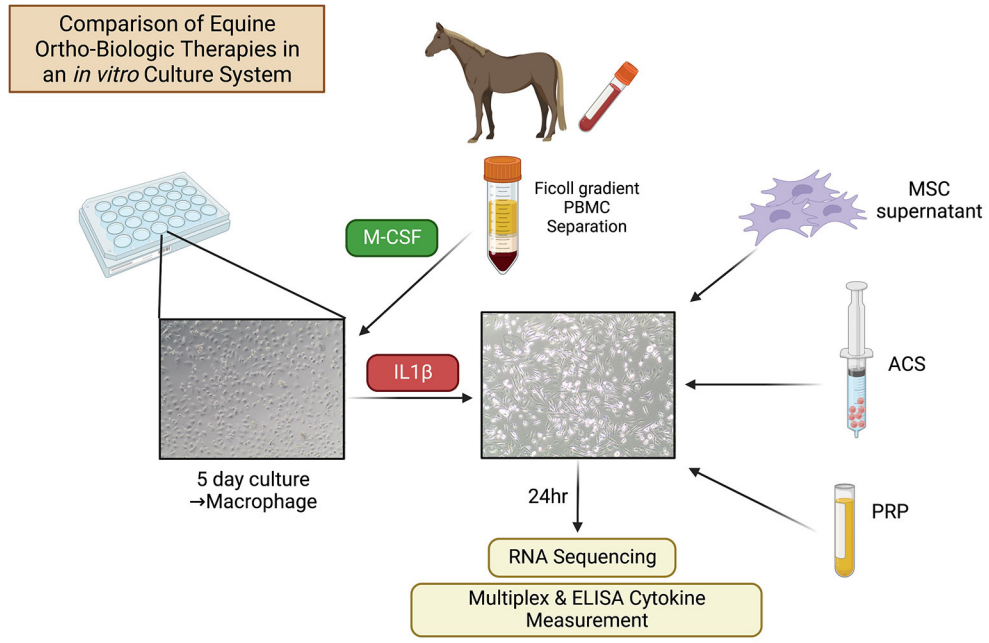

FIGURE 1
Schematic of study design. Equine macrophages were generated from PBMC by adherence of the monocytes, followed by treatment with M-CSF. After 5 days in culture, macrophages were stimulated with IL-1ß (10 ng/ml) and orthobiological therapies (MSC-CM, PRP, ACS) from $n = 3$ donor horses were added to macrophage cultures at 1:3 cell ratio. After 24 h of treatment, macrophages were washed with PBS and cultured in complete media an additional 24 h, at which time supernatants were collected for cytokine analysis and macrophage cells were collected for RNA sequencing.

To prepare autologous conditioned serum (interleukin 1 receptor antagonist; IRAP), blood (60 ml) was drawn and incubated according to manufacturers' instructions (IRAP II, Arthrex, Naples, FL, USA 34108). Aliquots (1 ml) of IRAP were frozen at −80°C for later use in immunoassays. To prepare platelet rich plasma lysate, blood was drawn, processed according to manufacturers' instructions (Arthrex ACP Double Syringe System, Naples, FL, USA 34108), and frozen at −80°C in 1 ml aliquots for use in co-culture assays.

Monocyte-derived macrophage cultures

To generate macrophage cultures, equine peripheral blood mononuclear cells were isolated from whole blood of two horses by density gradient centrifugation (Ficoll-Paque TM plus, GE Healthcare Bio-Sciences) and cultured in macrophage media (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, non-essential amino acids, and penicillin/streptomycin antibiotics; SigmaAldrich) with human M-CSF (PeproTech, Rocky Hill, NJ USA 80553) at 30 ng/ml to stimulate differentiation into macrophages in 5 days, as previously described (33).

Equine monocyte-derived macrophages were stimulated with IL-1ß (10 ng/ml) and OTs (MSC-CM, PRP lysate, ACS) were added at the same time to macrophage culture media at a ratio of 1:3 OT to complete growth media in culture (i.e., 25% OT in culture media). The ratio of OT to growth media was determined in a pilot study titrating OT to growth media to determine the maximum volume at which OT could be added in culture media while still maintaining macrophage cell viability over 80% following 24 h in culture. Controls included IL-1ß stimulated and unstimulated macrophages. Following transient addition of treatments for 24 h in culture, macrophages were washed three times with phosphate buffered saline (PBS) and cultured an additional 24 h in macrophage culture media. At that time, culture supernatants were collected and assessed by multiplex bead assay (23 cytokines) and ELISA immunoassay (PGE-2, TGF-ß) to characterize the macrophage response. Macrophages were collected in RNA lysis buffer (350 µl/sample) and frozen at −20°C until RNA isolation was performed.

ELISA for cytokine and PGE quantification

An ELISA was used to measure the concentration of prostaglandin E2 (PGE-2 high sensitivity ELISA kit, Enzo Life Sciences, Inc. Farmingdale, NY 11735) and TGF-β (Human/Mouse/Rat/Porcine/Canine TGF-ß1 quantikine ELISA, R&D Systems, Inc. Minneapolis, MN 55413) in culture supernatants. Fluorescent bead-based multiplex assay (Milliplex MAP Equine Cytokine/Chemokine Magnetic Beads Multiplex Assay, Millipore Sigma, Burlington, MA, 01803) was used to quantify the concentrations of 23 analytes [Eotaxin/CCL11, FGF-2, Fractalkine/CS3CL1, G-CSF, GM-CSF, GRO, IFN, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12 (p70), IL-13, IL-17a, IL-18, IP-10, MCP-1, RANTES/CCL5 and TNFα] in cell culture supernatants.

RNA sequencing

RNA was extracted from frozen samples using the RNeasy kit (Qiagen Germantown, MD) according to manufacturer's instructions and sent to Novogene Corporation Inc. (Sacramento, CA) for RNA sequencing. RNA quality was determined by bioanalyzer (Agilent Technologies, Santa Clara, CA) to have RIN (RNA integrity number) of over 9.0 for all samples. mRNA was enriched using oligo

(dT) beads, followed by cDNA library generation using TruSeq RNA Library Prep Kit (Illumina, San Diego, CA). Sequencing was performed on Illumina Novaseq 6000 machine using 150 bp paired end reads.

Data analysis

To analyze cytokine data, raw data was plotted and visually assessed for normality prior to statistical analysis. Cytokine data was then modeled individually using a linear mixed model [function *lmer* from the lme4 (34) and lmerTest (35) packages] with donor as a random effect to account for differences in donor cell lines. Each of the treatment groups (MSC-CM, ACS, PRP lysate, and negative control) were then compared individually with the positive control using estimated marginal means (package emmeans) (36) and Dunnett's test for multiple comparisons (37). When multiple treatments were found to be significantly different from the positive control, the comparisons were expanded to include evaluation of differences between all groups and Tukey's method for *p*-value adjustment was applied. For the cytokine secretion assays, orthobiologic treatment was modeled as the sole fixed effect. Statistical analyses, graphical analyses and graph generation were performed using Prism software v8.4.1 (GraphPad Software Inc., La Jolla, CA) and R version 4.1.2 "Bird Hippie" (R Foundation for Statistical Computing, Vienna, Austria) (38). For all analyses, statistical significance was assessed as $p < 0.05$.

To analyze RNA sequencing data, demultiplexed Fasq reads generated from Novogene were analyzed using Partek® Flow® software, v10.0 (Partek Inc. Chesterfield, MO). Reads were trimmed for Phred score of 20, adapters removed using cutadapt (39). Trimmed reads were aligned using STAR 2.7.3 using EquCab3.0 and annotated with Ensembl EquCab3.0.107. Feature counts were generated with HTseq (40). Differential analysis was computed using counts normalized to CPM, using DESeq (41). Pathway analysis was performed with GSEA (Gene Set Enrichment Analysis) v4.2.1using Hallmark pathways (42).

Results

Impact of OTs on cytokine and PGE secretion from IL-1ß activated macrophages

We first evaluated the impact of OTs on cytokine secretion by IL-1ß activated equine macrophages. This approach was based on the fact that IL-1ß is one of the key cytokines associated with cartilage degeneration in OA, and a cytokine known to strongly activate synovial macrophages (30). Given evidence that macrophages activated by inflammatory cytokines such as IL-1ß may be the key mediator cell for driving progressive OA, we modeled the impact of OTs on modulating cytokine production by activated equine macrophages, beginning with analysis of cytokine secretion.

Cytokine concentrations were measurable for ten cytokines *via* multiplex immunoassay (IL-1ß, IL-4, IL-6, IL-8, IFN-γ, IP10, GRO, IL-10, TNFα, and RANTES) and for PGE-2 *via* ELISA (Supplementary material 1). Levels of the remaining 14 cytokines assessed were below the detection limit of the multiplex assay (FGF-2, eotaxin, G-CSF, IL1α, GM-CSF, fractalkine, IL-13, IL-5, IL-18, IL-17A, IL-2, IL-12, and MCP-1) or ELISA (TGF-ß). Significant differences were seen between treatment groups for IL-1ß, IL-6, IL-10, IP-10, GRO, and PGE-2 (Figure 2). Levels of IL-1ß were significantly higher in supernatants collected from IL-1ß treated macrophages compared to control media ($p < 0.0001$). All treatments reduced levels of IL-1ß as compared to the IL-1ß positive control with no significant differences between groups (MSC-CM $p < 0.0001$, PRP $p < 0.0001$, ACS $p < 0.0001$). Treatment with PRP lysate induced a significant reduction in GRO compared to IL-1ß treated control ($p = 0.04$) while inducing increasing levels of PGE-2 ($p < 0.0001$) with other treatments showing no significant changes in those specific cytokines. Treatment with PRP lysate and ACS resulted in lower levels of cytokines IL-6 (PRP $p < 0.0001$, ACS $p < 0.0001$), IL-10 (PRP $p = 0.0002$, ACS $p = 0.015$) and IP-10 (PRP $p < 0.0001$, ACS $p = 0.005$) relative to IL-1ß treated controls. The PRP lysate and ACS groups also had significantly lower levels of IL-6 (PRP lysate $p = 0.007$, ACS $p = 0.03$) and IL-10 (PRP lysate $p = 0.0006$, ACS $p = 0.04$) as compared to MSC-CM, while levels of IP-10 were not different between the ACS and MSC-CM groups. There were no statistical differences between orthobiologic treatment groups for the remaining biomarkers assessed that achieved measurable levels by multiplex assay (IL-4, IL-8, IFN-γ, RANTES, and TNF-α). Cytokine levels in unconditioned control media were below the detection limit of the multiplex assay.

Transcriptomic analysis to understand the impact of OTs on activated macrophage immune pathways

To further understand how OTs may modulate the function of IL-1ß activated macrophages, we next used RNA sequencing to interrogate the transcriptomic responses of macrophages exposed to three different OTs (Figure 3; Supplementary materials 2–4). Such an analysis can provide a much more comprehensive understanding of the impact of OT treatment on specific gene expression by macrophages, but more importantly on immune and other pathways that may be relevant to modulation of OA progression.

As a first step in this analysis, we compared IL-1ß activated macrophage transcriptomes to those of non-activated macrophages (Figures 3, 4). After the addition of IL-1β, RNA sequencing analysis indicated a visible separation from untreated macrophage samples by PCA (principal component analysis) (Figure 3A). Moderate changes in transcriptome were detected (Figure 4), including the upregulation of 94 genes with fold change ≥2 or ≤-2 and significant FDR (false discovery rate) adjusted *p*-value of ≤0.05 (Figure 4A) and 73 significantly downregulated genes (Figure 3B). The most upregulated genes (Figure 4B) in IL-1ß activated macrophages included those related to inflammatory immune system process (SLAMF9 and PPBP), response to inflammatory stimuli (TRIB3, GPR84, and DDIT4), and metabolic process (CHAC1, PSAT1, PEAK3, and CEBPD). Downregulated genes mapped to categories including cell signaling (EPS8, GLI1, and WNT1) and to biological regulation of cellular construction, signaling, adhesion and differentiation (CDH5, CREB5, NR2F1, and STC1). Pathway analysis revealed that IL-1ß triggered significant downregulation of pathways involved in EMT transition, NFκβ signaling and upregulation of UV (DNA breakage) response, E2F targets, protein folding (structural) and G2M checkpoint (Figure 4C). Thus, the use of transcriptomic analysis provided important new and previously unpublished insights into

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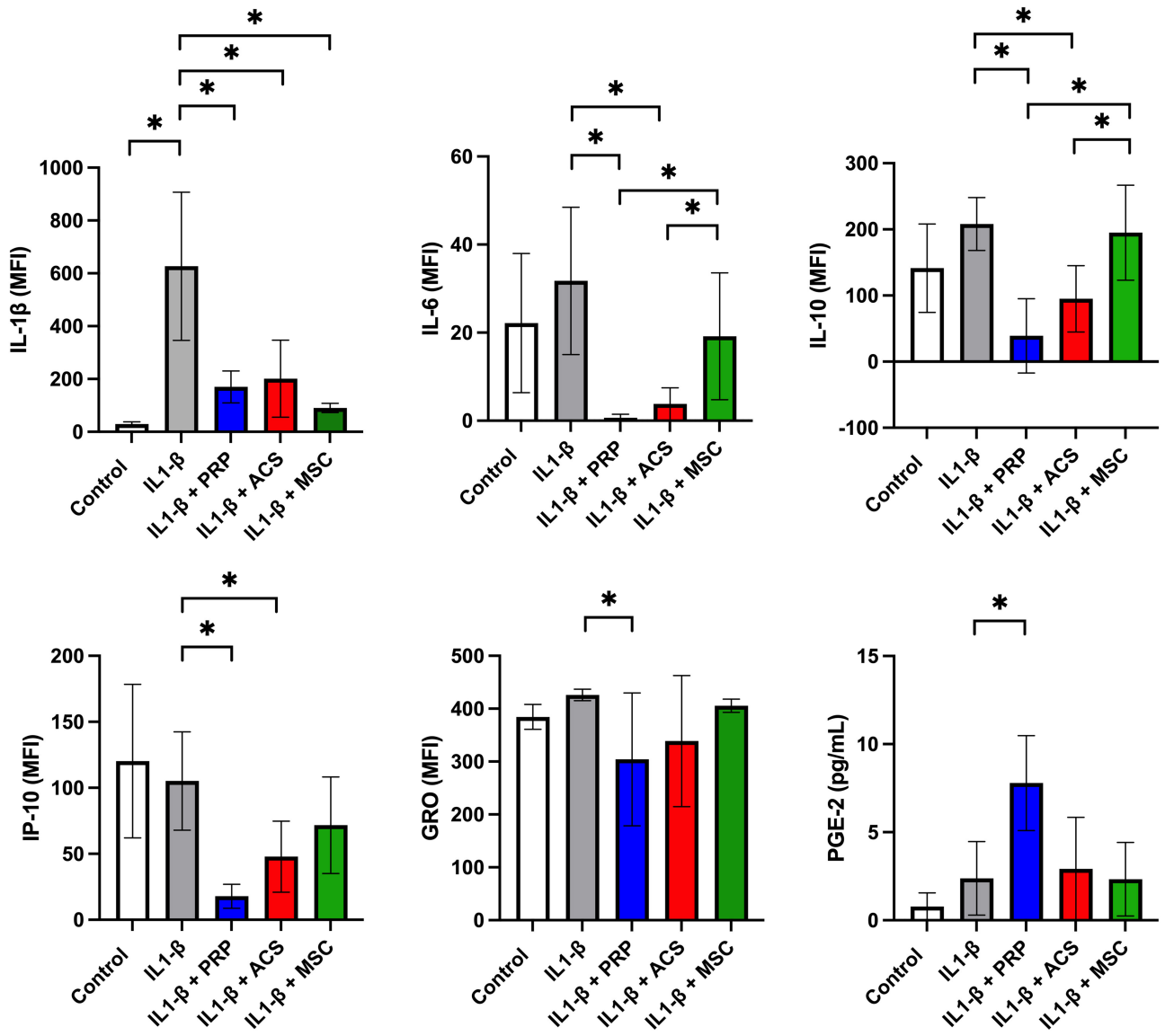

FIGURE 2
Effect of orthobiologic treatment on macrophage cytokine secretion. Orthobiologic therapies (platelet rich plasma or PRP, autologous conditioned serum or ACS, or mesenchymal stromal cell conditioned media or MSC) were added to culture media with equine monocyte derived macrophages and IL-1ß (10 ng/ml) for 24 h. Controls included unstimulated and IL-1ß stimulated macrophages. After 24 h, macrophages were washed in phosphate buffered saline and cultured an additional 24 h and culture supernatants collected and assessed for cytokine levels *via* multiplex immunoassay (23 cytokines) and ELISA immunoassay (PGE-2 and TGF-ß). Cytokine levels were measurable for ten cytokines *via* multiplex immunoassay (IL-1ß, IL-4, IL-6, IL-8, IFN-γ, IP10, GRO, IL-10, TNFα, and RANTES) and for one cytokine (PGE-2) *via* ELISA. Significant differences were seen between treatment groups for GRO, IL-1ß, IL-6, IL-10, IP-10, and PGE-2. There were no statistical differences noted for the remaining biomarkers assessed that achieved measurable levels by multiplex assay (IL-4, IL-8, IFN-γ, RANTES, and TNF-α). Levels of the remaining 14 cytokines assessed were below the detection limit of the multiplex assay (FGF-2, eotaxin, G-CSF, IL1α, GM-CSF, fractalkine, IL-13, IL-5, IL-18, IL-17A, IL-2, IL-12, and MCP-1) or ELISA (TGF-ß). Bars are mean and standard deviation of three biological replicates over two time points. *Statistical significance assessed at $p < 0.05$.

how equine macrophages respond to activation by relevant joint inflammatory cytokines.

Impact of OT treatment with MSC-CM on activated macrophage transcriptomes

We next conducted a series of studies to compare the impact of OT treatment on the activated macrophage transcriptome. The first OT evaluated was MSC, using CM from the MSC cultures to modulate IL-1ß activated macrophage immune responses. This analysis revealed that treatment of macrophages using supernatant collected from MSC cultures produced a smaller scale but anti-inflammatory change relative to that of the other OTs tested (Figures 3A, B). The PCA plot demonstrates MSC supernatant treated macrophages had 122 significantly differentiated genes relative to IL-1β conditioned macrophages (Figure 5A) with *p*-value (unadjusted) ≤0.05 and fold change ≥2. No significant genes were found when using FDR adjusted *p*-value. Out of these 122 significant DEGs, 75 were unique to the MSC-CM treatment (Figure 3B). The top 15 up or downregulated genes included biological processes such as immune system process (ENSECAG00000015109), stimulus

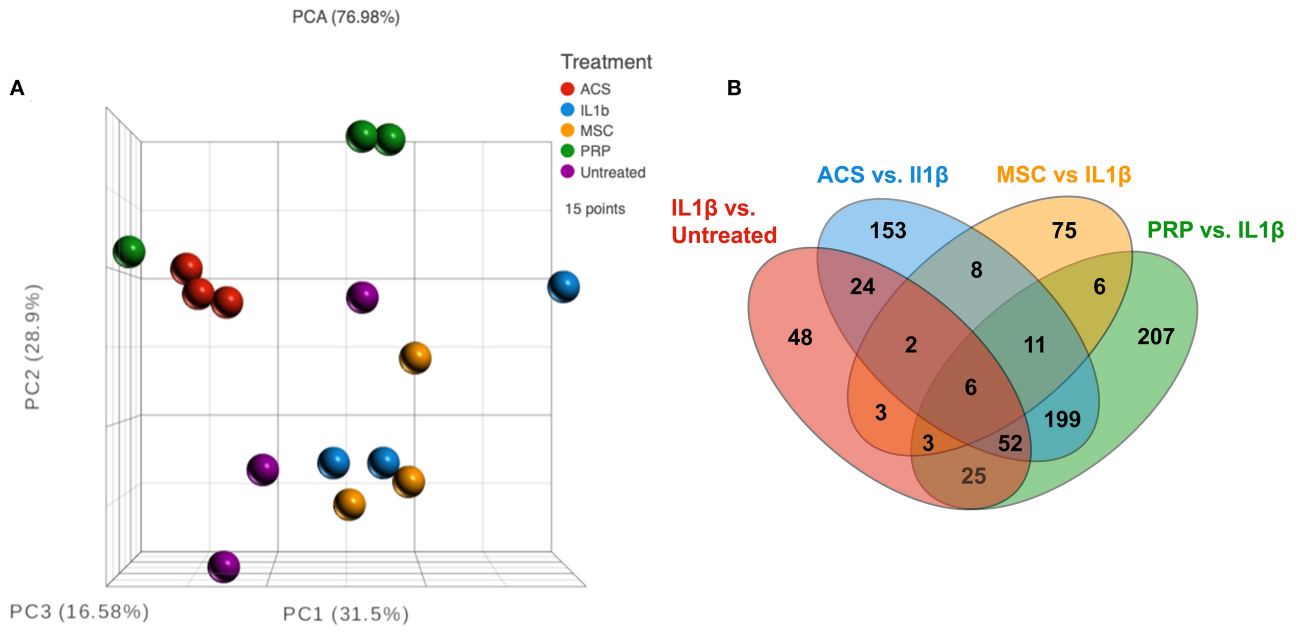

FIGURE 3
RNA sequencing analysis comparing three different types of treatment on macrophages. **(A)** Principle component analysis (PCA) or normalized counts from equine macrophages. Red dots show $n = 3$ technical replicate of macrophages treated with IL-1ß only, and purple show technical replicates of untreated macrophage. Other treatments include $n = 3$ biological replicates of orthobiologics including MSC supernatant (yellow), ACS (blue), and PRP (green), all treatments collected from $n = 3$ donor horses. **(B)** Venn diagram of differentially expressed genes from each comparison group, IL1-ß vs. untreated (red), IRAP vs. IL-1ß (blue), MSC vs. IL-1ß (yellow) and PRP vs. IL-1 ß (green). All comparisons except MSC were filtered by FDR adjusted *p* value of 0.05, MSC groups filtered using unadjusted *p* value of 0.05.

response (IL-1ß, GNGT2), cytoskeletal reorganization, binding and signaling (IL-31, SMC1B, TSACC, RERG, INKA2) (Figure 5B). Overall, MSC-CM treatment caused a significant downregulation of inflammatory pathways such as type 1 and type 2 interferon response, TNF-α and IL-6 signaling (Figure 5C). This anti-inflammatory response can be seen in the downregulation of genes such as CXCL10, IL-1RN, IL-17 receptor, which are known as common mediators of chronic inflammation in OA joints (43).

Macrophage transcriptomic response to treatment with PRP lysate

We next evaluated the impact of PRP lysate on activated macrophage transcriptomes. This analysis revealed that when compared to MSC-CM conditioned macrophages, PRP lysate treated cultures produced markedly larger changes in the transcriptome (Figure 3A), with 207 unique DEGs that were not found in the other treatment groups (Figure 3B). Differential analysis revealed a total of 564 significantly different (FDR $\leq$ 0.05 and fold change $\geq$ 2 or $\leq$-2) genes (Figure 6A). Many of the downregulated genes in the PRP lysate treated group included inflammation related genes such as IL-1RA, SLAMF9, ENSECAG00000022247 (Figure 6B). These genes mapped to inflammatory pathways including type 1 and type 2 interferon signaling, complement and coagulation, as well as MTOR. All of which are molecular mechanisms implicated in the inflammatory response in OA pathogenesis (44). However, the PRP lysate treatment also generated upregulation of inflammatory response according pathway analysis, with significant upregulation of pathways such as TNF-α, IL-2 signaling, and Myc targets (Figure 6C). These pathways were not found to be significant in the IL-1β alone group and are therefore a unique reaction of the macrophages responding to the proteins contained in the PRP.

Upregulation of multiple inflammatory pathways following treatment of activated macrophages with ACS

We assessed the responses of IL-1ß activated macrophages to treatment with ACS. In contrast to the MSC-CM treatment, the ACS treatment induced a similar response to PRP lysate, creating a greater degree of change in the macrophage transcriptome, with a total of 564 significantly differentially expressed genes (FDR $\leq$ 0.05 and fold change $\geq$ 2 or $\leq$-2) (Figure 7A). Out of all the significant DEGs, 268 of these genes were shared by the PRP treated macrophages (Figure 3B) whereas only 27 were shared with MSC-CM treated macrophages. Although there were genes associated with catalytic activity (RNASE6, DNMT3L), most of the upregulated genes are commonly associated with inflammatory responses such as CCL22, CCL17, ENSECAG00000031387 or CX3CL1, TIMP3 (Figure 7B). Matching the individual gene profile, the significant pathways included upregulation of inflammatory responses, IL-2 signaling, TNFα and KRAS signaling as well as hypoxia (Figure 7C). Finally, similarly to PRP lysate treatment, ACS also induced a downregulation of MTOR signaling and type 1 interferon signaling, demonstrating some potential to induce favorable changes in the OA joint environment.

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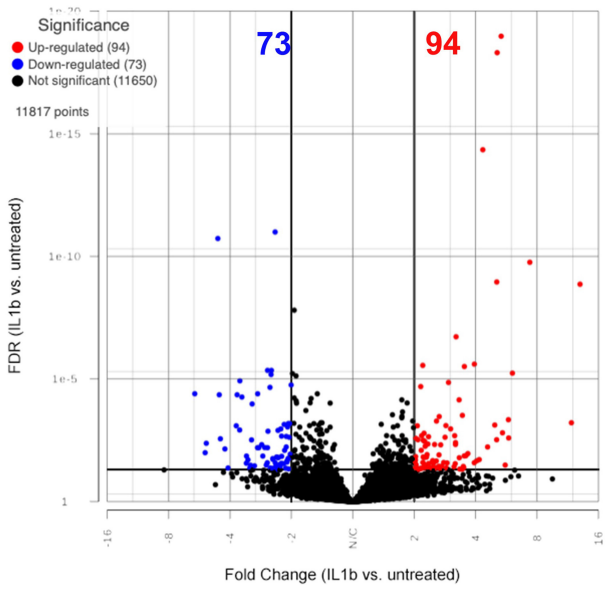

B

Gene ID	Description	FDR (IL1b vs. Untreated)	Fold change (IL1b vs. Untreated)
CHAC1	Glutathione-specific gamma-glutamylcyclotransferase 1	1.37E-09	13.01
CDKN1C	Cyclin-dependent kinase inhibitor 1C	6.15E-04	11.81
GPT2	Alanine aminotransferase 2	1.75E-10	7.38
PPBP	Platelet basic protein	5.83E-06	6.06
ENSECAG00000005179	Free fatty acid receptor 3	2.56E-03	5.81
CSF3	Granulocyte colony-stimulating factor	4.61E-04	5.80
SLAMF9	SLAM family member 9	3.28E-02	5.59
A2ML1	Alpha-2-macroglobulin-like protein 1	1.58E-03	5.42
CEBPD	CCAAT/enhancer-binding protein delta	1.05E-19	5.34
DDIT4	DNA damage-inducible transcript 4 protein	4.92E-19	5.11
PSAT1	Phosphoserine aminotransferase	1.11E-09	5.08
PEAK3	Protein PEAK3	3.03E-03	5.08
GPR84	G-protein coupled receptor 84	7.63E-04	4.97
KLC3	Kinesin light chain	5.97E-03	4.58
TRIB3	Tribbles homolog 3	4.48E-15	4.34
EPS8	Epidermal growth factor receptor kinase substrate 8	2.74E-02	-3.33
IGSF9B	Protein turtle homolog B	5.49E-05	-3.50
ESM1	Endothelial cell-specific molecule 1	1.20E-05	-3.58
GLI1	Zinc finger protein GLI1	1.21E-03	-3.59
XIRP1	Xin actin-binding repeat-containing protein 1	4.44E-05	-3.69
DNAAF1	Dynein axonemal assembly factor 1	8.18E-04	-3.73
GPRC5A	Retinoic acid-induced protein 3	4.29E-02	-4.09
NR2F1	COUP transcription factor 1	7.19E-03	-4.24
WNT1	Proto-oncogene Wnt-1	2.77E-03	-4.46
CDH5	Cadherin-5	4.44E-05	-4.52
ARC	Activity-regulated cytoskeleton-associated protein	1.87E-11	-4.59
CREB5	Cyclic AMP-responsive element-binding protein 5	4.22E-03	-5.23
MTMR11	Myotubularin-related protein 11	1.02E-02	-5.30
STC1	Stanniocalcin-1	4.03E-05	-5.95

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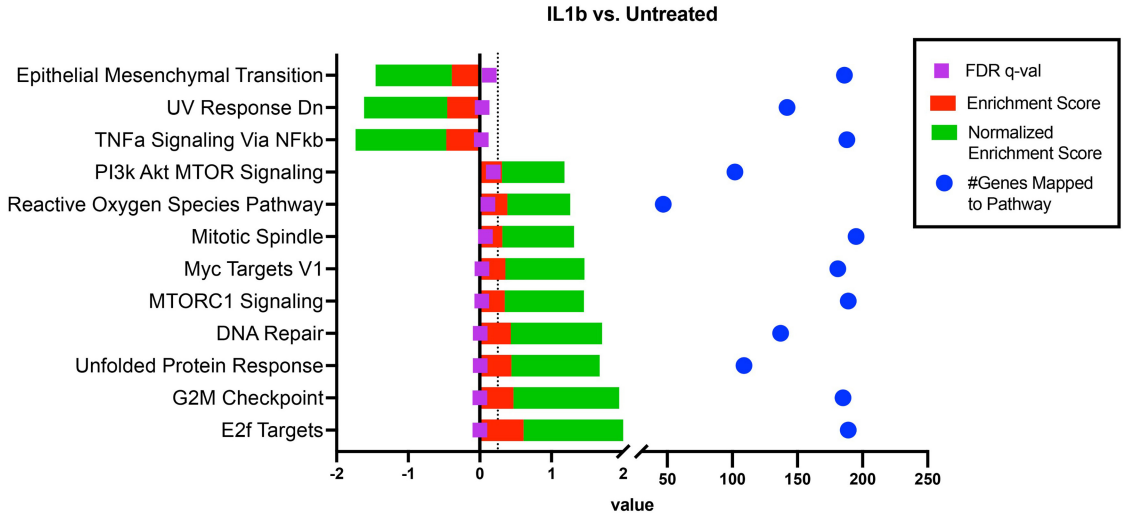

FIGURE 4
Differential gene expression and pathway analysis of IL-1ß stimulated macrophages compared to untreated. Differential gene expression and pathway analysis of IL-1ß stimulated macrophages compared to untreated. **(A)** Volcano plot of IL-1ß treatment vs. untreated. X axis shows fold change and y axis shows FDR adjusted *p*-value, with significantly upregulated genes shown in red dots and significantly downregulated genes in blue dots. Significance defined as FDR ≤ 0.05 fold change ≥ 2 or ≤ -2. **(B)** List of genes, description, FDR value and fold change of top 15 upregulated and downregulated genes in differential analysis results from IL-1ß vs. untreated samples. **(C)** GSEA (gene set enrichment analysis) results using normalized counts from $n = 3$ IL-1ß vs. untreated macrophages. FDR *p*-values shown in purple with dotted line on FDR = 0.25 for significance. Red bars show enrichment score (ES), green bars show Normalized enrichment score. Blue dots show total genes found in pathways. Pathways computed using hallmarks gene sets v2022.1.

Impact of OT treatment on macrophage polarization

Finally, we evaluated the ability of various OT treatments to affect macrophage polarization; recognizing that polarization is represented by a dynamic state beyond the traditional assessment of M1 vs. M2 (45, 46). Assessment of up- or downregulation of genes associated with M1 versus M2 macrophage phenotypes. These findings are summarized in Supplemental materials 5–7. Gene set lists from GSE5099 specific to *in vitro* derived M1 or M2 human monocyte derived macrophages were used to generate heat maps for genes expressed in the equine macrophages. Macrophages treated with orthobiologics present a mixed phenotype, with both up and down regulation of "M1 up" genes as well as

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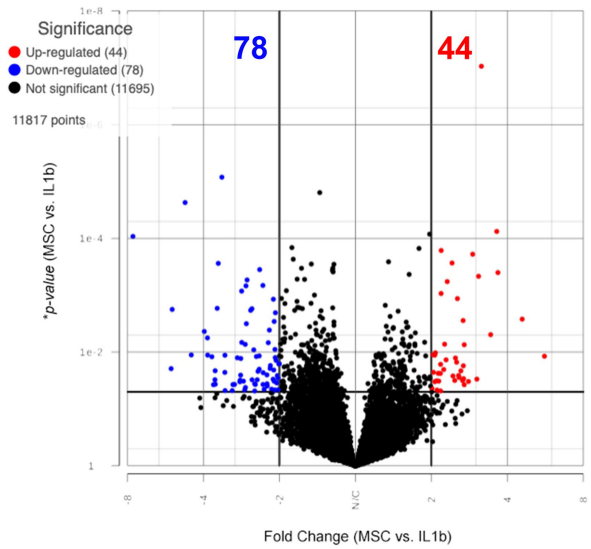

B

Gene ID	Description	FDR (MSC vs. IL1b)	Fold change (MSC vs. IL1b)
H2BC19	Histone H2B	7.57E-01	5.61
ENSECAG00000033981	Ig-like domain-containing protein	5.04E-01	4.58
TSACC	TSSK6-activating co-chaperone protein	1.88E-01	3.67
ENSECAG00000033460	Uncharacterized LOC111770210	1.26E-01	3.63
MTMR11	Myotubularin-related protein 11	6.11E-01	3.44
AOX1	Aldehyde oxidase	1.15E-03	3.16
ENSECAG00000027943	USP6 N-terminal-like protein	2.03E-01	3.08
ENSECAG00000030236	Interferon induced transmembrane protein 10	8.16E-01	3.03
DNAAF1	Dynein axonemal assembly factor 1	1.79E-01	2.92
RERG	Ras-related and estrogen-regulated growth inhibitor	8.16E-01	2.81
INKA2	PAK4-inhibitor INKA2	6.89E-01	2.70
ENSECAG00000028111	Ig-like domain-containing protein	8.16E-01	2.70
KCNK13	Potassium channel subfamily K member 13	8.16E-01	2.68
ENSECAG00000040533	Ig-like domain-containing protein	8.16E-01	2.68
CD160	CD160 antigen	5.25E-01	2.67
GFRA4	GDNF family receptor alpha-4	8.40E-06	-3.38
GNGT2	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-T2	2.75E-04	-3.49
IL1B	Interleukin-1 beta	1.70E-03	-3.53
PRXL2B	synthase	3.67E-02	-3.59
RARRES2	Retinoic acid receptor responder protein 2	2.13E-02	-3.60
NKAIN1	Sodium/potassium-transporting ATPase subunit beta-1-interacting protein 1	3.06E-02	-3.60
SMC1B	Structural maintenance of chromosomes protein 1B	3.75E-02	-3.65
STAP2	2	1.21E-02	-3.70
ENSECAG00000001923	Ig-like domain-containing protein	1.13E-02	-3.85
ENSECAG00000038866	Pentaxin	5.63E-03	-3.86
CHRDL2	Chordin-like protein 2	4.34E-03	-3.97
FAM216A	Protein FAM216A	1.12E-02	-4.47
CSF3	factor	2.34E-05	-4.73
ENSECAG00000015109	Ig-like domain-containing protein	1.96E-02	-5.37
IL31	Interleukin-31	9.24E-05	-7.61

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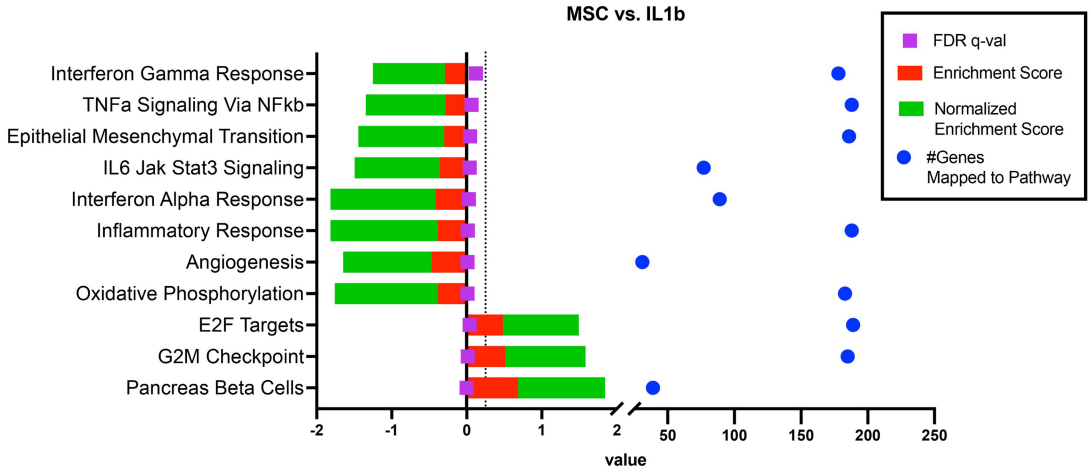

FIGURE 5
Differential gene expression and pathway analysis of MSC supernatant conditioned macrophages compared to IL-1ß stimulated. **(A)** Volcano plot of MSC treatment vs. IL-1ß. X-axis shows fold change and y-axis shows unadjusted adjusted *p*-value. Significance defined as *p*-value ≤ 0.05 fold change ≥ 2 or ≤ -2. **(B)** List of genes, description, *unadjusted *p*-value and fold change of top 15 upregulated and downregulated genes in differential analysis results from MSC supernatant treated vs. IL-1ß stimulated samples. **(C)** GSEA results using normalized counts from $n = 3$ MSC supernatant treated vs. IL-1ß stimulated macrophages. Legend shown in box to right.

"M1 dn". Macrophages treated with orthobiologics also showed mixed expression of "leukocyte activation genes" which would be important for modulation of the *in vivo* inflamed joint environment. Looking at a more condensed gene list of commonly known macrophage phenotyping genes (dot plots, Supplemental material 6), for example IFNG is downregulated in IL1b and MSC groups, IL6 is downregulated in PRP and ACS groups. Whereas IL13 and IL4 (M2 gene) is upregulated in PRP and ACS group.

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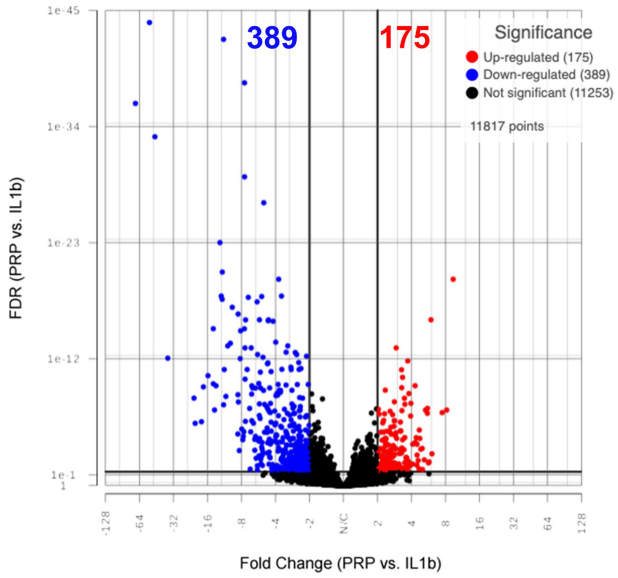

B

Gene ID	Description	FDR (PRP vs. IL1b)	Fold change (PRP vs. IL1b)
ESM1	Endothelial cell-specific molecule 1	2.95E-20	9.36
CCBE1	Collagen and calcium-binding EGF domain-containing protein 1	7.22E-08	8.19
STC1	Stanniocalcin-1	1.26E-07	7.48
NMU	Neuromedin-U	1.01E-03	6.07
CCL22	C-C motif chemokine 22	2.05E-16	5.95
ENSECAG00000039383	WAP domain-containing protein	5.84E-03	5.72
DSG2	Desmoglein-2	7.23E-03	5.65
DNAAF1	Dynein axonemal assembly factor 1	1.47E-07	5.53
NTNG2	Netrin-G2	7.36E-08	5.32
ABCC6	ATP-binding cassette sub-family C member 6	3.79E-03	5.20
CCDC8	Coiled-coil domain-containing protein 8	3.11E-02	5.00
CD1A1	CD1a1 molecule	3.15E-04	4.98
CCL17	C-C motif chemokine 17	3.71E-04	4.84
IL17A	Interleukin-17A	3.52E-05	4.74
MATN4	Matrilin-4	5.67E-06	4.69
ENSECAG00000017042	Membrane-spanning 4-domains subfamily A member 6A	6.56E-37	-69.44
CD163	Scavenger receptor cysteine-rich type 1 protein M130	1.44E-44	-52.17
ENSECAG00000038794	Membrane-spanning 4-domains subfamily A member 4A	9.46E-34	-46.71
ENSECAG00000002438	Signal regulatory protein beta 2	8.89E-13	-35.94
IGFBP4	Insulin-like growth factor-binding protein 4	5.39E-09	-21.05
SLAMF9	SLAM family member 9	1.30E-06	-20.54
ENSECAG00000038866	Pentaxin	9.21E-07	-18.08
CMKLR2	chemerin chemokine-like receptor 2	4.59E-10	-17.37
VCAM1	Vascular cell adhesion protein 1	4.03E-11	-15.83
CD163L1	Scavenger receptor cysteine-rich type 1 protein M160	1.43E-15	-14.21
GPBAR1	G-protein coupled bile acid receptor 1	2.37E-10	-14.20
ADAM28	Disintegrin and metalloproteinase domain-containing protein 28	7.17E-08	-13.85
LILRA	Leukocyte immunoglobulin-like receptor	3.61E-10	-13.43
ENSECAG00000019222	Sialic acid binding Ig like lectin 11	1.00E-23	-12.40
ENSECAG00000022247	Apolipoprotein R	1.16E-18	-12.09

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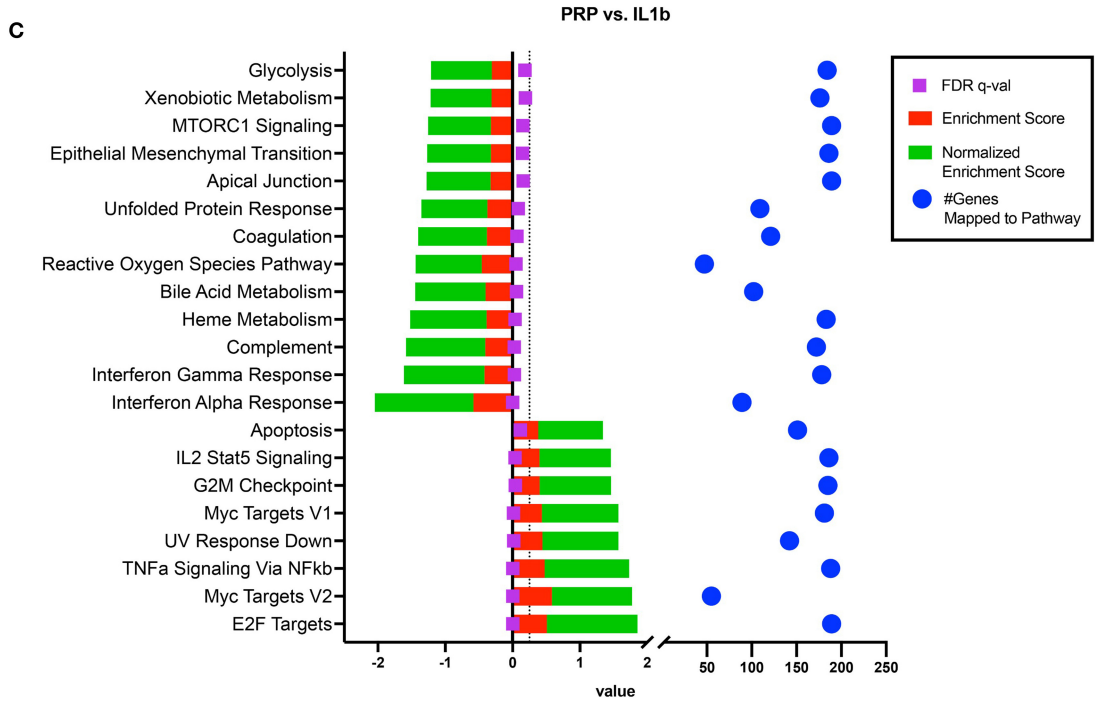

FIGURE 6
Differential gene expression and pathway analysis of PRP treated macrophages compared to IL-1ß stimulated. **(A)** Volcano plot of PRP treated vs. IL-1ß. X-axis shows fold change and y-axis shows unadjusted adjusted p-value. Significance defined as FDR adjusted p-value ≤ 0.05 fold change ≥ 2 or ≤ -2. **(B)** List of genes, description, FDR, and fold change of top 15 upregulated and downregulated genes in differential analysis results from PRP treated vs. IL-1ß stimulated samples. **(C)** GSEA results using normalized counts from $n = 3$ PRP treated vs. IL-1ß stimulated macrophages.

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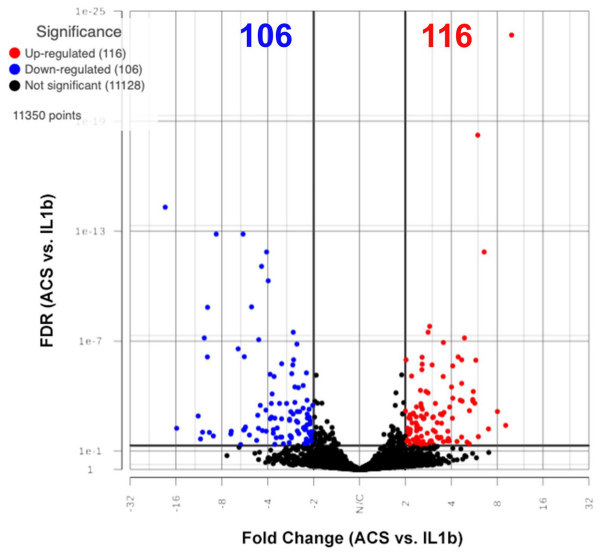

B

Gene name	Gene Description	FDR (IRAP vs. IL1b)	Fold change (IRAP vs. IL1b)
CCL22	C-C motif chemokine ligand 22	2.02E-24	9.95
STC1	stanniocalcin 1	3.99E-03	9.10
CD1A1	CD1a1 molecule	7.01E-04	8.03
CCBE1	collagen and calcium binding EGF domains 1	6.21E-03	7.02
TIMP3	TIMP metallopeptidase inhibitor 3	5.83E-19	5.97
CCL17	C-C motif chemokine ligand 17	1.11E-06	5.80
NR4A3	nuclear receptor subfamily 4 group A member 3	2.46E-04	5.71
NTNG2	netrin G2	5.65E-05	5.58
ESM1	endothelial cell specific molecule 1	1.56E-04	5.53
PARM1	prostate androgen-regulated mucin-like protein 1	6.29E-04	5.26
MANEAL	mannosidase endo-alpha like	4.08E-02	5.25
INHBA	inhibin subunit beta A	6.77E-08	4.88
GUCY1B1	guanylate cyclase 1 soluble subunit beta 1	1.89E-04	4.71
VEGFC	vascular endothelial growth factor C	1.74E-02	4.68
GPR158	G protein-coupled receptor 158	1.69E-04	4.59
CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	4.89E-03	-5.60
GPT2	glutamic--pyruvic transaminase 2	6.65E-03	-5.68
RAB3IL1	RAB3A interacting protein like 1	7.14E-07	-5.70
TMEM171	transmembrane protein 171	7.10E-03	-5.76
CEBPD	CCAAT enhancer binding protein delta	1.46E-13	-5.81
GPR84	G protein-coupled receptor 84	4.45E-02	-6.02
RNASE6	ribonuclease A family member k6	2.66E-07	-6.25
CCL24	C-C motif chemokine ligand 24	8.29E-03	-6.94
CD163	CD163 molecule	1.46E-13	-8.71
DNMT3L	DNA methyltransferase 3 like	1.50E-02	-9.10
A2ML1	alpha-2-macroglobulin like 1	7.35E-07	-9.96
CD163L1	CD163 molecule like 1	6.77E-08	-10.43
ENPP6	ectonucleotide pyrophosphatase/phosphodiesterase 6	9.36E-03	-10.71
IGFBP4	insulin like growth factor binding protein 4	1.22E-03	-11.44
SLAMF9	SLAM family member 9	5.73E-03	-15.83

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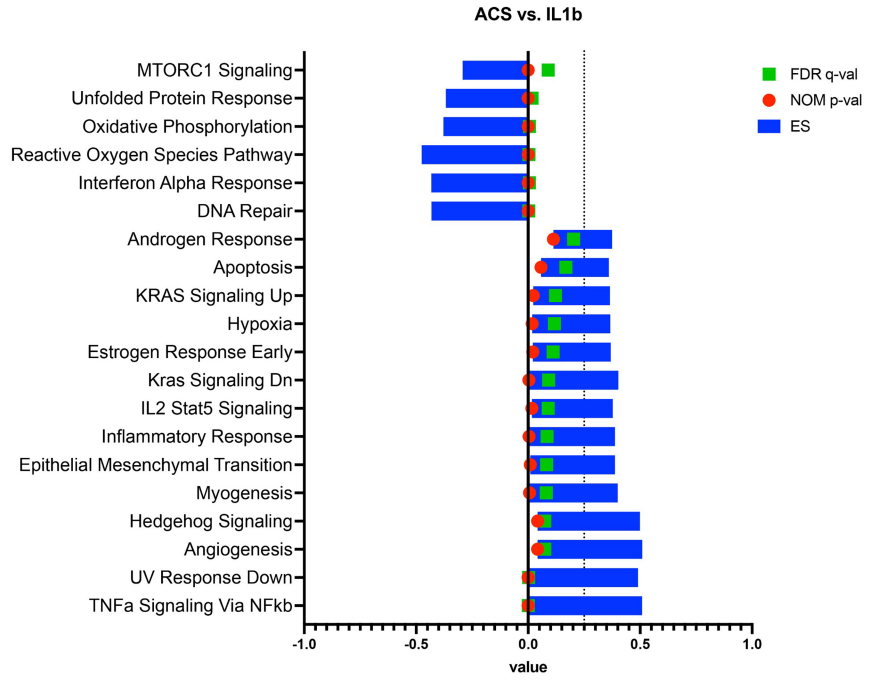

FIGURE 7
Differential gene expression and pathway analysis of ACS treated macrophages compared to IL-1ß stimulated. **(A)** Volcano plot of ACS treated vs. IL-1ß. X-axis shows fold change and y-axis shows unadjusted adjusted *p*-value. Significance defined as FDR adjusted *p*-value ≤ 0.05 fold change ≥ 2 or ≤ −2. **(B)** List of genes, description, FDR, and fold change of top 15 upregulated and downregulated genes in differential analysis results from ACS vs. IL-1ß stimulated samples. **(C)** GSEA results using normalized counts from $n = 3$ ACS treated vs. IL-1ß stimulated macrophages. Blue bars show ES, green FDR significant values, and nominal *p*-values shown in red.

Discussion

Orthobiologics have been increasingly used in the treatment of equine musculoskeletal disease in recent years, and it is therefore important to understand how these new treatments modulate joint inflammatory responses mechanistically (15, 16, 24, 28). Therefore, the primary goal of this study was to elucidate mechanisms of action of three commonly used biologic therapies on macrophage function and polarization, as synovial macrophages are recognized as one of the most immunologically active cells within the joint and in the progression of OA. Improved understanding of the effects of biologic therapies on key immune effector cells within the joint is fundamental to further designing trials with the correct biomarker endpoints to determine relative biological effects of treatments,

including cytokines in synovial fluid or sequencing of synovial fluid or tissue biopsies. Key findings of this study were that all treatments reduced levels of IL-1ß, while MSC-CM induced the greatest increase in anti-inflammatory IL-10 levels, and both PRP lysate and ACS induced lower levels of IL-6 and IP-10 relative to MSC-CM. These findings demonstrate potential beneficial effects of all treatments assessed relative to OA progression, albeit through different mechanisms. RNA sequencing revealed that MSC-CM downregulated inflammatory gene expression while PRP lysate and ACS directed a mixed response with upregulation of both pro- and anti-inflammatory gene pathways and mixed M1/M2 macrophage polarization. This study is the first to report the relative effects of three commonly used orthobiologic formulations in the modification of inflammation induced in macrophages, simulating conditions experienced in inflamed osteoarthritic joints. These findings will inform future studies examining the potential benefits of regenerative therapies *in vivo* in various models of OA in horses.

Multiple cytokines that were differentially secreted by macrophages following treatment with orthobiologics have been implicated to play a role or have prognostic value in assessing the severity of OA. Disruption of the cytokine balance toward a pro-inflammatory state in the pathogenesis of OA has been described to propagate a "vicious cycle" activating catabolic enzymes and contributing to further damage to cartilage, synovium, and intra-articular soft tissue structures (43). IL-1ß has been cited as one of the primary pro-inflammatory cytokines involved in the pathogenesis of OA, among other disease conditions (43), inducing catabolic events including cartilage degradation through mitogen-activated protein kinase (MAPK) signaling, reducing cartilage extracellular matrix *via* ERK activation, and inhibiting collagen synthesis through SOX-9 expression (47, 48). As noted, in this study, IL-1ß secretion was downregulated by all three orthobiologics assessed. Furthermore, concentrations of PGE-2 in synovial fluid have been evaluated to determine degree of joint inflammation as they are consistently elevated in naturally occurring (49) and experimental models of equine OA (50) and lameness in horses in general (51). While PGE-2 was not induced to a significantly greater extent in the IL-1ß stimulated macrophages vs. control, PGE-2 levels were found to be lowest in the MSC and ACS treated groups and were actually significantly upregulated in the PRP lysate group, consistent with the mixed induction of some pro-inflammatory pathways seen with PRP lysate on RNA sequencing analysis.

Interleukin-6 (IL-6) has historically been characterized as pro-inflammatory (52, 53) and documented to be upregulated in joints with osteoarthritis (54, 55), although more recent work has suggested that it may play a more immunomodulatory and not strictly a pro-inflammatory role (56–58). In humans undergoing knee arthroscopy, IL-6 and MCP-1 concentrations have been correlated to higher (worse) intraoperative International Cartilage Repair Society (ICRS) scores, were the greatest predictors of more severe cartilage lesions, and were associated with more prolonged pain postoperatively (59). Both IL-6 and IP-10 were further associated with greater hip OA pain and were detected in both the synovial fluid (IL-6 and IP-10) and synovium (IP-10) of OA vs. healthy patients, indicating distinct inflammatory processes may drive OA in specific joints or at specific time points in disease progression (60). In this study, IL-6 and IP-10 secretion were downregulated by PRP lysate and ACS in comparison to IL-1ß stimulated macrophages. Given the conflicting reports on the relative catabolic vs. pro-chondrogenic effects of IL-6 to equine cartilage, further studies on the global role of IL-6 in equine OA are warranted.

Interleukin-10 (IL-10) is broadly considered to be an anti-inflammatory cytokine through multiple pathways and is primarily synthesized by immune cells and to a lesser extent by chondrocytes within the joint, where it plays a role in cartilage extracellular matrix turnover (61). In this study, MSC-CM maintained levels of IL-10 close to those of the positive control and to significantly higher levels than that induced by treatment with PRP lysate or ACS. In addition, RANTES (regulated upon activation, normal T cell expressed and secreted) has further been associated with recruitment of macrophages and PGE-2 generation following injection in rodent models and reported as a mediator of acute and chronic inflammation (59). In humans, RANTES levels were among the strongest predictors, along with platelet-derived growth factor and vascular endothelial growth factor, of postoperative improvement regardless of initial injury or degree of cartilage degradation at the time of surgery (59). This is interesting in the context that, of the treatments assessed, MSC-CM upregulated RANTES most, resulting in greater secretion of RANTES by macrophages compared to PRP lysate, although no treatment was significantly different from the positive or negative control. These findings shed some light on the relative effects of orthobiologic agents in the context of IL-1ß induced inflammation and highlight the concept that the pathogenesis of OA involves activation of signaling pathways by multiple cytokines (43).

This study represents the first in-depth look at how equine macrophages respond to IL-1ß, a relevant inflammatory cytokine in osteoarthritis progression, quantified *via* transcriptomic analysis. Macrophages were co-cultured with recombinant IL-1ß to model the inflammatory synovial environment that impairs healing and exacerbates OA progression, as IL-1ß has been commonly associated with the osteoarthritic synovial environment as a pro-inflammatory cytokine, in addition to IL-6 and TNF-α (43). In response to IL-1ß stimulus, the ROS pathway, and several key leukocyte activation genes such as TLR1, TLR9, TLR2, IL4, IFNGR1, IL-13 etc. (GO:0045321), were upregulated in IL-1ß treated macrophages compared to the control unstimulated macrophages. Overall, the most upregulated genes were related to inflammatory immune system (SLAMF9, PPBP, TRIB3, GPR84, and DDIT4) and metabolic processes (CHAC1, PSAT1, PEAK3, and CEBPD). Simultaneously, downregulated genes mapped to categories including impaired cell signaling and biological regulation of cellular adhesion and differentiation. Thus, the transcriptomic analysis techniques employed here provided previously unreported insights into how equine macrophages respond to activation by relevant joint inflammatory cytokines, with relevance to the interaction and response of joint cells to orthobiologic treatments in osteoarthritis.

Macrophage polarization states are key in regulation of inflammation in the osteoarthritic joint (62–64). The upregulation of anti-inflammatory genes demonstrated following MSC-CM treatment represents a polarization toward an M2 macrophage phenotype and is consistent with previous reports discussing the importance of MSC-macrophage crosstalk in the maintenance of homeostasis in inflammatory microenvironments and the role of macrophage phenotype switching from M1 to M2 in tissue repair (62, 63) (Supplementary materials 5–7). Published datasets of gene expression include key patterns (65) seen in polarized

macrophages *in vitro* that define the transcriptomic response beyond the dichotomy of M1 and M2 (66). While it is recognized that the findings of this study may not be directly compared to human data sets on resting macrophages given species differences and the induced IL-1ß inflammation modeled here, these previous reports provide a baseline from which initial comparisons may be drawn. In this study, the IL-1ß treated macrophages co-cultured with PRP upregulated many genes previously classified as M1 or pro-inflammatory oriented; for example, 41% of the M1 genes found in GSE5099 and several of the "leukocyte activation genes" found in GO:0002269 (leukocyte activation involved in inflammatory response) such as IFN-γ, IL-13, and IL-4, were upregulated, suggesting that PRP lysate polarized macrophages toward an M1 profile in these pathways (Supplementary material 2). Transcriptome abundance, a more sensitive measure than protein levels, further indicated upregulation of TNF-α; of note, these outcomes may lack correlation if protein levels were not high enough to detect *via* multiplex assay or if the stimulus was not sufficient to trigger release but was strong enough to alter transcript abundance. However, overall pathway analysis for PRP lysate-treated samples revealed a global transcriptome that points to a downregulation of the immune response, type I and II interferon pathways, complement and ROS pathways, indicating (as with MSC-CM) the potential utility of PRP lysate to resolve chronic inflammation during OA pathogenesis. Finally, PRP treatment also resulted in the largest number of "uniquely" differentially expressed genes (207 as seen on VENN diagram, Supplementary material 3) of orthobiologic therapies assessed, some of which mapped to angiogenesis, integrin signaling, TGF-ß, and other pathways that produce downstream effects that could potentially contribute to the amelioration of tissue damage and inflammatory cell infiltrate. These results highlight the importance of the non-biased approach used here to analyze transcriptomic response to therapy and investigate the mechanisms of action by which orthobiologic therapies exert an effect.

Treatment of macrophages with ACS also created an apparent shift in the macrophage transcriptome, and, similarly to that seen with PRP lysate treatment, downregulated several inflammatory pathways including ROS, oxidative phosphorylation, and the type II interferon response. The 153 genes unique to the ACS treatment (Figure 3) present sets of genes that, although inflammatory, could be beneficial to recruit innate immune cells to the site of inflammation (toll like receptor (TLR) signaling, Wnt signaling, integrin, glycolysis, DNA replication, and EGF receptor), which may further contribute to the initial phase of tissue repair in traumatic injury. In contrast to MSC-CM, ACS treatment also upregulated several inflammatory pathways (TNF-α, IL-2, Stat 5 signaling, and apoptosis), along with multiple M1 leukocyte activation genes (TNF-α, IFN- γ, IL4, and FOXP1). Similarly to PRP lysate-treated macrophages, approximately 44% of M1-associated genes (GSE5099) were upregulated compared to the 62% M1-associated upregulated genes in the IL-1ß alone group (Supplementary Figure S2). Despite the upregulation of multiple M1 genes, it has been previously reported that the M2 macrophage response encompasses a dynamic spectrum of transcriptomic states ranging from the classic M2 tissue resident macrophage to M2a, b, c and d subtypes depending on which receptor(s) are activated (IL-4, TGF-ß, or glucocorticoids) and their respective downstream effects (67). For example, the M2a phenotype has been found to upregulate peroxisome proliferator-activated receptor (PPAR), STAT6 and STAT3 pathway genes, which were all upregulated in the ACS group. These transcriptomic shifts and plasticity of macrophage polarization further highlight the advantages of using an impartial approach (i.e., bulk RNA sequencing) to investigate cell product derived therapeutics for the treatment of joint diseases.

Caveats to study design include small donor horse sample size, inherent variability in orthobiologic composition between individual donors and products available, and assessment of cytokine concentrations and differential gene expression at a single time point. Different donor horses were used to develop orthobiologic treatments vs. monocyte-derived macrophage cultures simply due to availability at the time the studies were performed. It is further acknowledged that the model employed is not proposed to represent the spectrum of conditions encompassing OA, and likely did not fully capture the chronic inflammatory response seen in longstanding degenerative joint disease nor the potential for orthobiologic agents to exert a longer-term effect in mitigation of disease progression as culture media and macrophages were only assessed at a single time point. The peripheral blood monocyte-derived macrophage model employed is recognized to not fully represent the complexity and spectrum of synovial macrophage phenotypes. In addition, activation of macrophages in this model may have been enhanced through combined stimulation using both IL-1ß and TNF-α, both cytokines found to be elevated in OA, as has been recently described (68). Interpretation of transcriptomic data cannot be used to predict net outcomes in comparing OT treatments but does give us the most in-depth evaluation possible as to what processes are invoked through OT treatment with emphasis on overall pathways, rather than individual genes. Finally, it is acknowledged that substantial variability exists between preparation and composition of orthobiologic therapies, including differences between manufacturers, culture techniques such as serum source and media components for mesenchymal stromal cells, and individual donor factors including the health, time of day and environment of the donor prior to tissue donation (69, 70). For example, the PRP lysate product used (Arthrex ACP) represents a relatively low platelet concentrate with lower levels of platelet derived growth factor (PDGF) and other related growth factors than other commercially available products; it is recognized that other formulations with higher platelet concentrations may have yielded different results. Furthermore, characterization of the PRP lysate and ACS products used here were not supplied, which is recognized as a limitation. Finally, cultures were performed with conditioned media from MSC rather than cells themselves, which was done in an attempt to standardize comparisons between products as much as possible, and it is hypothesized that co-culture with cells would have only accentuated the findings reported. While this study represents an initial comparison of three treatments that are currently commonly employed in equine practice, but it is further acknowledged that multiple others (e.g., alpha-2 macroglobulin, autologous protein solution, adipose derived MSC or MSC-CM, amnion, and urinary bladder matrix) exist, in addition to other formulations of the products investigated here, and that comparison of effect and mechanism of action in future *in vitro* and *in vivo* studies is warranted.

In summary, these findings indicate that commonly used equine orthobiologic therapies exert their actions through various mechanisms including induction of differential cytokine production and gene expression from resident joint tissues that may be beneficial

in treatment of osteoarthritis, and further highlight the benefits of employing a non-biased approach to transcriptomic and cytokine analysis to investigate mechanisms of action of these treatments. These studies begin to address a critical gap in our understanding of the relative immunomodulatory properties of regenerative therapies commonly used in equine practice to treat musculoskeletal disease and will serve as a platform from which further *in vivo* comparisons of orthobiologic therapies may build.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository and accession number(s) can be found below: GEO public genomics data repository, and the accession number is GSE224326.

Ethics statement

The animal study was reviewed and approved by Colorado State University Institutional Animal Care and Use Committee.

Author contributions

Study conception and design: LP, LC, SD, LB, LG, and GG. Acquisition of data: LP, LC, LB, RI, and GG. Data analysis and interpretation: GG, LP, LC, SD, LB, and LG. Drafting of manuscript: LP, GG, and LC. All authors contributed to and approved the submitted version of the manuscript.

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Conflict of interest

LP, LG, LC, and SD acknowledge that they hold stock options in eQCell Inc. (LP, LG, and SD), Validus (SD), and ART Advanced Regenerative Therapies (LG), and have filed provisional patents covering immune activated MSC technology for treatment of musculoskeletal disease (SD, LP, LC, and LG).

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1109473/full#supplementary-material

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Induced pluripotent stem cells from domesticated ruminants and their potential for enhancing livestock production

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Ruminant livestock, including cattle, sheep, goat, and buffalo, are essential for global food security and serve valuable roles in sustainable agricultural systems. With the limited availability of embryonic stem cells (ESCs) from these species, ruminant induced pluripotent stem cells (iPSCs) and iPSC-like cells provide a valuable research tool for agricultural, veterinary, biomedical, and pharmaceutical applications, as well as for the prospect of translation to human medicine. iPSCs are generated by reprogramming of adult or fetal cells to an ESC-like state by ectopic expression of defined transcription factors. Despite the slow pace the field has evolved in livestock species compared to mice and humans, significant progress has been made over the past 15 years in using different cell sources and reprogramming protocols to generate iPSCs/iPSC-like cells from ruminants. This mini review summarizes the current literature related to the derivation of iPSCs/iPSC-like cells from domesticated ruminants with a focus on reprogramming protocols, characterization, associated limitations, and potential applications in ruminant basic science research and production.

KEYWORDS

ruminants, cellular reprogramming, characterization, induced pluripotency, stem cells

Introduction

The world population is projected to reach 9.8 billion by 2050 and 11.2 billion by 2100 (1). As a result, the demand for livestock commodities to support global food security is expected to double by 2050 (2). In both industrialized and developing agricultural systems, current livestock production practices are insufficient to fulfill projected world needs. To address this issue, the genetics of animal development, conformation, and disease resistance are being studied with the goal of improving the efficiency of animal food production. In addition, knowledge of livestock genetics has the potential to refine veterinary practices and contribute to biomedical and pharmaceutical applications that may be translatable to human medicine.

Embryonic stem cells (ESCs) are pluripotent cells typically derived from the inner cell mass of blastocyst-stage embryos (3). Ruminant ESCs can (i) provide material for genomic testing, (ii) be used to select desirable genetic traits, and (iii) be engineered to improve desirable genetic traits, each of which has the potential to expand our current knowledge of livestock genetics. Ruminant ESCs, however, are difficult to obtain and have proven hard to maintain in culture for research purposes.

Induced pluripotent stem cells (iPSCs) are pluripotent cells created by reprogramming differentiated cells. The creation of iPSCs from mouse (4), and shortly thereafter from humans (5, 6), opened new avenues for basic science research while also significantly improving the feasibility of producing and analyzing stem cells from other mammalian species. iPSCs have been produced from a wide range of eutherian mammals, including several types of domesticated ruminant species such as cattle, sheep, goats, and buffalo. With the limited availability of bona fide ESCs from these ruminants, iPSCs, which closely resemble ESCs, provide a practically limitless source of pluripotent stem cells.

The production of iPSCs from domesticated ruminants has the potential to benefit both agriculture and biotechnology. Here, we describe the methods utilized to create, characterize, and maintain, ruminant pluripotent cell lines. This mini review refers to these cell lines as iPSCs regardless of the extent to which they have been characterized (Table 1), and thus, represent authentic iPSCs. Moreover, we discuss the limitations of these cells and explore possibilities for enhancing livestock production.

Limited availability of embryonic stem cells from domesticated ruminants

The derivation and maintenance of stable ESCs from domesticated ruminants is challenging and complicated. Over the years, there have been numerous and contradictory reports of the successful generation of ESCs from domesticated ruminants (7). However, stable, well-characterized ruminant ESCs are extremely limited in supply. The poor success rates in developing and maintaining ESCs from ruminants compared to mice or humans can be attributed to the differences in these animals' developmental processes and the need for specific culture conditions. Fundamental biological differences between species, the time point differences utilized to isolate ESCs, differences in the genes and molecular pathways that govern the pluripotency network, and poorly defined pluripotency states (naïve vs. prime) in ruminant species, may necessitate protocols designed specifically for handling ESCs from each species. In addition, the long-term culture of ruminant ESCs while maintaining full pluripotency is challenging and requires further refinements.

Nevertheless, efforts have been made to establish (putative) ruminant ESCs by applying standard or modified culture systems developed for murine and human ESCs. For instance, a culture system containing fibroblast growth factor 2 (FGF2) and an inhibitor of the canonical Wnt-signaling pathway, which was successfully used to create human ESCs, was employed to derive pluripotent cell lines from cow blastocysts with stable morphology, karyotype, pluripotency marker expression and epigenetic features (8). Likewise, ovine ESCs have been derived using similar conditions (9). The generation of caprine ESCs (10), caprine ESC-like cells (11), buffalo ESCs (12, 13) and buffalo ESC-like cells (14), has been reported as well. However, most of these cells did not maintain robust self-renewal capacity and did not develop into bona fide ESC lines capable of undergoing germline transmission.

Generation and characterization of induced pluripotent stem cells from domesticated ruminants

Since the first reports were published in 2011, numerous studies describe the production of ruminant iPSCs and iPSC-like cells using a variety of cell sources, reprogramming systems, reprogramming factor combinations, and culture conditions. Moreover, these cultures have been characterized *in vitro* and/or *in vivo* to various degrees, and are referred to as iPSCs in this review, regardless of the extent to which they have been characterized and, thus, to what extent they represent authentic iPSCs. Table 1 illustrates variations and similarities in the generation and characterization of domesticated ruminant iPSCs across studies.

General criteria to characterize induced pluripotent stem cells

Measuring pluripotency is a fundamental component of every stem cell-based study. Assays testing pluripotency *in vitro* include relative quantification of the expression of pluripotency genes at the mRNA level and immunocytochemistry to detect specific pluripotency markers at the protein level. Moreover, embryoid body (EB) formation assays to test the ability of the cells to form three embryonic germ-layers can be conducted. Teratoma formation in immunodeficient mice is widely used as an index of pluripotency, as it assesses the capability of the cells to differentiate into the three embryonic germ layers *in vivo*, and it provides a reliable and comprehensive validation of the functional pluripotency of the cells (15).

Descriptions of the assays used to characterize each ruminant cell line discussed are detailed in Table 1.

Induced pluripotent stem cells from cattle

Several groups have reprogrammed bovine cells from various developmental stages and tissues, including non-conventional cell sources such as amnion-derived cells, Wharton's jelly cells, and multipotent stem cells such as neural stem cells and mesenchymal stromal cells (MSCs). These groups primarily relied on bovine, human, or murine reprogramming factors consisting of POU class 5 homeobox1 *(OCT4),* SRY-box transcription factor 2 *(SOX)2,* KLF transcription factor 4 *(KLF4)* and MYC proto-oncogene *(c-MYC)* (OSKM) or OSKM plus Lin-28 homolog A *(LIN28)* and Nanog homeobox *(NANOG)* (OSKMNL) (Table 1). Variations such as overexpression of Lysine demethylase 4A (KDM4A) or forced expression of SV40 large T antigen, together with reprogramming factors (16, 17) or antigen reprogramming with micro RNAs (18), have also been employed. Generally, reprogramming elements were delivered via viral vectors, but the use of transposon systems encoding reprogramming factors (19) and electroporation of plasmid DNA containing a single reprogramming gene have been explored as well (20). The majority of bovine iPSCs were maintained in a dual-factor culture medium consisting of both FGF2 and leukemia inhibitory factor (LIF) for proliferation in an undifferentiated state.

TABLE 1 Summary of induced pluripotent stem cells (iPSCs) generated from cattle, sheep, goat, and buffalo.

Reprogramming cell sources	Reprogramming method	Culture conditions	Pluripotency marker expression (protein, *gene*)	Transgene(s) detected after viral reprogramming	Highest reported passage	Differentiation		References
						In vitro	*In vivo*	
Cattle								
Embryonic fibroblasts	Retroviral system; Bovine OSKMLN; Human OSKM	DMEM-F12, 20% KSR, Human FGF2, Mouse LIF; MEF feeders	ALP, SOX2, SSEA-1, SSEA-4, TRA-1-60; *OCT4, SOX2*	Yes	16	EB	Teratomas	(70)
Skin fibroblasts	Lentiviral System; Human OSKM defined-factor fusion proteins	DMEM, 15% FBS, LIF, FGF2; MEF feeders	ALP, OCT4, NANOG, SSEA1; *OCT4, KLF4, NANOG*	Yes	40	EB	Teratomas	(21)
Testicular cells	Electroporation of OCT4	DMEM, 10% FBS, Human LIF; MEF feeders	ALP, OCT4, NANOG, SOX2, SSEA-1, SSEA-4; *OCT4, SOX2, MYC, KLF4, STAT3, SUZ12, DNMT1, MEF2A*	ND	15	Ectodermal, mesodermal, and endodermal precursors	Teratomas	(20)
Mammary epithelial cells; Skin fibroblasts	Retroviral system; Mouse OSKM	DMEM, 10% FBS, LIF. BFGF; MEF feeders	ALP, OCT4, LIN28; *OCT4, KLF4, SOX2, NANOG, LIN28, REX1*	Yes	20	ND*	Teratomas	(61)
Fetal fibroblasts	Transposon systems; Sleeping Beauty; Mouse OSKM; PiggyBac; Human OSKMNL	DMEM-F12, 20% KSR, Human FGF2, Human LIF	ALP, OCT4, SSEA-1, SSEA-3, SSEA-4; *OCT4, KLF4, SOX2, C-MYC, NANOG, REX1*	Yes	40	ND	Teratomas	(22)
Amnion-derived cells	Transposon systems; PiggyBac; Doxycycline-inducible OSKM	MEM/F12, 20% KSR, Human FGF2, Bovine LIF, MEK/ERK inhibitor, GSK3 inhibitor, Forskolin	ALP, OCT4, NANOG; *OCT3/4, NANOG, REX1, ESRRβ, STELLA, SOCS3*	Yes	70	EB	Naïve state-like iPSCs, Contributed to ICM of blastocysts and tissues	(19)
Neural stem cells	Lentiviral system; Bovine miR-302/367	ES culture medium; MEF feeders	ALP, OCT4, NANOG; *OCT4, SOX2, NANOG*	ND	Not mentioned	ND	Teratomas	(18)
Embryonic fibroblasts	Lentiviral system; Human OSKM	DMEM/F12, 20% KSR, Human FGF2, Human LIF	ND	Yes	12	ND	ND	(71)
Embryonic fibroblasts and Wharton's jelly cells	Lentiviral system; Human OSKM/OSKMN; Retroviral system; Bovine OSKM/OSKMN	DMEM-F12, 15% KSR, Human FGF2, Human LIF; MEF feeders	ALP, SSEA1	ND	3	ND	ND	(72)

(Continued)

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TABLE 1 (Continued)

Fetal fibroblasts and adipose-derived mesenchymal cells	Lentiviral system; Human OSKM; Mouse OSKM	KO DMEM-F12, 20% KSR, Human FGF2, Mouse LIF, Human LIF	ALP, OCT4, NANOG	ND	50	EB	ND	(23)
Fetal fibroblasts	Lentiviral system; Mouse OSKM	KO DMEM-F12, 20% KSR, 5% or 20% Oxygen, Human FGF2, Mouse LIF, MEK inhibitor, GSK-3 inhibitor; MEF feeders	ALP, SOX2, OCT4; *SOX2, OCT4, STELLA*	Yes	25	EB	ND	(73)
Fetal fibroblasts	Lentiviral system; Mouse OSKM, SV40LT; Bovine Nanog	DMEM, 10% FBS, Human FGF2, Human LIF; MEF feeders	ALP, SSEA1; *OCT4, SOX2, NANOG, ESRRB, KLF4, STST3*	Yes (at passage 2); No (at passages 10 & 20)	22	EB	Teratomas	(16)
Mesenchymal stem cells	Retroviral system; Mouse OSKMLN; KDM4A	DMEM/F12 and neutral basal medium, Human FGF2, CHIR-99021, Activin A; MEF feeders	ALP, OCT4, NANOG, SOX2,; SSEA3, SSEA4, TRA-1-60; *OCT4, NANOG, SOX2*	Yes (in early passages); No (at passages 10-17)	70	EB	Naïve-like iPSCs incorporated into mouse embryos and integrated into extra-embryonic tissues	(17)
Fetal fibroblasts	Lentiviral system; Mouse OSKM	KO DMEM-F12, 20% KSR; MEF feeders	ALP, SOX2, OCT4, NANOG; *SOX2, OCT4, NANOG, ESRRβ, STELLA, LIF, OTX2*	Yes	30	EB iPSCs contributed to the ICM region of day 7 blastocysts	ND	(74)
Sheep								
Adult fibroblasts	Lentiviral system; Human OSKMLN, SV40LT; Tet-on inducible	DMEM/F12, 20% KSR, DOX; MEF feeders	ALP, SSEA-1, TRA-1-60, TRA-1-81, REX1, E-cadherin; *OCT4, SOX2, NANOG*	Yes	31	EB	Teratomas	(24)
Fetal fibroblasts	Lentiviral system; Mouse OSKM; Tet-on inducible	KO DMEM, 20% KSR, Human FGF2; MEF feeders	ALP, OCT4, SOX2, NANOG, SSEA-4; *SOX2, NANOG, KLF4*	Yes	20	EB	Teratomas	(25)
Embryonic fibroblasts	Retroviral system; Human OSKM	DMEM, 20% FBS, Human FGF2, Mouse LIF; MEF feeders	OCT4, NANOG; *OCT4, SOX 2*	Yes	17	EB	Teratomas Contributed to the ICM of blastocysts	(30)
Embryonic fibroblasts	Retroviral system; Mouse OSKM	KO DMEM, 20% KSR, Human FGF2, and DMEM, 15% FCS, Mouse LIF; SNL feeders	ALP, NANOG	Yes	23	EB	Teratomas Contributed to live-born chimeric lambs	(31)
Fetal fibroblasts	Retroviral system; Mouse OSKM	KO DMEM, 20% KSR, Human FGF2; MEF feeders	ALP, OCT4, FGFR2; *OCT4, SOX 2*	Yes	40	EB	SCNT to create embryos failed	(75)

(Continued)

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TABLE 1 (Continued)

Kidney cells	Lentiviral system; Human OSKMLN, SV40LT, Human TERT, p53 RNAi, ASF1A	DMEM/F12, 20% KSR, Human FGF2, Vitamin C, VPA; MEF feeders	ALP, SOX2, OCT4, NANOG, REX1, SSEA-1, TRA1-60, TRA1-81, E-Cadherin; *NANOG, OCT4, SOX2, TDGF1, DAX1, ERAS, DNMT3b, DPPA4, GDF3*	Yes (in early passages); No (by passage 53)	30	EB	Teratomas	(28)
Fibroblasts from 15-day old sheep	Transposon systems; PiggyBac; Bovine OSKM, Porcine NANOG, Human LIN28, SV40LT, Human TERT; DOX-inducible	KO DMEM, 15% FBS, Human FGF2, Human LIF, Vitamin C; STO feeders	ALP, SOX2, OCT4, NANOG; *OCT4, NANOG, SOX2, KLF4*	Yes	32	EB Chimeric contribution to early blastocysts of sheep and mice and E6.5 mouse embryos	ND	(26)
Fetal fibroblasts	Plasmid vector carrying synthetic precursor miRNAs to induce mature miR-302s/367 expression	DMEM/F12, 20% KSR, Human FGF2, Vitamin C, VPA	miR-302s/367 did not reprogram cells into iPSCs; Inhibition of proliferation and apoptosis by targeting CDK2, E2F1, E2F2, and PTEN in the cell cycle and PI3K-Akt pathways	ND	Not mentioned	NA**	NA	(27)
Kidney cells	Lentiviral system; Human OSKMLN, SV40LT, Human TERT; Overexpression; of miR-200c-141; Tet-on inducible	DMEM/F12, 20% KSR, Human FGF2, Vitamin C, VPA; MEF feeders	ALP, OCT4, SOX2, NANOG, REX1; *OCT4, SOX2, NANOG, DAX1*	Yes	10	EB	ND	(29)
Goat								
Fetal ear fibroblasts	Lentiviral system; Human OSKM	DMEM/F12, 20% KSR, Human FGF2; MEF feeders	ALP, OCT4, NANOG; *OCT4, SOX2, cMYC, NANOG, KLF4*	ND	17	EB	Teratomas	(76)
Fetal fibroblasts	Lentiviral system; Mouse OSKM; Tet-on inducible	KO DMEM, 20% KSR, Mouse LIF, Vitamin C, VPA; MEF feeders	ALP, OCT4, SOX2, NANOG, SSEA-1, TRA-1-60, TRA-1-81; *OCT4, SOX2, NANOG, KLF4, LIN28, REX1*	Yes (in early passages); No (by passage 15)	15	EB	Teratomas	(77)
Fetal fibroblasts	Lentiviral system; Bovine OSKMLN in combination with aMIR302/367cluster	DMEM/F12, 20% KSR, Mouse LIF, Human FGF2; MEF and SNL feeders	ALP, OCT4, SOX2, NANOG; *OCT4, SOX2, KLF4*	Yes	30	EB	Teratomas	(34)
Embryonic fibroblasts	Lentiviral transduction; Human OSKM, +/- PRMT5	KO DMEM, 20% KSR, Human LIF, Human FGF2; MEF feeders	ALP, OCT4, C-MYC, SSEA1, SSEA4; *SOX2, KLF4, OCT4, C-MYC, NANOG*	ND	4	EB	ND	(78)
Fetal fibroblasts	Retroviral transduction; Mouse OSKM	KO DMEM, 15% FBS, Mouse LIF, Human FGF2; MEF feeders	ALP, OCT4, NANOG, SSEA1; *OCT4, REX*	Yes	20	ND	ND	(79)
Embryonic fibroblasts	Transfection with mRNA OSKM	KO DMEM, 20% KSR, Human FGF2	ALP, OCT4, SOX2, KLF 4, NANOG, CDX2, REX, SSEA-1, TRA-1-60, TRA-1-81; *OCT4, SOX2, NANOG, DAX1, GDF3*	Yes	22	EB	ND	(32)

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TABLE 1 (Continued)

Ear fibroblasts	Chemical induction with small molecules CHIR98014, Forskolin, VPA, Tranylcypromine, ALK5 inhibitor, TTNPB, 3-DZnep	KO DMEM-F12, Neurobasal medium, N-2 supplement; Matrigel-treated plates MEF feeders	ALP, OCT4, SOX2; *OCT4, SOX2, NANOG, CDH1, TDGF, DAX1*	NA	Not mentioned	EB	ND	(33)
Buffalo								
Fetal fibroblasts	Retroviral transduction; Buffalo OSKM	DMEM, 20% FBS, Human FGF2, Human LIF; MEF feeders	ALP, OCT4, SOX2, NANOG, SSEA-1, SSEA-4, TRA-1-81, E-cadherin; *OCT4, SOX2, NANOG, LIN28*	Yes (in early passages); No (by passage 10)	10	EB	Teratomas	(37)
Fetal Fibroblasts	Chicken egg extract at various concentrations	KO DMEM/F12, 20% FBS, Human LIF, Human FGF2; BFF feeders	ALP, OCT4, NANOG, SSEA-1, TRA-1-60, TRA-1-81; *OCT4, NANOG, SOX2, KLF4, C-MYC*	NA	2	ND	ND	(36)
Fetal Fibroblasts	Lentiviral transduction; Mouse OSKM; VPA	KO DMEM/F12, 20% FBS, Human LIF, Human FGF2; BFF feeders	ALP, OCT4, NANOG, SSEA-1, TRA-1-60, TRA-1-81; *OCT4, NANOG, SOX2, KLF4, C-MYC*	Yes (in early passages); No (by passage 15)	18	EB	ND	(80)
Adipose-derived mesenchymal stem cells	Retroviral plasmids; Mouse OSKM; Hypoxic (5% O2) or normoxic; VPA	DMEM, 20% FBS, Human FGF2, Human LIF	CT4, NANOG, SSEA-4, TRA-1–81; *OCT4, NANOG*	ND	9	EB	Teratomas	(35)
Fetal Fibroblasts	Transposon systems; PiggyBac; Human SOKMNL	DMEM/F12, 20% KSR, Human LIF, Human FGF2	ALP, OCT4, NANOG, SOX 2 SSEA-1, SSEA-4, SSEA-5, TRA-1-81; *OCT4, NANOG, SOX2, KLF4, C-MYC, LIN28*	ND	15	EB	ND	(81)
Fetal Fibroblasts	Lentiviral transduction; Mouse OSKM	KO DMEM/F12, 20% FBS, Human LIF, Human FGF2; MFF feeders	ALP; *OCT4, SOX2, KLF4, c-MYC, REX1, TRA1-81*	Yes	15	EB	ND	(82)
Fetal skin fibroblasts	Transposon systems; PiggyBac; Buffalo OSKM	DMEM, 5% FBS	OCT4, SOX2; Activation of LIF, Activin, BMP4 and SMAD1/5/9	ND	Not mentioned	EB	ND	(83)

*ND, not done; **NA, not applicable.

ALP, Alkaline Phosphate; ASF1A, Anti-Silencing Function 1A Histone Chaperone; BFF, Bovine Fetal Fibroblasts; BMP4, Bone Morphogenetic Protein 4; CDH1, Cadherin 1; CDK2, Cyclin-Dependent Kinase 2; C-MYC, MYC Proto-oncogene; DAX1, Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X gene 1; DMEM, Dulbecco's Modified Eagle Medium; DNMT1, DNA Methyltransferase 1; DNMT3B, DNA Methyltransferase 3 Beta; DOX, Doxycycline; DPPA4, Developmental Pluripotency Associated 4; E2F1, E2F Transcription Factor 2; E2F1, E2F Transcription Factor 1; EB, Embryoid bodies; ERAS, ES Cell Expressed Ras; ERK, Extracellular signal-regulated kinases; ES, Embryonic Stem; ESRRB, Estrogen-Related Receptor Beta; FGF2, Fibroblast growth factor 2; FGFR2, Fibroblast Growth Factor Receptor 2; GDF3 Gene, Growth Differentiation Factor 3; GSK3, Glycogen Synthase Kinase 3; ICM, Inner Cell Mass; KDM4A, Lysine Demethylase 4A; KLF4, Krüppel-like factor 4; LIF, Leukemia Inhibitory Factor; LIFr, Leukemia Inhibitory Factor receptor; LIN28, Lin-28 homolog A; MEF, Mouse Embryonic Fibroblasts; MEF2A, Myocyte Enhancer Factor 2A; MEK, Mitogen-Activated Protein Kinase Kinase; MEM, Minimum Essential Medium; miRNAs, microRNAs; NANOG, Homeobox protein NANOG; OCT4, Octamer-binding transcription factor 4; OSKM, OCT4, SOX2, KLF4, C-MYC; OSKMLN, OCT4, SOX2, KLF4, C-MYC, NANOG, LIN28; OTX2, Orthodenticle Homeobox 2; PRMT5, Protein Arginine Methyltransferase 5; PTEN, Phosphatase and Tensin Homolog; REX1, Reduced Expression gene 1; RNAi, RNA interference; SCNT, Somatic Cell Nuclear Transfer; SMAD, Suppressor of Mothers Against Decapentaplegic; SNL, SNL feeder cells; SOCS3, Suppressor of Cytokine Signaling 3; SOX2, SRY-Box Transcription Factor 2; SSEA-1, Stage-Specific Embryonic Antigen-1; SSEA-4, Stage-Specific Embryonic Antigen-4; STAT3, Signal Transducer and Activator of Transcription 3; STO, Sandos inbred mouse (SIM)-derived 6-thioguanine- and ouabain-resistant cells; SUZ12, SUZ12 Polycomb Repressive Complex 2 Subunit; SV40LT, SV40 large T antigen; TDGF1, Teratocarcinoma-Derived Growth Factor 1; TERT, Telomerase Reverse Transcriptase; TRA-1-60, T cell Receptor Alpha locus; TRA-1-81, Podocalyxin-Like Protein-1; VPA, Valproic acid.

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Characterization of these bovine iPSCs demonstrated the expression of endogenous pluripotency factors such as ZFP42 Zinc Finger Protein *(REX1),* Estrogen related receptor beta *(ESRRB)* and Developmental pluripotency associated 3 *(STELA)* at the transcript level, and ALP, Fucosyltransferase 4 (SSEA-1) and LIN28 at the protein level. Two studies demonstrated bovine iPSC longevity by maintaining cultures for more than 40 passages (21, 22). Both groups supplemented the culture medium with FGF2 and LIF, and one added kinase inhibitors (22). Another group reported the maintenance of bovine iPSCs for over 50 passages using FGF2 and LIF supplemented culture medium (23). In addition, bovine iPSCs cultured for over 70 passages was achieved with a culture medium containing doxycycline, histone methyltransferase, and WNT inhibitors (17, 19). Additionally, few groups demonstrated epigenetic validation of iPSCs by showing demethylation of *NANOG* and *OCT4* promoter regions in the host cells' genomes (17, 18, 21). Finally, many cultures were not subjected to experimentation to demonstrate differentiation into EBs *in vitro* and/or formation of teratomas *in vivo*, measures of functional pluripotency (Table 1). While most studies did not focus on the pluripotency state, two research groups reported the generation of bovine naïve-like iPSCs (16, 19), and both naïve state-like and primed state-like status of these cells was achieved using culture conditions for mouse and human iPSCs (17, 19).

Induced pluripotent stem cells from sheep

Two independent groups published the first reports of ovine iPSCs in 2011 (24, 25). They used viral vectors to introduce OSKM or OSKM plus additional pluripotency genes into fibroblasts collected at different stages of development and showed that the generated iPSCs expressed multiple pluripotency markers and formed EB and teratomas. Since these two initial reports, additional groups have introduced pluripotency genes into ovine cells using a PiggyBac transposon system (26) and microRNAs (27). In addition to ovine fibroblasts, kidney cells have also been used for reprogramming (28, 29). A variety of culture media has been used across ovine iPSC studies, and different pluripotency markers have been assessed (Table 1). Most ovine iPSC cultures could form EB, some formed teratomas, and other cultures were found to contribute to early blastocysts (26, 30) and live-born chimeric lambs (31).

Induced pluripotent stem cells from goats

All caprine iPSCs produced thus far were derived from fetal, embryonic, or adult fibroblasts. In most of the cases, lentiviral or retroviral vectors containing OSKM or OSKM plus additional pluripotency factors were utilized (Table 1). Caprine iPSCs have also been produced *via* mRNA transduction (32) and chemical induction using small molecules (33). Many different culture conditions have been used, which either included LIF or FGF2, or both. Most of the caprine iPSC cultures displayed a colony morphology like mouse iPSCs, stained positively for alkaline phosphatase (ALP), and exhibited goat-specific pluripotency markers at the transcriptional and/or protein level. Although most caprine iPSC cultures could develop into EBs, very few were able to induce teratomas *in vivo*. One study showed that directed differentiation of caprine iPSCs resulted in the *in vitro* production of trophoblast-like cells, yolk-sac endoderm-like cells and neuronal cells (34).

Induced pluripotent stem cells from buffalo

Almost all buffalo iPSC cultures have been generated using fetal fibroblasts as the reprogramming cell source, except for one study which used adipose-derived MSCs (Table 1). In addition to viral or non-viral delivery of buffalo, mouse, or human reprogramming factors (OSKM or OSKMNL), the epigenetic modifier valproic acid has been used to enhance the reprogramming efficiency (35). Chicken egg extract added to the culture medium was also shown to be adequate to generate putative buffalo iPSCs colonies (36). Although most of these buffalo iPSC cultures were able to differentiate into EBs, only one group reported generation of *in vivo* teratomas and epigenetically validated buffalo iPSCs (35, 37).

Limitations of ruminant induced pluripotent stem cells

Technical barriers to iPSC generation and maintenance, safety concerns when using iPSC *in vivo*, and the cost of creating and sustaining iPSC lines for therapeutic use, all contribute to the slow rate of progress in the field of iPSC research across species.

Technically, the core genes required for the establishment of pluripotency are different between mammals and are expressed at different stages of development (38). As a result, pluripotency factors other than OSKMNL need to be tested and timing of the introduction of pluripotency genes must be optimized, to determine the best methods for establishing pluripotent cell lines from ruminants. When pluripotent iPSC lines are created successfully, permanent expression of viral transgenes can interfere with differentiation into desired cell types (39). Non-viral methods of introducing pluripotency factors to target cells might circumvent this issue, but such methods have not been well-explored in ruminants to date.

For the *in vivo* use of iPSCs, safety concerns are at the forefront, primarily the risk of (i) harmful immune reactions to allogeneic cells, (ii) random integration of transduction material into the recipient's genome and (iii) differentiation of iPSCs leading to tumorigenesis. Immune reactions may be avoided by using iPSCs derived from autologous cells or altering MHC genes in iPSCs to make them less immunogenic (40). However, each of these strategies has its drawbacks. Autologous cells are not practical for large-scale, commercial treatments, and administering foreign cells that can completely avoid the host immune response introduces a risk of unchecked, inappropriate cell growth. Non-viral methods to induce pluripotency would avert the random integration of transgenes, but as mentioned above, these methods are not yet well-developed. The risk of tumor development could be reduced by differentiating iPSCs *in vitro* before administering them as a treatment (41) or by introducing a drug-inducible "suicide" gene,

that can be turned on to prevent tumor growth (42). Such methods, however, are not fully optimized and currently not used *in vivo*.

The cost of generating and maintaining iPSCs for research is not trivial. The expenses required to create marketable iPSC-derived meat and other animal products in controlled laboratory environments (43), as well as those involved in commercializing iPSC-based therapies (44), are tremendous. Governments and private companies must be assured that ruminant iPSCs are a useful resource worth investing in if progress is to be made in the fields of food production, and veterinary, biomedical, and/or pharmaceutical applications.

Potential applications of ruminant induced pluripotent stem cells for research and enhancing livestock production

iPSCs derived from ruminant somatic cells have the potential to (i) improve agriculture, (ii) enhance veterinary, biomedical, and pharmaceutical practices, and (iii) provide knowledge that may be translatable to human medicine (Figure 1).

Cellular agriculture

Finding alternatives to conventional farming practices is crucial, given the rising reliance on animal products for human nutrition. Compared to plant-based food sources, conventionally produced animal-based material has a larger environmental footprint, requires more soil and water, and leads to the emission of more greenhouse gases (45). Moreover, antibiotic overuse in livestock farming results in the emergence of antimicrobial-resistant bacterial strains, a significant human health concern (46). Cellular agriculture, defined as the production of animal-sourced food from cultured cells, has the potential to replace traditional farming with more environmentally friendly practices. The production of meat *in vitro* using iPSCs is proposed as a clean and prominent alternative to reduce the global burden of the livestock industry (47). In 2012, meat derived from bovine stem cells was used to create the first lab-made hamburger (48). Moreover, a commercial meat producer in the UK reported the first lab-made strips of bacon and pork belly in 2020 (49). In addition, production of cell-based seafood from fish cells and tissue cultures is also becoming popular to address the challenges associated with industrial aquaculture systems and marine capture (50). More recently, laboratory-grown meat, slaughter-free chicken, received clearance from U.S. Food and Drug Administration for human consumption (51). Additionally, generation of other iPSC-derived animal products, such as skin and fur, could reduce our dependence on industrial farming and minimize the associated environmentally harmful effects.

Genetically modified (transgenic) ruminant livestock

The recent advancements in iPSC generation along with targeted genome editing technologies, especially the CRISPR-Cas9 system (52), have facilitated the introduction of desired genetic modifications and, combined with somatic cell nuclear transfer (SCNT) or blastocyst complementation, represent a powerful platform for transgenic animal production (53, 54).

Genetically modifying ruminants can enhance growth rates and production, improve nutrients in animal products, increase disease resistance, and enhance reproductive efficiency and fecundity. Moreover, transgenic ruminant livestock, especially those animals used for milk production such as cattle, buffalo, and goats, that are generated by genetically modified iPSCs could be used as bioreactors to produce therapeutic proteins of pharmaceutical interest. To this end, cloned transgenic cattle, which produce recombinant proteins in milk such as human coagulation factor IX has been reported (55). Additionally, transgenic ruminant livestock has the potential to significantly reduce the environmental footprint of livestock husbandry by increasing productivity and efficiency through transgenesis, which results in reduced use of land and water resources while safeguarding the soil and groundwater.

Reproduction and conservation

Significant paradigm changes in reproduction have been made possible by prominent developments in stem cell biology. Germ cells have been derived successfully from mouse stem cells (56) and although protocols for differentiating buffalo embryonic stem cells into germ cells (57) and animal embryo-stem cell livestock laboratory breeding systems have been proposed (58), the differentiation of ruminant iPSCs to functional gametes *in vitro* has not been achieved yet.

Derivation of ruminant iPSCs may open the possibility of *in vitro* breeding. For example, selected cell lines could be differentiated to create functional gametes, which would then be used to create a new generation of embryos through *in vitro* fertilization. Such breeding schemes could substantially reduce generation intervals, enhance selection intensity, achieve more genetic gain, and preserve rare ruminant breeds and highly valuable genotypes. In addition, these cells could be expanded for the banking of genetic material and be used as donor cells for SCNT.

Disease modeling

Ruminant diseases are widespread and have detrimental consequences on the herd and consumer health (59). The lack of appropriate ruminant disease models hampers the study of disease pathogenesis and the development of strategies to control these diseases. iPSCs are valuable tools for tissue and disease modeling, as well as preclinical therapeutic development in both human and mouse models (60). However, the use of iPSCs for ruminant disease modeling is currently limited, in part because differentiating ruminant iPSCs into clinically relevant lineages has not been well explored. One study demonstrated the potential of using iPSC technology for generating bovine mammary tissue *in vitro* (61). In this study, bovine iPSCs were successfully generated from the bovine mammary epithelium, and mammary epithelium-derived-iPSCs were differentiated back to a mammary phenotype characterized by epithelial cells expressing cytokeratin

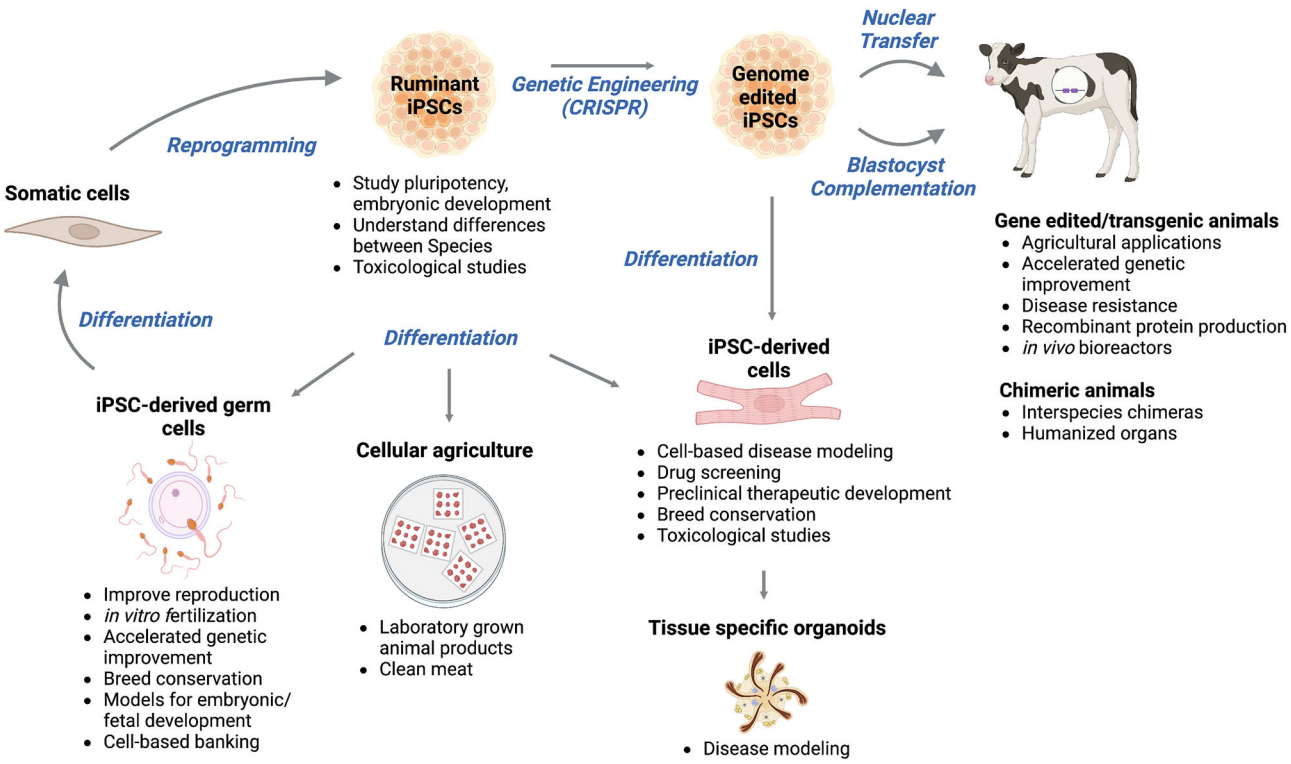

FIGURE 1
Applications and future usages of induced pluripotent stem cells (iPSCs) from ruminant livestock. Ruminant iPSCs are generated by the reprogramming of somatic cells. Ruminant iPSCs are a useful tool for studies of pluripotency, embryonic development, and understanding species differences, and can be genetically modified to create transgenic animals for various agricultural, veterinary, biomedical, and pharmaceutical applications. Ruminant iPSCs and/or gene-edited iPSCs have the potential to be used for the generation of interspecies chimeras and humanized organs. They can be the basis for disease modeling, drug screening, preclinical therapeutic development, and toxicological studies. Tissue organoids derived from ruminant iPSCs can be used to model diseases and identify effective treatment options. Ruminant iPSCs can also contribute to cellular agriculture as a source of laboratory-grown animal products. Successful generation of ruminant iPSC-derived germ cells can lead to improved reproduction, genetic improvement, breed conversation, and can be used as tools to study embryonic and/or fetal development. Figure was created using Biorender.com.

14, cytokeratin 18, and smooth muscle actin, after treatment with progesterone (61). These studies could be complemented by generating iPSC-derived mammary organoids that can be used to explore the pathogenesis and prevention of important bovine udder diseases. Additionally, rare genetic disorders such as bovine citrullinemia and bovine leukocyte adhesion deficiency found in Holstein-Friesian cattle (62), and Chediak-Higashi syndrome found in Hereford, Brangus, and Japanese black cattle (63), may be studied using bovine iPSCs models, based on the unique advantages that iPSC cultures have shown in the modeling of rare human genetic disorders (64).

Toxicology studies

Endocrine-disrupting chemicals (EDCs) may significantly impact the reproductive functioning of ruminant livestock, which greatly impacts agricultural production (65). Bovine iPSCs have been used to study the effects of phthalate esters, synthetic organic chemicals used in the plastic industry (20). These esters were found to significantly downregulate androgen receptors on iPSCs, which supported apoptosis (20). Ruminant iPSCs may also be used in toxicological studies investigating how pharmaceuticals, potential toxins, teratogens, and EDCs affect livestock species and humans.

Chimera formation and growth of human organs

A composite organism of at least two genetically distinct cell populations is called a chimera. With the use of iPSC technology, the production of chimeric ruminants would allow for the genetic engineering of farm animals to improve traits of agricultural importance and the generation of biomedical models. Reports on interspecies ruminant chimeras such as sheep-goat (66) and cattle-buffalo (67) are already available. Moreover, human-ruminant chimeras could be created for use as models to study human organ development and disease pathogenesis, as well as to meet the increasing demand for and reduce the shortage of human organs. For example, human iPSCs have been engrafted in cattle pre-implantation blastocysts (68). However, enormous technical challenges and complex ethical issues must be considered and overcome before producing human organs in

ruminants or any other mammals becomes feasible. The risks of human consciousness, human traits, and the creation of human gametes by such chimeras are the primary ethical concerns (69), and any attempt to create human-ruminant chimeras must be thoroughly risk-assessed, technically evaluated, and closely supervised.

Conclusions

This mini review summarizes the work carried out to generate, maintain, and characterize iPSCs and iPSC-like cells derived from somatic cells of domesticated ruminants. Despite their undeniable potential in agriculture, conservation biology, biotechnology, and as models for preclinical research, iPSC cultures from ruminant livestock species have yet to be fully optimized. Developing uniform (i) reprogramming protocols, (ii) characterization criteria, and (iii) methods for the long-term maintenance of ruminant iPSCs needs to be prioritized to establish stable, well-defined ruminant iPSC lines that can be used to improve animal and human well-being.

Author contributions

PW and RH: original draft preparation and editing. GV: conceptualization and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

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Systematic review and meta-analysis of positive long-term effects after intra-articular administration of orthobiologic therapeutics in horses with naturally occurring osteoarthritis

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Equine veterinarians face challenges in treating horses with osteoarthritic joint pain in routine veterinary practice. All common treatment options aim to reduce the clinical consequences of osteoarthritis (OA) characterized by persistent synovitis and progressive degradation of articular cartilage. A range of joint-associated cell types and extracellular matrices are involved in the not yet entirely understood chronic inflammatory process. Regeneration of articular tissues to re-establish joint hemostasis is the future perspective when fundamental healing of OA is the long-term goal. The use of intra-articular applied biologic therapeutics derived from blood or mesenchymal stroma cell (MSC) sources is nowadays a well-accepted treatment option. Although this group of therapeutics is not totally consistent due to the lack of clear definitions and compositions, they all share a potential regenerative effect on articular tissues as described in *in vivo* and *in vitro* studies. However, the current stage of science in regenerative medicine needs to be supported by clinical reports as in fact, *in vitro* studies as well as studies using induced OA models still represent a fragment of the complex pathomechanism of naturally occurring OA. This systemic review aims to determine the long-term effect of orthobiologic therapeutics in horses suffering naturally occurring OA. Thereby, a meta-analysis of randomized controlled trials (RCTs) is conducted to describe the efficiency and safety of intra-articular applied orthobiologics in terms of lameness reduction in the long-term. Using the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines, thirteen studies met the inclusion criteria for the systemic review. Four of those studies have further been evaluated by the meta-analysis comparing the long-term effect in lameness reduction. Each study was examined for risk of bias. For data evaluation, a random-effects model was used, describing the overall outcome in a forest plot. The I^2 statistic was used to assess heterogeneity. Results indicate, that orthobiologic therapies represent an effective long-term and safe OA treatment option. Due to the inhomogeneity of included studies, no statements are provided

addressing specific orthobiologic therapies, affected joints, OA stage and horse's intended use. Future clinical trials should follow standardized study designs to provide comparable data.

KEYWORDS

horse, degenerative joint disease, regenerative medicine, orthobiologics, autologous blood products, mesenchymal stromal cells (MSC), review—systematic, meta-analysis

1. Introduction

Osteoarthritis (OA) is an intensively researched condition in human and equine patients characterized by persistent articular inflammation leading to chronic synovitis, progressive destruction of articular cartilage, and consequently to a permanent loss of function and joint pain (1–3). Causative and stimulating factors of OA are still not fully investigated. In horses, the etiology of OA is assumed to be mainly post-traumatic. Therefore, OA in horses can be understood as the result of a failed repair of damaged articular and periarticular tissues. However, not only the nature of the initial structural tissue damage (repetitive microtrauma vs. single severe trauma), but also the degree and course of imbalance of the joint homeostasis seem to determine OA manifestation and progression (4).

In equine patients, joint related diseases including OA are considered the most common cause of lameness, as being involved in approximately 60% of all lameness cases (5–7). More than 70% of racehorses population suffer from lameness due to articular inflammation during their career (8, 9). However, the occurrence of OA is not only linked to high-speed and high-performance sport horse disciplines, such as horse racing (10–12) and show jumping (13, 14), but also to the increasing age of the patients (15–17). In OA-affected horses, the prognosis for long-term return to exercise and work on intended use varies between 30 and 50% and depends on the disease stage, the affected joints, and the horse's work level (17, 18).

In daily clinical practice, equine veterinarians face the challenge of treating OA as a persistent and chronic disease potentially affecting all joint associated tissues (10, 19, 20). Often the subsequent treatment choice is based on the veterinarian's personal experience, the owner's economic feasibility and the intended use for the horse in relation to disease stage. Although a broad spectrum of varying therapeutic concepts is stated (21, 22), conventional treatment options are limited in terms of modifying or reversing disease progression, thereby potentially being inferior in the long-term treatment success. However, the development of successful long-term treatment options is difficult, due to the intricate pathomechanisms of OA initiation as well as progression and the involvement of various cell types and extra-cellular matrices.

Recent studies have shown that biologic therapeutics derived from blood and mesenchymal stromal cell (MSC) sources hold a potentially regenerative potential for articular tissues *in vitro* (23–26) and *in vivo* (27–29). Beneficial clinical effects described after an intra-articular administration of biological therapeutics include reduction of lameness and joint effusion (30–32). It is assumed that clinically relevant effects of intra-articular administered blood products and MSCs in OA-affected joints in part are attributed to locally effective growth factors, cytokines, as well as secretomes and exosomes from delivered cells, which further innate on-site cell regeneration (33–35). Although the group of these so named orthobiologics or orthobiologic therapeutic agents is not totally consistent due to differences in manufacturing, processing and application, they all share potential regenerative effects on the described articular tissues proven *in vitro* (26, 36, 37) and *in vivo* (38–40) studies.

After more than 20 years of clinical experience in equine medicine, the use of intra-articularly applied orthobiologic therapeutics is considered as a safe and recognized treatment option for osteoarthritic joints today (41, 42). Yet, existing studies, which form the basis of our knowledge about the efficacy of orthobiologic therapeutics in equine medicine, differ in fundamental study design parameters like the availability of placebo groups or the type of researched OA (naturally occurring vs. experimentally induced OA). Consequences drawn from these studies are at best implemented in the treatment of clinical cases and provide evidence-based treatment concepts for equine OA. However, due to the heterogenicity of therapeutic products (blood-derived, tissue-derived), processing methods and components used (cell-free, blood-derived cells, tissue-derived cells), and treatment regimens (single injection, multiple injections), an unacceptably high number of subjects would be required to draw definitive conclusions. Therefore, the application of quantitative statistical methods summarizing primary data from clinical and experimental trials *via* meta-analysis is a useful tool to draw conclusions from a cohort of studies. The aim of the present study is to conduct a systematic review of current literature in the field of the intra-articular application of orthobiologic therapeutics in naturally occurring equine OA. Furthermore, a meta-analysis of *in vivo* and controlled studies has been carried out to assess the long-term effect of orthobiologic therapeutics on naturally OA-affected joints in horses.

2. Materials and methods

2.1. Definition of orthobiologic therapies

The present systematic review focuses on the following two intra-articularly applicable orthobiologic therapeutic concepts for equine OA.

2.1.1. Mesenchymal stromal cells

Caplan described the first approaches of stem cell therapy in 1991, proposing potential differentiation into desired tissues (43). The characteristic differentiation potential of these cells has laid the foundation to prove therapeutic concepts in various fields of medicine where tissue regeneration and restoration are the aimed effects (44–49). In the process of clinical stem cell application, orthopedic diseases such as OA were becoming an inherent part of scientific interest (35, 50). The common term "stem cell" is nowadays used in popular science and increasingly replaced by the more scientific expression of a "multipotent mesenchymal stromal cell (MSC)" because specific stem cell characteristics (51) [long *in vivo* survivability, ability for self-replication and multipotent differentiation into certain tissue types (43)] are insufficiently accurate to prove in therapeutic purposes. However, the term "MSC" is not used uniformly and is not subject to a clear definition. Due to increasing impact of MSCs *via* paracrine effects, the term "medicinal signal cell" has been proposed in recent publications (52, 53).

MSCs can be derived from mesenchymal tissues such as blood, bone marrow and adipose tissue, but do not represent a homogeneous stem cell population (41). In horses, commonly used MSC sources are fat, harvested from subcutaneous adipose tissue at the tail base (lipectomy) (54, 55), bone marrow obtained by puncturing the sternum (56) or venous blood (40, 57). Following tissue harvesting, the process of MSC isolation and cultivation under laboratory conditions requires several weeks to obtain cell numbers usually used for intra-articular applications (41). Besides these autologous cultivated MSCs, commercially available MSC therapeutics are approved by the European Medicines Agency (EMA). Currently, two off the shelf MSC therapeutics are available, one of which uses chondrogenic induced MSCs dissolved in allogeneic plasma (40, 57–59), whereas the other product uses MSCs derived from the umbilical cord (60, 61). These therapeutics contain a defined number of allogeneic MSCs from donor equids. A further alternative to commercially available ready-to-use products is the in-house production of therapeutics from tissue sources like blood, bone marrow or adipose tissue, usually received from the equine patient (autologous) (42). These so-called point-of-care products are readily available through a fast, standardized process of cell separation and MSC enrichment by medical devices (27, 62). Depending on the tissue sources and processing, the final solution contains a variety of different cell types in a mixed population of blood and adipose progenitor cells as well as differentiated cells (41, 42). The proportion of MSC-like cells within the final product is regarded low and not defined (63). With regard to obtain a high number of defined MSCs from the stated tissue sources, MSC isolation and cultivation has to be performed under laboratory conditions (autologous cultivated MSCs) (64, 65). As a result, several millions MSCs are available for application (66). The time between tissue sampling to MSC harvesting calculates several weeks, which must be considered for autologous treatment regimes.

2.1.2. Autologous blood products

Autologous blood products represent a wide range of therapeutics due to the variety of blood processing methods and individual blood components (33, 67). Basically, two groups of blood derived applicable therapeutics can be stated: (1) cell-based and (2) cell-free autologous blood products. For blood processing, commercially available medical devices are provided to equine practitioners.

Cell-based autologous blood products aim to increase the concentration of certain blood cells, mainly platelets, within the applicable therapeutic agent to transmit the regenerative potential of platelet containing growth factors into the joint (68, 69). Depending on the respective blood platelet number and the processing method, the increase in platelet concentration varies widely among products (70). The amount of transmitted growth factors and cytokines depends on the total number of applied platelets, on the injected solution and whether the therapeutic cells are solved in plasma or in a non-blood based injectable solution (71, 72). Platelet rich plasma (PRP) is one of the best-known representatives of this therapeutic group, with a defined 3- to 5-fold increase in platelet concentration in autologous plasma (73, 74). PRP is produced using a double-centrifugation method (41). Alternative processing methods such as single-centrifugation techniques and filtration provide therapeutics with deviating values of platelets and leucocytes from PRP (33, 75). In horses, cellular autologous blood products were commonly used in cases with tendon and ligament injuries (76). However, their use in joint-related diseases is described, and positive outcomes are documented, particularly in combination with MSC-treatments (40, 77). The therapeutic effects have not yet been clarified in detail, since not only growth factors play a pivotal role in tissue regeneration.

Cell-free, serum-based therapeutics represent another group of autologous blood products. After extended coagulation of the patient's blood at 37°C and subsequent centrifugation, the final orthobiologic therapeutic substance provides the full blood cell secretome (26, 78). In addition to the already serum-diluted cytokines, growth factors and proteins, the extended coagulation phase also stimulates *de novo* synthesis of proteins, which enrich the final product to a so far not totally defined extended secretome (79, 80). The mode of action of the acellular autologous blood products is in many aspects not fully defined (81, 82). The often referred increase of anti-inflammatory interleukin-1 receptor antagonist (IL-1Ra) concentration is only partially responsible for the described positive clinical effects (30, 83). The mechanism of action of enriched IL-1Ra as therapeutic agent is to block the receptors and therefore prevent the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) released by the intra-articular inflammatory process from binding (33).

2.2. Inclusion criteria

A distinction was made between systematic analysis and meta-analysis. To obtain a general overview, all experimental studies with a follow-up time of more than 6 months were examined in the systematic review. In the meta-analysis, only randomized and controlled trials (RCTs) with a follow-up period of more than 6 months were examined according to the following

inclusion and exclusion criteria. To present the results as clearly as possible, the PICO method was used. (a) Population: horses with naturally occurred OA; (b) Interventions: intra-articular therapy by MSCs alone or by MSCs in combination with autologous blood products, or autologous blood products alone; (c) Comparison: degree of lameness before and after intra-articular treatment (comparison of success rate, horses working on competition, horses working at trainings level, lame free horses); (d) Outcome: degree of lameness and adverse effects; (e) Study designs: for the systematic review all experimental studies were included, for the meta-analysis randomized controlled trials were included.

2.3. Exclusion criteria

The following studies were excluded: (a) treated animals other than horses and diseases other than OA; (b) use of treatment method other than intra-articular; (c) no clear lameness diagnostics used; (d) not published in English or German; German language was included as this is the authors mother language and articles could be assessed in detail (e) no complete replication of quantitative data of the treated animals (for example individual degree of lameness).

2.4. Search strategy

The following research platforms were used (listed according to weighting): PubMed, Google Scholar and CAB direct. Literature searches were carried out using the following keywords: "horse/equine," "joint/osteoarthritis," "intra-articular," "regenerative therapy," "return/performance." The search terms could be summarized with the Boolean operators "AND" or "OR" (84). The research was conducted between January 2021 and March 2022. A comprehensive literature search on orthobiologic based joint therapies in horses was undertaken, including all studies published in English and German. This initial investigation summarized 271 findings, of which all studies were examined according to inclusion and exclusion criteria. Subsequently, this initial investigation delivered 86 results. In addition, the reference list of all 86 papers were manually checked for research-relevant studies. To ensure that no meta-analyses relevant to this topic were available, a hit query was performed on PubMed using the two keywords "horse" and "meta-analysis." The response resulted in 79 meta-analyses. This compares to 18 matches with three real meta-analyses in 2017 (85). These results prove clearly that meta-analysis is becoming more and more relevant in evidence-based medicine. None of these 79 meta-analyses deals in a similar or identical way with the issue investigated in this research. The subsequent table lists the most important studies, sorted by intra-articular administered products, in horses with naturally occurring OA compared to induced OA. In addition, the study duration is indicated >6 months (Table 1).

2.5. Data extraction

To meet the aim of the topic, only *in vivo* studies were analyzed. For the systematic review, all experimental studies were included regardless their level of evidence or design, with and without a control group. The control group was defined as another horse, another leg (contralateral limb) or another treatment method. The following data were examined and listed according to the following aspects: author, year of publication, type of study (RCTs/ No-RCTs), sample size, treatment protocol, treated joint/joints, placebo-controlled, adverse reactions, follow-up time, lameness evaluation (horses working at trainings level/lame free horses/horses working on competition level/success rate).

For the intra-articular treatment regimen with orthobiologic therapeutics, there were no specifications regarding diagnostic methods, treatment frequency, dosage, and preparation of the appropriate therapeutics (intra-articular therapeutics with MSC and/or autologous blood products are allowed). In addition, studies with any joint with naturally occurring OA were included in the systematic review; there were no specifications on a specific localization. Finally, all studies were evaluated based on the lameness examination and classified into either a positive or a negative outcome. The positive outcomes were divided into two groups: horses working at training level and horses returning to competition. Horses with a negative outcome did not respond to treatment or had a relapse during the observation period. For the meta-analysis, the two positive outcome groups (horses working at training level/lame free and horses returning to competition) were combined due to a lack of study numbers.

2.6. Quality assessment

Each study in the systematic review was examined for the following 7 bias characteristics: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), other source of bias. Regarding each aspect, the studies were classified as high risk, low risk or unclear risk according to the PRISMA guidelines (93). For a better visualization, a traffic light table with "high risk" in red, "low risk" in green, and "unclear risk" in yellow was created. The classification into the category "unclear risk" occurs when relevant details for the classification into bias are not sufficiently substantiated in the respective study (94).

2.7. Statistical analysis

Using the PRISMA guidelines, 13 studies met the inclusion criteria for the systematic review (93). To compare dichotomous outcomes *via* meta-analysis, an odds ratio (OR) with 95% confidence interval (CI) was calculated using the R program (95). For data evaluation a random-effects model was used describing the overall outcome. Each study with its estimated effect size and corresponding confidence interval is graphically represented in the

TABLE 1 Summary of all studies on naturally occurring OA included in the systematic review.

Mesenchymal stromal cells									
References	Type of study	Number of horses	Orthobiologic therapeutic agent and Number of horses per group (n)	Treated joint	Control group	Adverse reactions	Time follow up, number of horses completed follow up (n)	Outcome: Training level/lame free	Outcome: Competition level
Broeckx et al. (86)	Randomized multicenter double blinded and placebo-controlled study	75	IVP Group (allogenic blood-derived, chondrogenic induced MSCs with equine allogeneic plasma),	Fetlock	Yes	No	1 year $n = 75$	IVP: 19/50 (37%) Placebo: 2/25 (8%)	IVP: 23/50 (47%) Placebo: 0/25 (0%)
Magri et al. (31)	Prospective blinded placebo-controlled study	28	Allogenic umbilical cord-derived MSCs	MCP 16 MTP 6	Yes	Owner detected adverse effects to MSC injection were recorded in 18% of the horses	6 months $n = 22$	8/22 (36%)	5/22 (23%)
Broeckx et al. (77)	Preliminary study	20 (4 × 5)	Group 1: PRP Group 2: allogenic blood-derived, native MSCs Group 3: allogenic blood-derived, native MSCs + PRP Group 4: allogenic blood-derived, chondrogenic induced MSCs + PRP	Fetlock	Yes	No	6 months $n = 20$	Group 1: 0/5 (0%) Group 2: 4/5 (80%) Group 3: 3/5 (60%) Group 4: 4/5 (80%)	
Broeckx et al. (40)	Pilot study	165	Group 1: allogenic blood-derived, native MSCs + PRP, $n = 49$ Group 2: allogenic blood-derived, chondrogenic induced MSCs + PRP, $n = 116$	Coffin (43) Pastern (34) Fetlock (58) Stifle (30)	No	One week after treatment 3 horses had moderate flare reaction	18 weeks Group 1: $n = 25$ Group 2: $n = 66$	Group 1: 11/25 (44%) Group 2: 32/66 (49%)	Group 1: 9/25 (36%) Group 2: 24/66 (36%)
Ferris et al. (18)	Prospective case series	33	Autologous bone marrow-derived MSCs	Stifle	No	3 horses with transient joint flare	24 months $n = 33$	11/33 (33%)	14/33 (42%)
Autologous blood products									
References	Type of study	Number of horses	Treatment and number of horses per group (*n*)	Treated joint	Control group	Adverse reactions	Time follow up, number of horses completed follow up (n)	Outcome: Training level/lame free	Outcome: Competition level
Fürst et al. (87)	Prospective randomized controlled trial	30	Group B: GOLDIC® gold-induced autologous-conditioned serum $n = 16$ Group A: betamethasone and hyaluronic acid $n = 14$	Coffin (9) Pastern (1) Fetlock (4) Carpus (8) Tarsus (4) Stifle (3) Shoulder (1)	Yes	Group B: 3/16 mild to moderate (lameness for 24 hours; increased swelling) Group A: 2/14 (joint flare after anesthesia)	> 6 months n group B = 16 n group A = 13	Group B: 3/16 (19%)	Group B: 10/16 (63%) Group A: 6/13 (46%)

(Continued)

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TABLE 1 (Continued)

Warner et al. (88)	Retrospective case series	26	ACS	Coffin	No	No	>2 years	4/26 (15%)	8/26 (31%)
Tyrnenopoulou et al. (89)	Placebo controlled study	15	PL	Coffin	Yes	No	1 year	0/10 (0%)	
Bembo et al. (90)	Preliminary clinical study	8	Combination of autologous micro-fat and PRP	Fetlock (7) Carpus (1)	No	No	5–10 months	7/8 (88%)	0/8 (0%)
Bertone et al. (75)	Prospective randomized masked placebo controlled clinical trial	40	APS	MCP (12) MTP (3) Carpus (6) Tarsus (1) Stifle (18)	Yes	No	52 weeks $n = 38$	17/38 (45%)	
Pichereau et al. (91)	Retrospective study	20	PC	Fetlock	No	No	1 year	2/20 (10%)	14/20 (70%)
Jöstingmeier (92)	Prospective study	54	Group 1: Na-Hyaluronat (Hylartil®) and Betamethasone (Celestovet®) $n = 27$ Group 2: ACS $n = 27$	Coffin	Yes	No	6 months	Group 1: 17/27 (63%) Group 2: 24/27 (89%)	
Carmona et al. (32)	Preliminary pilot clinical study	4	PC	Coffin (1) Fetlock (1) Tarsus (1) Stifle (1)	No	No	1 year	0/4 (0%)	

ACS, Autologous conditioned serum; APS, Autologous protein solution; IRAP, Interleukin 1- Receptor- Antagonist- Protein; IVP, Investigational veterinary product; MCP, Metacarpophalangeal joint; MSC, Mesenchymal stromal cells; MTP, Metatarsophalangeal joint; OA, Osteoarthritis; PC, Autologous platelet concentrate; PL, Platelet lysate; PRP, Platelet rich plasma.

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TABLE 2 Studies included in the systematic review demonstrating different source of bias.

References	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other source of bias
Broeckx et al. (86)	Low	Low	Low	Low	Low	Low	Low
Magri et al. (31)	Low	Low	Low	Low	Low	Low	Low
Broeckx et al. (77)	Low	High	High	High	Low	Low	Low
Broeckx et al. (40)	High	High	High	High	Low	Low	Unclear
Ferris et al. (18)	High	High	High	High	Low	Low	High
Fürst et al. (87)	Low	High	High	High	Low	Low	Low
Warner et al. (88)	High	High	High	High	Low	Low	High
Tyrnenopoulou et al. (89)	Low	High	High	High	Low	Low	Unclear
Bembo et al. (90)	High	High	High	High	Low	Low	Low
Bertone et al. (75)	Low	High	High	High	Low	Low	Low
Pichereau et al. (91)	High	High	High	High	Low	Low	Unclear
Jöstingmeier (92)	High	High	High	High	Low	Low	Unclear
Carmona et al. (32)	High	High	High	High	Low	Low	High

forest plot. Furthermore, the forest plot illustrates the extent to which the result from the individual study varies (96, 97). This variability is referred to as heterogeneity and is assessed by I^2 in the following meta-analysis. Heterogeneity was determined to be significant at $I^2 > 50\%$ or $p < 0.1$. A result was considered significant with $p < 0.05$.

2.8. Meta-analysis

In the meta-analysis, the results of lameness evaluation at different time periods of the studies were presented in the individual sections. In the short-term follow-up periods, one additional placebo-controlled and randomized trial was examined for better comparability (57). These will be discussed separately. All long-term studies are listed in the last row of the forest plot.

3. Results

3.1. Risk of bias

With all instruments that measure the risk of bias in clinical trials, it must be considered that they do not present an exact measurement method. Instead, it is an estimation in which the result always contains a subjective component. The purpose is to compare similar and homogenous treatment groups affected only by random variabilities (75).

All studies in the systemic review were assessed against the listed seven criteria and classified as high, low, or unclear risk (Table 2). The traffic light system (Figure 1) was used to illustrate the overall risk achieved by each study. Six studies avoided selection bias by randomly assigning participants (31, 75, 77, 86, 87, 89). Secrecy of the randomization scheme and blinding of veterinarians and patient owners was met by only two studies, both demonstrate a low risk of bias (31, 86). Blinding of treatment was achieved by generating two groups of examining and dispensing veterinarians at both study sites and owner's absence at administering the agent (86). For comparison, in the other study, the syringe was blinded so that owners and veterinarians did not know which treatment regime was selected. Blinding was maintained throughout the entire duration of the study (31).

All studies in the systematic review reported study discontinuations and missing outcome data. Therefore, almost all studies were considered to have a low risk of incomplete results and selective reporting. In addition, most studies used an owner questionnaire for long-term follow-up. In summary, eleven studies are at a high risk of bias (18, 32, 40, 75, 77, 87–92), due to the lack of blinding. The risk of bias graph shows the authors' assessment of each item in percentage (Figure 2). Overall, <25% of the studies included in the systematic review were found to be at a low risk of bias.

3.2. Systematic review

The flowchart (Figure 3) shows the detailed systematic analysis after initial electronic and manual research, with a total of 271 studies. This resulted in 28 studies being assessed for the qualitative synthesis after an initial review. These studies were further assigned to the defined orthobiologic therapies when treatment of equine OA was the scientific focus (Table 3). Following this, the biologic cell source of the selected 28 studies was assessed and listed

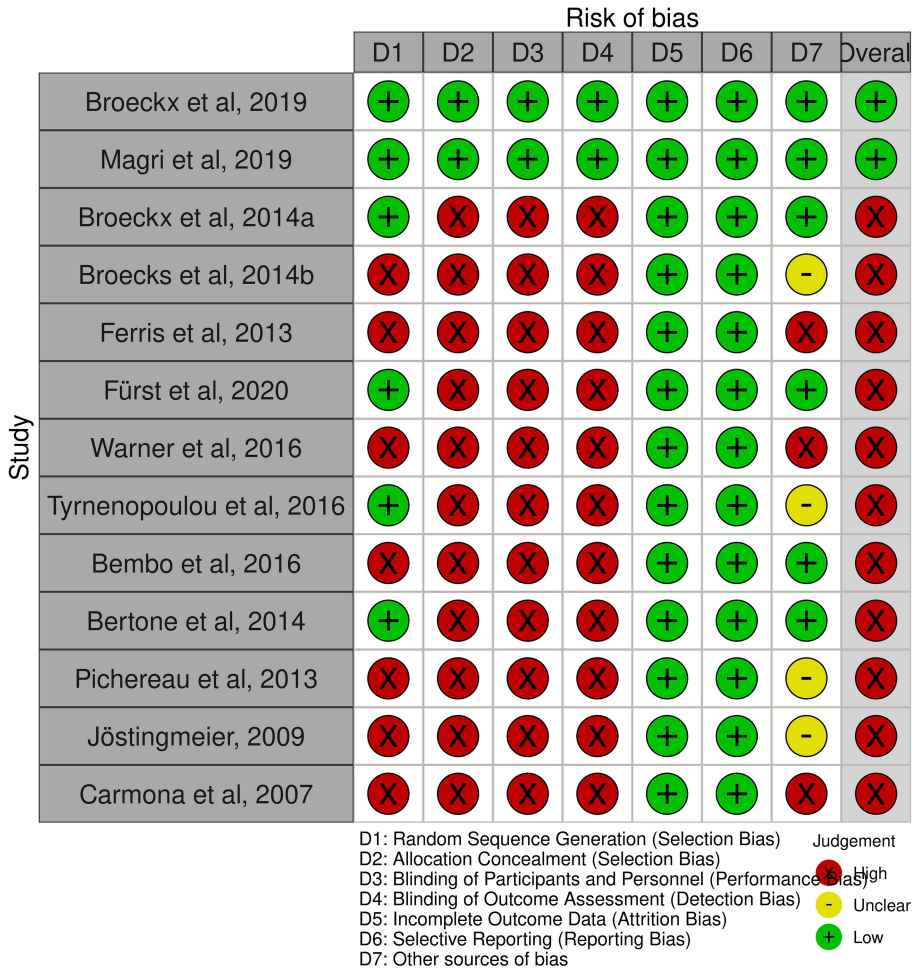

FIGURE 1
Summary of risk of bias (98).

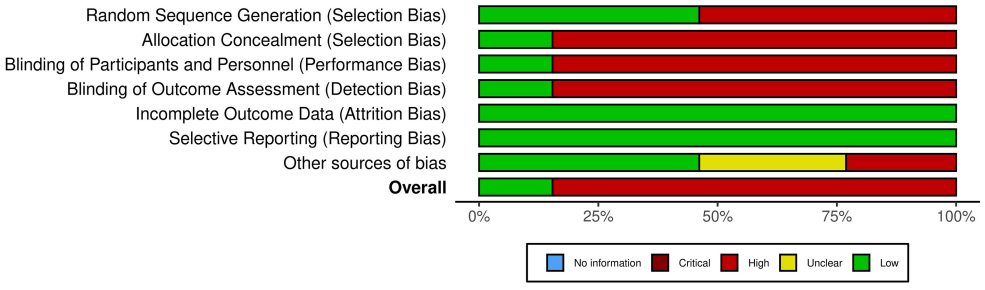

FIGURE 2
Risk of bias (98).

(Table 4). This states, that based on their sources of orthobiologic therapeutics, 4 studies were included using blood-derived MSCs either non-induced (native) or chondrogenic-induced (40, 57, 77, 86), 5 studies focused on bone marrow-derived MSCs (18, 29, 99–101) from which 2 studies also included MSCs derived from adipose tissue for comparison (100, 101). Five studies included adipose tissue-derived MSCs only (27, 55, 100–102). The effect of umbilical cord-derived MSCs were studied in 2 publications (31, 61). Eight studies describe the use of cell-based autologous blood products as orthobiologic therapeutics (19, 32, 75, 89–91, 103, 104) and 6 studies a cell-free final therapeutic product (30, 81, 87, 88, 92, 105). The following results were obtained: 8 studies used MSCs as a therapeutic agent for naturally occurring OA (18, 31, 40, 55, 61, 77, 86, 102); 6 studies examined the effect of MSCs after inducing

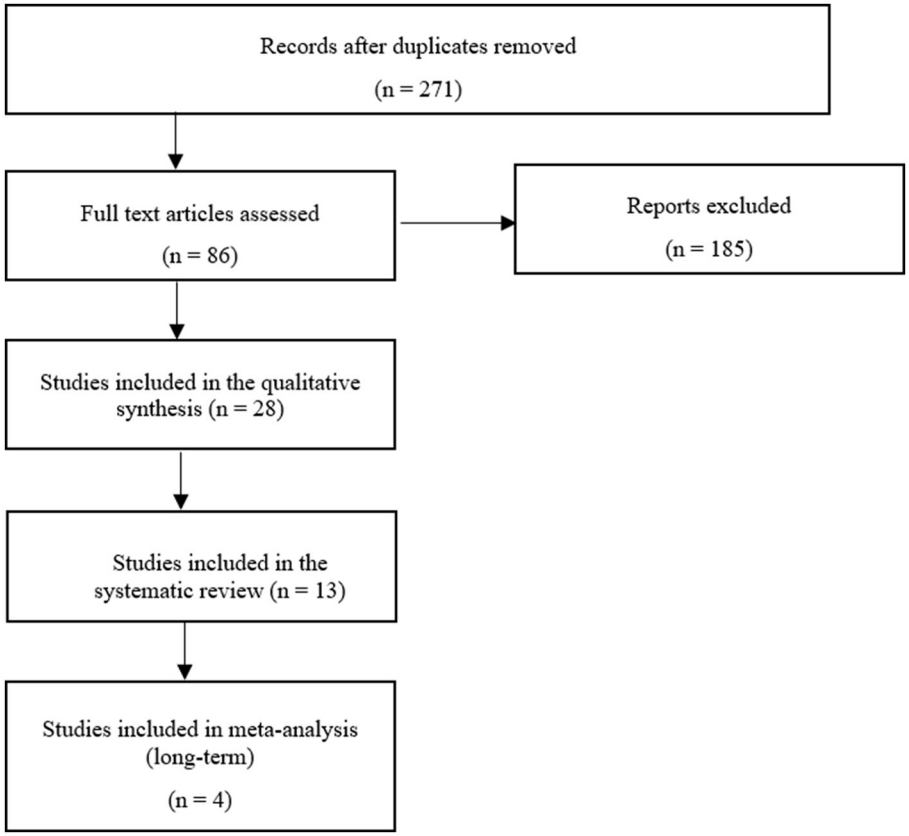

FIGURE 3
Flow chart showing the methods used for the systemic search (93).

TABLE 3 Numbers of studies from qualitative synthesis including naturally occurring OA compared to induced OA, trials with a placebo group, and studies with a follow-up time over 6 months.

Product	Naturally occurring OA	Induced OA	Placebo	Outcome > 6 months
Mesenchymal stromal cells (MSC)	8	6	12	7
Autologous blood products	13	1	7	8
Total number qualitative synthesis	28			

OA, Osteoarthritis.

OA (27, 29, 57, 99–101); 14 studies treated with autologous blood products, with 1 study inducing OA (30) while the remaining studies examined naturally occurring OA (19, 32, 75, 81, 87–92, 103–105). Of the 14 MSC-related studies, 12 were placebo controlled (27, 29, 31, 55, 57, 61, 77, 86, 99–102) and 7 studies had an outcome with patient follow-up at least 6 months after treatment initiation (18, 27, 29, 31, 77, 86, 99). Comparatively, of the 14 groups treated with autologous blood products, 7 were placebo controlled (19, 30, 75, 87, 89, 92, 103) and 8 studies had a long-term follow-up (32, 75, 87–92). After screening the studies with the specified inclusion and exclusion criteria, 13 studies were examined for systematic analysis, and listed in Table 1 (18, 31, 32, 40, 75, 77, 86–92).

Table 5 lists all 15 studies from the quantitative synthesis that were not included in the systematic review due to the lack of information on the individual degree of lameness. Therefore, an average value was given for the whole group. Other reasons for exclusion were short observation periods, induced OA, or an overall too short observation time.

The age, sex, breed, and disposition of the horses selected for the investigations varied among the studies. Most of the trials in the systematic review examined the effect of orthobiologic therapeutics for the coffin or fetlock joint (Table 1). Five of the 13 studies treated naturally occurring OA with MSCs (18, 31, 40, 77, 86), and 1 study subdivided the treatment groups into f4 subgroups (PRP; native MSCs, native MSCs with PRP; chondrogenic-induced MSCs with PRP) (77). This study used allogenic peripheral blood as MSC source and labeled isolated, non-induced MSCs as "native." Due to lack of placebo-controlled studies, the PRP-subgroup was compared with the MSC-subgroups of different sources in combination with PRP in the following meta-analysis. The remaining studies treated horses with non-induced ("native") MSCs, chondrogenic-induced MSCs, umbilical cord-derived MSCs and bone marrow-derived MSCs. None of the

TABLE 4 Numbers of studies from qualitative synthesis demonstrating the source and composition of orthobiologic therapeutic agents.

Product	Blood-derived	Bone marrow-derived	Umbilical cord-derived	Adipose tissue-derived	Blood cell-based	Blood cell-free
Mesenchymal stromal cells (MSCs)	4 (40, 57, 77, 86)	5 (18, 29, 99–101)	2 (31, 61)	5 (27, 55, 100–102) +2 (100, 101)		
Autologous blood products					8 (19, 32, 75, 89–91, 103, 104)	6 (30, 81, 87, 88, 92, 105)

MSCs, Mesenchymal stromal cells.
Two studies (100, 101) are double reported due to the use of both, bone marrow-derived and adipose tissue-derived MSCs.

5 reviewed studies using adipose-derived MSCs as orthobiologic agent met the inclusion criteria for meta-analysis (27, 55, 100–102). In general, all MSC-studies demonstrated a heterogenous group regarding manufacturing and processing methods of the particular cell source. Considering possible side effects, 3 studies observed a mild to moderate inflammatory response after intra-articular treatment with MSCs (18, 31, 40). At the final examination, all patients felt well. Therefore, no general side effects were concluded.

Eight of the 13 studies examined treatment outcomes with autologous blood products. Of these, 3 studies used autologous conditioned serum (ACS) products (87, 88, 92). The remaining studies used cellular autologous blood products with a high platelet-rich content (32, 75, 89–91). Mild side effects such as self-limiting local swelling and lameness were noted in 1 ACS study (87).

Concerning the post-treatment, every study designed a particular rehabilitation program. All horses received a 1- (86, 89) to 8-week (31) hand-walking program at the end of treatment, followed by individual retraining. Most studies graded the severity of lameness according to the AAEP (American Association of Equine Practitioners) scoring system (18, 32, 75, 77, 86–90).

Although several placebo-controlled studies were included, most of them lack long-term follow-up or control was not maintained throughout the entire duration of the study. For example, in one RCT, horse owners in the placebo group were offered treatment with autologous protein solution (APS) 14 days after the placebo treatment. The randomized controlled study was well-structured, but the observation time of the control group was too short to be included in our meta-analysis. The APS group improved significantly after the treatment compared with baseline or control group scores (75). In total, 5 studies were placebo controlled over the entire observation period, 4 of which were randomized (77, 86, 87, 89). These studies have also been included in the meta-analysis.

In summary, an average of 65% improvement in lameness grade was achieved after the treatment with intra-articular applied orthobiologic therapeutics, regardless of which therapeutic agent was used (Figure 4). Eleven studies showed a general positive effect after treatment, with horses working at trainings level or horses returning to competition (Table 1). Two outliers could be detected, that showed initial improvement in the first 7–8 months after treatment but then returned to their initial degree of lameness (32, 89). In both studies, the majority of horses responded positively at the beginning and maintained their high level of performance over a period of at least 6 months. Furthermore, horses showed no adverse reactions. This outcome suggests that platelet lysate (PL) and autologous platelet concentrate (PC) can be an efficient short-term therapy for horses suffering from OA (32, 89) (Figure 5). Looking at the average proportions without outliers, 80% of the horses involved in the studies showed lameness reduction after treatment with orthobiologic therapies (Figure 5).

As previously stated, in 1 study treatment groups were divided into 4 subgroups, and interestingly, the group treated with chondrogenic induced MSCs had the most successful result, with 80% lameness-free horses and horses working at trainings level (77). Another pilot study compared non-induced (native) MSCs with chondrogenic induced MSCs, both in combination with PRP. This resulted in a higher average score for the beneficial effects using chondrogenic induced MSCs. However, the result was statistically non-significant (40).

Promising results with MSCs and ACS were justified over a 24-months follow-up period (18, 88). After intra-articular administration of MSCs postoperatively after arthroscopy of the stifle, 42% of horses returned to their previous level of work, and 33% returned to work after a mean follow-up period of 24 months (18). In a retrospective study from Warner et al. 31% of the horses returned to their previous level of work and 15% performed at exercise level after a period of at least 2 years following the ACS treatment of the coffin joint (88). Both studies were not blinded and without a control group, which significantly limits their validity. However, both studies provide indication of a long-term effect of MSC and ACS treatment in OA.

3.3. Meta-analysis

Four RCTs (77, 86, 87, 89) out of the 13 trials were included in the long-term meta-analysis with a follow-up time >6 months. The control groups were treated with saline (86, 89), other orthobiologic therapeutic agents (PRP) (77) or corticosteroids and hyaluronic acid (87). All studies included horses of different breed, sex, age, and level of performance. Moreover, the diagnosed and treated OA occurred in different joints, ranging from low to high motion joints (Table 1). Due to the scarcity of studies, no restrictions were made.

Figure 6 demonstrates a forest plot with outcomes at different time points. The focus was the set inclusion criteria of a follow-up period >6 months. Three studies (77, 86, 87) showed a positive

TABLE 5 Studies from literature review that are not included in the systematic review because of incomplete data referring to the set inclusion criteria.

References	Title	Exclusion criteria
McIlwraith et al. (29)	Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects	Defects arthroscopically created; second-look arthroscopy at 6 months; lameness effects were not reported for each horse individually
Mariñas-Pardo et al. (55)	Allogeneic adipose-derived mesenchymal stem cells (Horse Allo 20) for the treatment of osteoarthritis associated lameness in horses: characterization, safety and efficacy of intraarticular treatment	Follow up 90 days; lameness effects were not reported for each horse individually
Frisbie et al. (101)	Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis	Osteoarthritis was induced arthroscopically; follow up 70 days; lameness effects were not reported for each horse individually
Broeckx et al. (57)	The use of equine chondrogenic-induced mesenchymal stem cells as a treatment for osteoarthritis: A randomized, double-blinded, placebo-controlled proof-of-concept study	Osteoarthritis was induced using an osteochondral fragment-groove model; follow up to week 11 *Study was included in the meta-analysis as short-term study due to the randomized, double-blinded, placebo-controlled study design*
Frisbie et al. (100)	Evaluation of bone marrow derived stem cells and adipose derived stromal vascular fraction for treatment of osteoarthritis using an equine experimental model	Osteoarthritis was induced arthroscopically; follow up 8 weeks; lameness effects were not reported for each horse individually
Pradera Muñoz (61)	Efficacy and safety study of allogeneic Equine Umbilical Cord derived Mesenchymal-Stem Cells (EUC-MSCs) for the treatment of clinical symptomatology associated with mild to moderate degenerative joint disease (osteoarthritis) in horses under field conditions	Follow up 63 days; many data were lost during the 2 years follow up; *Study included in the meta-analysis as short-term study due controlled, blinded, randomized study design*
Mirza et al. (104)	Gait Changes Vary among Horses with Naturally Occurring Osteoarthritis Following Intra-articular Administration of Autologous Platelet-Rich Plasma	Horses did not respond to intra-articular anesthesia with a consistent pattern of gait changes as expected from responses; lameness effects were not reported for each horse individually
Frisbie et al. (30)	Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis	Osteoarthritis was experimentally induced; follow up 70 days; lameness effects were not reported for each horse individually
Weinberger (105)	Klinische Erfahrungen mit der Anwendung von ACS/ORTHOKIN/IRAP beim Pferd Clinical experience with the application of ACS/ORTHOKINE/IRAP in horses	Not placebo controlled; follow up time 12 weeks
Nicpoń et al. (102)	Therapeutic effect of adipose-derived mesenchymal stem cell injection in horses suffering from bone spavin	Insufficiently detailed case numbers about working at trainings level or success rate
Abellant et al. (103)	Intraarticular platelet rich plasma (PRP) therapy evaluation in 42 sport horses with OA	Publication in IVIS only; not published in a peer reviewed journal
Smit et al. (19)	Clinical findings, synovial fluid cytology and growth factor concentrations after intra-articular use of a platelet-rich product in horses with osteoarthritis	Follow up 56 days; no lameness evaluation because due to unforeseen external factors
Barrachina et al. (99)	Assessment of effectiveness and safety of repeat administration of proinflammatory primed allogeneic mesenchymal stem cells in an equine model of chemically induced osteoarthritis	Follow up 6 months; lameness effects were not reported for each horse individually
Marques-Smith et al. (81)	Is clinical effect of autologous conditioned serum in spontaneously occurring equine articular lameness related to ACS cytokine profile?	Follow up mean 48 days; lack of control
Yamada et al. (27)	Mesenchymal stem cells enhances chondral defects healing in horses	Experimentally induced OA; lameness effects were not reported for each horse individually

impact of orthobiologic therapeutics compared to their control groups.

One study reported a regression to its initial lameness level after an observation period of 1 year (89). In this study, no side effects were noted in the first 6 months after treatment and 9 out of 10 horses treated with PL returned to their normal activity. Lameness recurred from the 7th month, and all horses relapsed to their initial degree of lameness at the end of the study period. This study illustrates the correlation between duration of follow-up and recurrence of lameness. Within 6 months, horses returned to their previous level of performance. However, all horses relapsed to their initial degree of lameness, therefore only a temporary positive effect could be observed.

The forest plot illustrates the common effect model and the random effects model and whether heterogeneity could be stated as significant. Data demonstrating $I^2 > 50\%$ were assigned to the

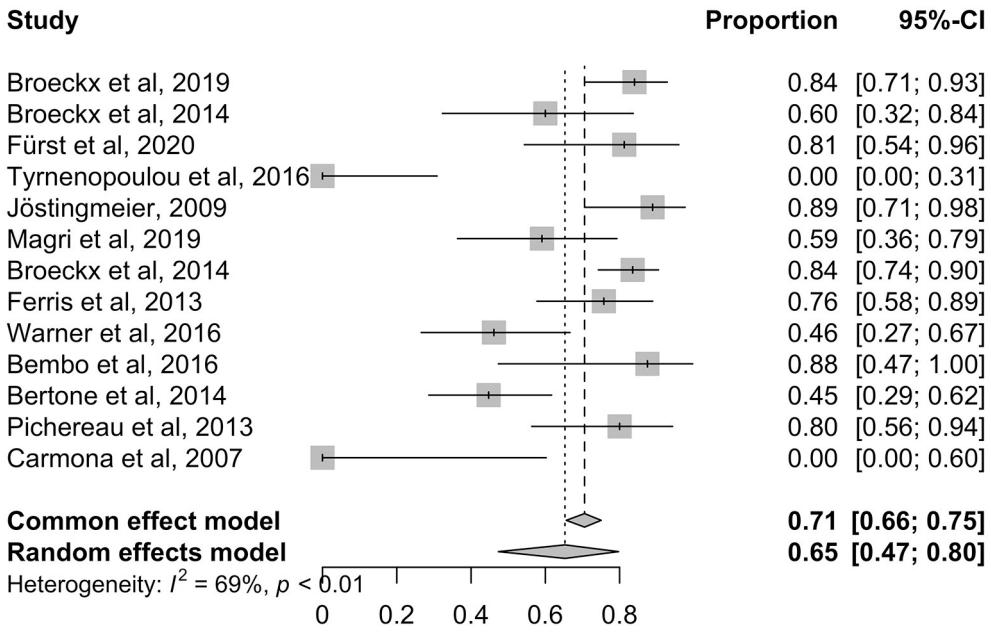

FIGURE 4
Shows the ratios of the individual studies in the systematic analysis graphically with outliers.

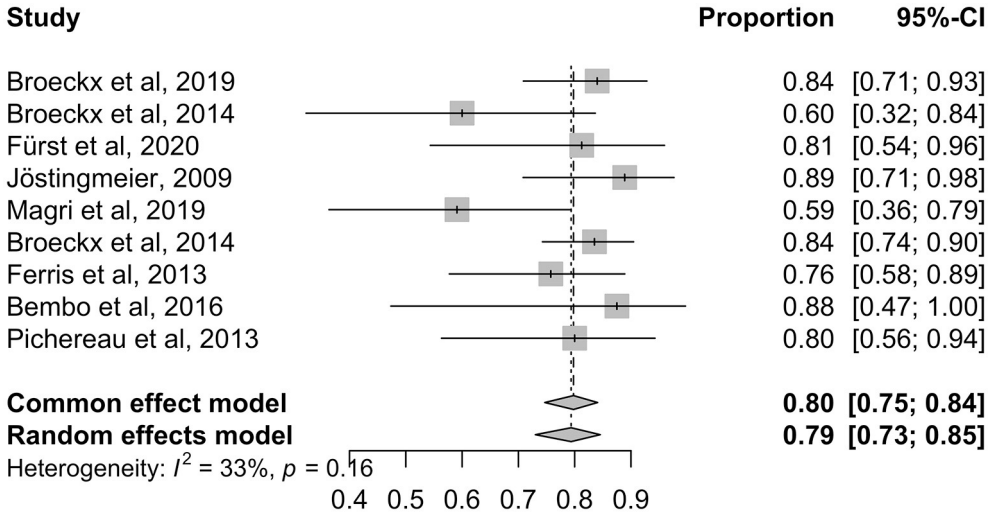

FIGURE 5
Shows the ratios of the individual studies in the systematic analysis graphically without outliers.

random effects model. As demonstrated in the last row of the plot, all long-term follow-up studies showed moderate heterogeneity with $I^2 = 55\%$ and $p = 0.11$ (Figure 6). A random effects model was used due to the assumption of moderate differences among study design and implementation in the clinical studies.

An odds ratio (OR) of 1 indicates no difference between the treatment and control group, whereas an OR > 1 indicates that lameness is more likely to be reduced in the experimental group. All studies with an OR values higher than 1 favor the experimental group (Figure 6: OR 17.02; 95% CI: 8.5474 to 33.8849 $p < 0.0001$). None of the studies crossed the line into ineffectiveness, suggesting that the treatment effect was estimated to be similar across studies.

The diamond square represents the average of all individual studies. If the limit of ineffectiveness is not exceeded, a significant difference in lameness reduction between the experimental and control groups is stated. It can be summarized, that the included orthobiologic therapeutics are safe and showed significant improvement in lameness reduction compared to their control groups. Three studies (77, 86, 87) showed a long and constant improvement over 6 months.

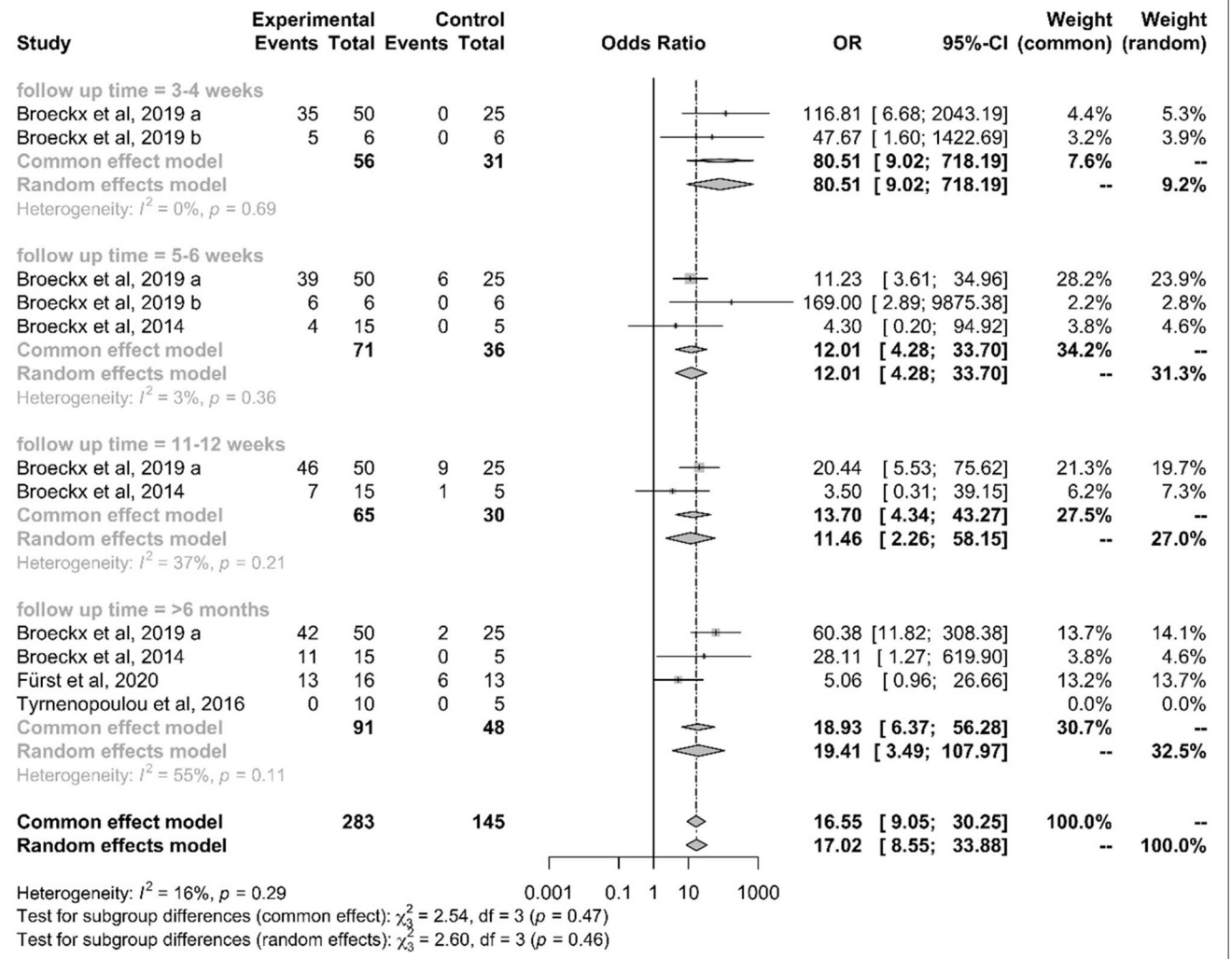

FIGURE 6
Forest plot showing results of selected studies using a meta-analysis to compare lameness reduction of experimental and control. The common effect model and the random effects model are shown. Depending on heterogeneity (I^2 > 50%) the random effects model was used for studies with long-term follow-up. The greater the squares, the more participants included the study. The size of the squares is proportional to the weight of the study. The whiskers correspond to the 95% confidence interval (CI).

One short-term RCT was included in the forest plot for comparative reasons (57). We assessed at what time point the trial showed significance for treatment with an orthobiologic therapy and how effective the short-term trial was. Treatment success with chondrogenic induced MSCs in an induced OA model was demonstrated to be a time dependent factor, with decreasing lameness levels from 2 weeks after treatment throughout the observation period of 11 weeks (57).

In summary, the use of intra-articular administered orthobiologic therapeutic agents show an incidence of lameness reduction by 73% compared to the control in the long-term follow-up, whereas in the control group lameness was reduced by 17% (77, 86, 87, 89). According to the included studies, horses with naturally occurring OA demonstrated a significantly reduced degree of lameness after intra-articular treatment with orthobiologic therapeutics compared with the control in the long-term follow-up.

3.4. Publication bias

Publication bias occurs when the probability of a study being published depends significantly on its outcome. This means that it is more likely, that a study will be published if the results are consistent with the hypothesis or if the study results are significant (106).

The occurrence of publication bias can be tested by creating a funnel plot. Ideally, the individual data points form a symmetrical, inverted funnel. On the x-axis, the treatment effect is plotted against the study size on the y-axis. The largest studies are located at the top of the graph and plotted near the average. The smaller studies are distributed on both sides of the average and lie close to the x-axis.

The funnel plot showed almost the desired symmetrical shape, with the studies close to the midline. It is important to note, that studies that conducted lameness examinations at different time points are considered as individual studies. For instance, a study

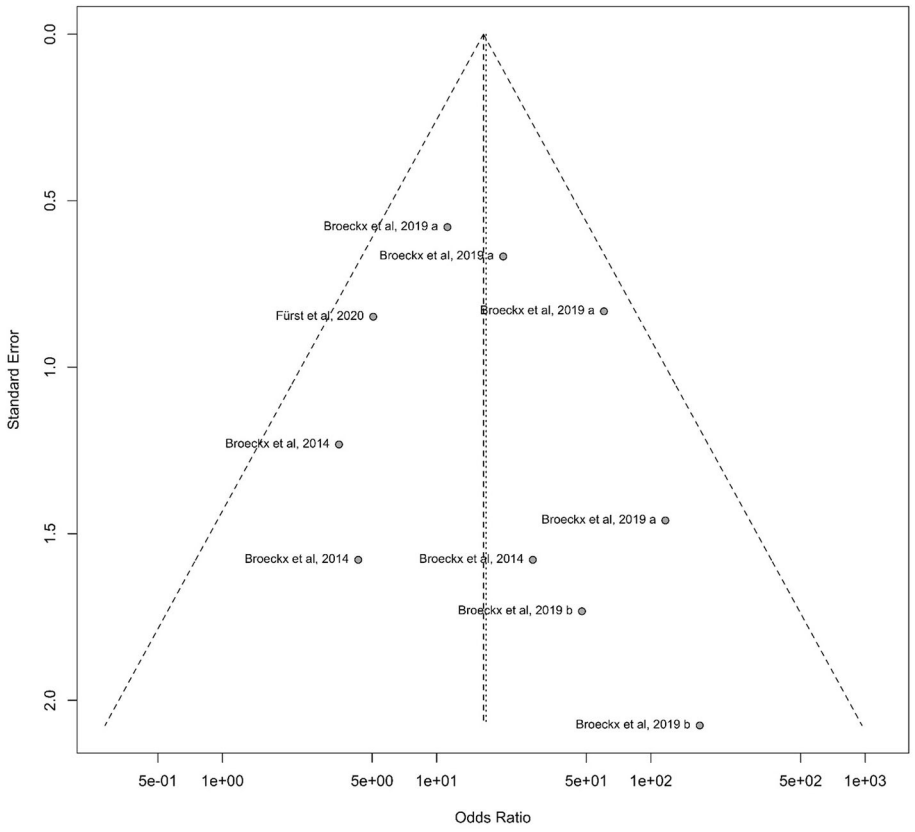

FIGURE 7
Funnel diagram demonstrating the standard error to the odds ratio according to the study design of participating horses. On the x-axis, the treatment effect is plotted against the study size on the y-axis. Largest studies are located at the top of the graph and plotted near the average. Smaller studies will spread on both sides of the average and lie close to the x-axis.

by Broeckx et al. (57) is plotted 4 times in the funnel plot, at each study time point. The hypothesis that studies with a smaller number of participants are more likely to be in the bottom range is correct. Overall, the publication bias can be classified as low (Figure 7).

4. Discussion

OA is a leading cause of pain, disability and economic impact on the health system worldwide (3, 107). The demand for regenerative medicine to treat OA is steadily increasing in human and veterinary medicine. Therefore, it is important to obtain an up-to-date state of knowledge and to compare previous studies using meta-analysis (108, 109). There are two main reasons why the equine model is a suitable model for human medicine. First, horses spontaneously develop chondral defects and age-/trauma-induced OA that are very similar to humans (15). Second, there are numerous *in vitro* and *in vivo* studies, some even with experimentally induced OA, in which the therapeutic index of orthobiologic therapeutics can be assessed (109).

To our knowledge, this is the first meta-analysis comparing orthobiologic therapies with its control group in long-term *in vivo* studies for the treatment of OA. Overall, many topic-related articles were recorded, but of the 86 articles fully screened, only 13 (15%) were useful for the systematic review after passing the inclusion and exclusion criteria. Finally, only 4 (5%) of these studies could meet the criteria for the meta-analysis (Figure 3). This shows that although there is a great research interest in this topic area only a few studies examine long-term success compared with a control group. The result of this meta-analysis showed comparable studies with a moderate heterogeneity, which overall demonstrate a positive result in terms of orthobiologic therapy (Figure 6). By demonstrating the therapeutic efficiency of the mentioned therapies in the long-term in clinical cases of OA, the application of such therapeutics in equine veterinary practice is justifiable.

Major limitations were, that the number of comparable studies that met the inclusion criteria were low. Most studies suitable for systematic review lacked a control group. Another shortcoming was the absence of a uniform treatment pattern in the controlled trials. All controlled studies treated with different placebos [saline (86, 89), other potentially regenerative agents (77), cortisone and hyaluronic acid (87)]. Compared to other meta-analysis and systematic reviews, the lack of an adequate placebo group was also the main point of criticism (70, 110). From an animal welfare perspective, it is unethical to not treat animals suffering from joint-related pain. Moreover, it is difficult to find a homogenous control group, in which all horses are treated with the same agent. However, it is almost impossible to convince horse owners to participate in a long-term study without them knowing whether they will be receiving a placebo or a treatment. Especially, since there is a real

chance that their horse will miss out on a potential therapy. In general, all privately owned horse owners wanted to be assured that everything was being done to get the horse well and back to work.

The lack of blinded study designs in RCTs is noticeable. Overall, only 2 long and 2 short-term studies were fully blinded (31, 57, 61, 86). Reasons for this include the high effort of blinding all medical staff and owners. In addition, it is often difficult to obtain permission from horse owners for placebo-controlled and blinded study participation for the entire study duration. The absence of blinding is often associated with excessive reasoning, especially when assessing subjective outcomes (111, 112). The lack of blinding is the main reason for the high risk of bias.

Another serious point of criticism is the difference of the joint localization. Due to the lack of studies, no restriction was made here, and all long-term studies could participate, regardless of the joint in which the OA occurred. The emphasis was placed on lameness reduction in a long-term follow-up. Of course, from a medical point of view, there is criticism on the comparability of the individual joints. No distinction was made between chronic or acute OA, mild or advanced OA. The absence of a homogenous concept shows the need for further studies. To avoid heterogenicity, a meta-analysis with naturally occurring chronic OA in the same joints would be useful.

The systematic analysis showed a positive result of 80% in all studies, except for the two outliers. In other words, over 80% of the horses treated with orthobiologic therapies showed a reduction in their degree of lameness. Lameness evaluation was uniformly investigated in 9 studies using the AAEP score (18, 32, 75, 77, 86–90); the other studies used their own clinical scores. In most studies, the endpoint survey was conducted using an owner survey. Although the owners' assessment is subjective, comparability can be established because health status and degree of lameness are collected in relation to the performance level before and after treatment.

Two short-term studies were double-blind, randomized, and placebo-controlled with a low potential for bias. This showed that a very safe study design is possible in studies with a shorter control period, as blinding can be maintained (57, 61). In summary, significant lameness improvement with orthobiologic therapy was observed in both groups from the 2nd (57) and 5th (61) week after treatment. In these models, accurate experimental design and maintenance of blinding is facilitated. However, most animal models are limited to a period of 8 to 12 weeks (Table 5). In addition, many studies reported only an average or mean values for lameness evaluation. Individual results are usually missing here (30, 57, 100, 101). Due to a missing randomization scheme and blinding, many studies show a high potential for bias.

Overall, moderate heterogeneity among the studies in the meta-analysis has been described. All product- and treatment-specific factors mentioned above have an unknown impact on treatment success. The aim of this study is to draw attention to the importance of a correct study design. The results indicate a significant improvement with orthobiologic therapies compared to their control for at least several months. However, due to the paucity of studies with long-term and placebo-controlled follow-up, no concrete statement can be made regarding effectiveness of specific orthobiologics, exemplary the preference of MSCs to autologous blood products and vice versa. However, equine practitioners can rely on a safe and effective treatment option when using orthobiologics but thereof no recommendation regarding specific products can be derived. In the future, more randomized, controlled, blinded studies and long-term studies are needed to make further informed conclusions. It is crucial to determine the exact composition and effect of all orthobiologic therapeutics in further studies to develop effective and standardized treatment protocols.

5. Conclusion

Apart from the limited and sometimes controversial findings, the systematic review and meta-analysis showed an overall support toward the orthobiologic therapeutic application. After treatment with orthobiologics, a beneficial effect on OA was demonstrated without significant adverse effects. Satisfactory effects were examined over a period of 6–12 months, with a high success rate. Limitations lie within the lack of homogeneous standardization protocols and outcome measurements. Future studies should focus on standardized study designs regarding patient details, treated joints and type of orthobiologic substances in RCTs to allow comparable conclusions about the long-term effect of intra-articular administered orthobiologic therapeutics.

Author contributions

AM, AT, YZ, and SR constructed the manuscript. AT, SR, and WB edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Cytokines in equine platelet lysate and related blood products

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In equine medicine, the use of regenerative therapeutics has gained growing attention, but is still a new and complex field with room for improvement. Platelet lysate (PL) can be used as therapeutic agent but is also a promising supplement for the culture of multipotent mesenchymal stromal cells. To enable a targeted use of PL both in clinic and laboratory, it is crucial to learn more details on its effective ingredients. While so far, mainly growth factor components have been analyzed in platelet-based products such as PL, the current study focuses on the content of cytokines in serum, plasma, platelet concentrate and PL. Blood was harvested from 20 clinically healthy horses and subjected to blood count and chemistry analysis, as well as to further processing to PL. Plasma and platelet concentrate were produced by a buffy-coat-based method and PL was produced from the concentrate by freeze-thawing. Samples from each horse were analyzed regarding interleukin (IL)-1β, −4, −6 and −10, interferon-γ and tumor necrosis factor-α concentrations using sandwich ELISAs. Cytokine concentrations in serum, plasma, concentrate and PL were similar and correlated significantly. However, there was a large inter-individual variability in cytokine concentrations between the different donor horses. The samples from some donor animals had overall very high cytokine concentrations, while samples from other donors had no measurable cytokine ingredient. This pattern was observed for all cytokines. There was a noticeable link between high cytokine concentrations in the blood products and abnormal findings in blood chemistry. Cytokine concentrations in samples from horses with abnormal findings were significantly higher than in samples from the remaining horses. The interindividual differences in cytokine concentrations could be highly relevant when using PL for therapy and cell culture, as the mode of action of the PL is likely changed depending on the presence of pro- and anti-inflammatory cytokines. Blood chemistry might be useful to predict cytokine concentrations in blood products.

KEYWORDS

platelet lysate, equine, IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-10

1. Introduction

Platelets play a crucial role in hemostasis and contain various cytokines, chemokines and growth factors, which are released after activation (1). These messenger substances cause a further release of soluble mediators, initiating signaling cascades e.g., for inflammation regulation, angiogenesis or tissue regeneration (2, 3). Besides their biological functions in the body, platelets are being harvested for biomedical purposes, which includes their use in regenerative medicine in the form of platelet concentrate or platelet lysate (PL).

Platelet concentrate is mainly being used for therapeutic purposes (4, 5). However, when further processed to PL by using freeze-thaw cycles to disrupt the platelets (6, 7), their contents are released (6, 8–10), and shelf life and storability of the blood product are improved (11). Therefore, PL can not only be used as a direct therapeutic agent for various indications such as tendon disorders, corneal defects or to support wound healing in horses and humans (12–14), but also as a cell culture supplement (15) which can replace the ethically critical fetal bovine serum (16, 17).

PL as cell culture supplement is particularly useful for the culture of multipotent mesenchymal stromal cells (MSC), which are being explored as therapeutic agent in their own right. MSC culture with addition of PL not only preserves their proliferation and basic properties, such as plastic adherence, expression of specific surface antigens, and trilineage differentiation potential (18), but also has a positive impact on their efficacy. Overall, PL supports the unfolding of diverse biological activities of MSC so that they can exert proregenerative, anti-inflammatory, antifibrotic, and immunomodulatory effects (10). Accordingly, we have previously demonstrated that equine PL, produced by a scalable buffy-coat method, supports equine MSC proliferation and increases their pro-angiogenic potency (19). However, it has not been fully elucidated which PL components contribute to these beneficial effects.

To date, PL has been analyzed predominantly regarding its growth factor contents, while the possible presence of inflammation mediators in platelet-based blood products has been widely disregarded so far. This is surprising, considering the discussion on platelet rich plasma leukocyte contents and considering that the mode of action of other blood products such as conditioned serum relies on (anti-)inflammatory mediators. Knowledge on the cytokine ingredients of platelet-based blood products would be tremendously helpful for their targeted therapeutic use (20, 21). Moreover, the presence of cytokines in PL could play a crucial role for MSC culture, as it could affect their mode of action, for example by inflammatory licensing (22).

To close this gap of knowledge, in this study, we aimed to characterize and quantify different cytokines in equine PL. We show that the cytokine levels vary heavily between individual horses and that abnormal findings in blood chemistry might be indicative of high cytokine levels.

2. Materials and methods

2.1. Donor health status

Blood was collected from 20 horses (4–15 years; 14 mares, 5 geldings, 1 stallion) as approved by the responsible authority (regional council Giessen, A14/2019). Beforehand, the donor health status was evaluated by general clinical examination and only animals that were free of abnormal clinical findings were included. In addition to the whole blood intended for platelet lysate

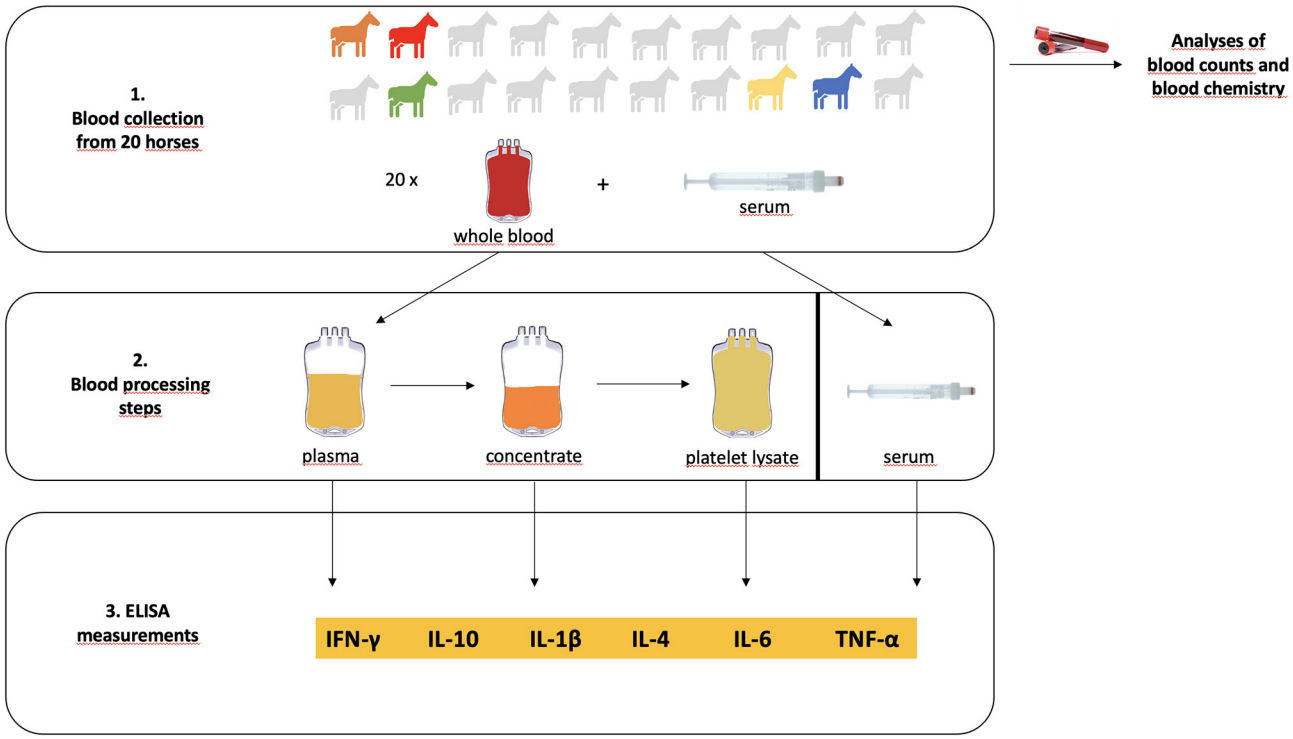

FIGURE 1
Overview of the study design. Blood was collected from 20 clinically healthy horses, of which 5 turned out to have mildly to moderately abnormal findings in blood chemistry (designated by the colored icons). Whole blood was processed to platelet lysate. Serum, plasma, concentrate and platelet lysate were then subjected to ELISA measurements of their cytokine contents.

production, blood was drawn into EDTA and lithium heparin as well as serum blood collection tubes and analyzed in the laboratory. This included complete blood counts and blood chemistry analysis using an ADVIA 2120i with Multispecies software MS 5.9 (Siemens Healthcare GmbH, Erlangen, Germany). With the latter, electrolytes, urea, creatinine, total protein, albumin, globulins, bilirubin, alkaline phosphatase, glutamate dehydrogenase, γ-glutamyltransferase, aspartate aminotransferase, creatine kinase and lactate dehydrogenase were measured.

2.2. Blood processing

Whole blood was collected and processed to produce PL as described in detail previously (6). Briefly, using a buffy-coat based approach, platelet concentrate was produced from whole blood collected in CPD-loaded blood collection bags by centrifugation and blood separation steps. The PL was then produced from this concentrate by freeze-thaw cycles, centrifugation and filtration. Samples from plasma, platelet concentrate and PL were frozen and stored at −80°C for cytokine analyses. Additionally, serum samples were handled accordingly.

2.3. Cytokine measurements

Cytokine concentrations were analyzed in serum, plasma, platelet concentrate and PL from each horse using Equine DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) for IFN-γ, TNF-α, IL-1β, IL-4, IL-6 and IL-10. The plates were read in an Infinite F50 plate reader and raw data were processed with the corresponding Magellan software (Tecan Ltd., Maennedorf, Switzerland).

First, different serum dilutions were tested for all horses in order to establish the experimental setup. The results showed considerable inter-individual differences in cytokine concentrations demonstrating the need to adjust the dilution factor for each donor. To estimate the possible influence of serum matrix effects in different serum dilutions, spiking experiments were performed. For this purpose, serum from horses with no detectable cytokine content was pooled and a dilution series (1:1, 1:10, 1:100, 1:1,000 in RD buffer) was prepared. These diluted serum samples were then spiked with cytokine standard, corresponding to the second highest concentration of the assay standard curve for each respective cytokine. The spiked samples were subjected to the remaining assay procedure according to the manufacturer's instructions.

The cytokine measurements in all blood products were performed for all cytokines using the ELISA assays according to the manufacturer's instructions. Blood product dilutions were adjusted individually, aiming to use the highest dilutions yielding results within the standard curve ranges in each case. Nevertheless, some samples had to be measured with no or low dilution. Samples with no detectable cytokine content in undiluted samples were assigned the value 0 for graphical presentation. The overall study design is shown in Figure 1.

2.4. Statistical analysis

Statistical analyses and graphical presentation of data were performed using IBM SPSS Statistics 26. Based on the results of the spiking experiment, we considered that the cytokine concentrations measured in blood product samples with low to medium cytokine content might be underestimated to some extent, as these samples could not be diluted enough to overcome the anticipated matrix effects. Therefore, for the statistical comparisons, cytokine concentrations were transformed into categories to circumvent calculations with inaccurate values. Category 1 corresponds to no detectable cytokine content, category 2 corresponds to the clusters with low to medium cytokine content, and category 3 reflects the clusters with high cytokine content. Non-parametric tests for paired samples were run to compare the different blood products, and non-parametric tests for unpaired samples were run to compare the donors with physiological blood chemistry and those with abnormal blood chemistry findings. In addition, possible correlations were evaluated according to Spearman.

3. Results

3.1. Donor health status

With respect to the general clinical findings, all horses were considered as healthy. Measurements of blood cells were within the reference range, except for very mild changes in two animals (18 and 19). Blood chemistry measurements were within the reference range in 11 horses, showed very mild changes in four horses (4, 5, 13 and 17) and mild to moderate elevations of either alkaline phosphatase, creatinine, lactate dehydrogenase in five horses (horses 1 and 18, horse 2, horses 12 and 19, respectively) (Table 1). The latter were considered as abnormal in the further analyses.

3.2. Impact of matrix effects

Putative matrix effects were observed for IFN-γ, TNFα, IL-4, IL-6 and, to a lesser extent, IL-10. For IL-1β, matrix effects appeared to be only marginal. In IFN-γ, TNF-α-, IL-4- and IL-6-spiked serum, the measured absorbance was reduced to <50% of the absorbance of the corresponding spiked reagent diluent assay buffer sample. In IL-10-spiked serum, the absorption was reduced to approximately 65%. Diluting the serum decreased this effect, but it was still evident in serum diluted 1:10 (Supplementary Figure 1).

3.3. Cytokines in different blood products

The concentrations of cytokines in the blood products were highly variable between individual horses. For each cytokine, there were a number of blood donors with no measurable concentration (category 1), donors with medium concentrations

TABLE 1 Findings in blood counts and blood chemistry.

Donor	Elevated parameter	Value	Reference values
1*	Alkaline phosphatase	296 U/L	<260 U/L
2*	Creatinine	168.6 µmol/L	76.8–146.7 µmol/L
4	Albumin	35.8 g/L	27.4–35.7 g/L
5	Albumin	35.8 g/L	27.4–35.7 g/L
12*	Lactate dehydrogenase	826 U/L	<640 U/L
13	Bilirubin, direct	7.5 µmol/L	3.3–7.4 µmol/L
17	Urea	7.4 mmol/L	3.0–7.1 mmol/L
18*	Eosinophils Alkaline phosphatase	0.82 x 10^9 cells/L 318 U/L	<0.7 x 10^9 cells/L <260 U/L
19*	Mean corpuscular hemoglobin Mean corpuscular hemoglobin concentration Lactate dehydrogenase	1.25 fmol 24.03 mmol/L 780 U/L	0.9–1.2 fmol 20.8–23.5 mmol/L <640 U/L

The table shows all parameters that were above the reference ranges given in Bauer and Keresztes (23). The donors with mild to moderate changes that were grouped as abnormal are marked with an asterisk.

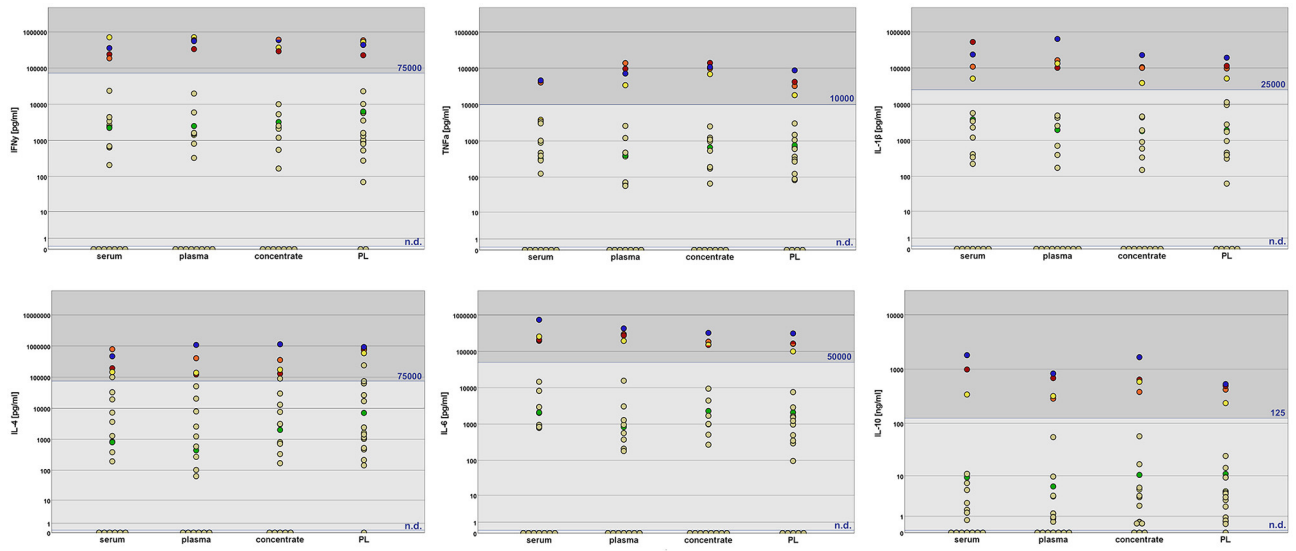

FIGURE 2
Cytokine concentrations in equine serum, plasma, platelet concentrate and platelet lysate (PL) as measured by sandwich ELISA. The assigned concentration categories are indicated by dark gray (category 3), gray (category 2) and light gray (category 1) background. The thresholds between category 2 and 3 are given in blue letters; category 1 corresponds to no detectable cytokine content (n.d.). Data from donors with abnormal findings in blood chemistry are highlighted in different colors (horse 1: orange; horse 2: red; horse 12: green; horse 18: yellow; horse 19: blue). The cytokine concentration categories were significantly higher in these animals as compared to the others ($p < 0.01$, except for IL-4 in PL).

(category 2), as well as donors with very high concentrations (category 3). Yet interestingly, the number of donors with no detectable cytokine content was reduced during blood processing to PL, suggesting some cytokine release during freeze-thawing of the concentrates in these samples (Figure 2). Nevertheless, considering all donors, cytokine concentrations remained similar between the different blood products, with no significant differences in concentration categories in the *post-hoc* tests. Instead, the cytokine concentration categories correlated between the different blood products. These correlations were very strong between serum, plasma and concentrate, and more moderate when these were compared with PL (Table 2).

Furthermore, the concentration categories of the different cytokines compared with each other correlated significantly, revealing that specific donors had either low, medium or high overall cytokine levels, widely irrespective of the type of cytokine. Again, these correlations were very strong in serum, plasma and concentrate, but more moderate in PL (Table 3).

TABLE 2 Correlations of cytokine concentration categories between the different blood products.

	Plasma	Concentrate	PL
IFN-γ			
Serum	$p < 0.001$ $r = 0.948$	$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.789$
Plasma		$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.770$
Concentrate			$p < 0.001$ $r = 0.789$
TNF-α			
Serum	$p < 0.001$ $r = 0.965$	$p < 0.001$ $r = 0.965$	$p < 0.001$ $r = 0.770$
Plasma		$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.857$
Concentrate			$p < 0.001$ $r = 0.857$
IL-1β			
Serum	$p < 0.001$ $r = 0.902$	$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.784$
Plasma		$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.828$
Concentrate			$p < 0.001$ $r = 0.861$
IL-4			
Serum	$p < 0.001$ $r = 0.960$	$p < 0.001$ $r = 0.954$	$p = 0.005$ $r = 0.600$
Plasma		$p < 0.001$ $r = 0.909$	$p = 0.026$ $r = 0.498$
Concentrate			$p = 0.004$ $r = 0.616$
IL-6			
Serum	$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.948$	$p = 0.011$ $r = 0.554$
Plasma		$p < 0.001$ $r = 0.948$	$p = 0.011$ $r = 0.554$
Concentrate			$p = 0.003$ $r = 0.631$
IL-10			
Serum	$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.784$	$p < 0.001$ $r = 0.710$
Plasma		$p < 0.001$ $r = 0.822$	$p < 0.001$ $r = 0.764$
Concentrate			$p < 0.001$ $r = 0.877$

3.4. Cytokine levels and donor health status

To find reasons and predictors for the high inter-individual variability in the blood product cytokine concentrations, we searched for links between cytokine contents and health status. Clearly higher cytokine concentrations (category 3 for most cytokines) had been detected in horses 1, 2, 18 and 19 as compared to the other horses. Interestingly, these were 4 out of the 5 horses that had shown elevated readings in the blood chemistry parameters, namely alkaline phosphatase, creatinine and lactate dehydrogenase (Table 1). The remaining horse (horse 12) with an abnormal blood chemistry finding, however, had middle range cytokine concentrations in its blood products (category 2). Nevertheless, cytokine concentration categories were significantly higher in the donor group with abnormal blood work results than in the donor group with physiological blood work results ($p < 0.01$ for all cytokines and blood products analyzed, except for IL-4 in PL).

4. Discussion

The primary aim of this study was to gain insight into the cytokine content of PL and related blood products. Anticipating the high inter-individual differences, we also aimed to identify putative predictors for cytokine content.

The high inter-individual differences in cytokine concentrations between the horses were the most conspicuous finding of the current study, which illustrated that it will be difficult to establish robust reference ranges for serum analysis or quality control thresholds for platelet concentrate and PL products. For serum, such variations are described in the literature, especially regarding IFN-γ concentrations due to vaccination or viral antigen exposure (24). In the current study, however, at the time of blood sampling, none of the horses had received any vaccinations or other medications in the last 2 weeks, and all had received standard vaccinations over the past years.

This study demonstrated that there is a relationship between high cytokine concentrations in blood products, including PL, and increased values in blood chemistry parameters (lactate dehydrogenase, alkaline phosphatase and creatinine). In accordance with this, previous studies showed that there are correlations for the cytokines IFN-γ and TNF-α with the levels of alkaline phosphatase and lactate dehydrogenase (25–27). Probably, the increase in cytokine concentration can be explained by inflammatory processes in the organs such as liver, kidney, muscle or bone, for which these enzymes are considered as indicators.

Cytokines serve as messengers in the regulation of immune response and inflammation. Traditionally, they are divided into pro- (IFN-γ, IL-1β, IL-6 and TNF-α) and anti-inflammatory cytokines (IL-4 and−10), which interact with each other (28, 29). Cytokines are mainly produced by TH1 and TH2 lymphocytes, natural killer as well as mast cells and macrophages, as a result of stimulation by antigens or signaling molecules (24). Due to their contribution to pathological processes, they are being explored as biomarkers for various diseases (30). The exact relationship of cytokine levels and blood chemistry changes in horses will need to be further explored. However, the findings of this study suggest the possibility that blood chemistry measurement might be a convenient tool to estimate the level of cytokines in donor blood and thus facilitate the targeted use of PL.

So far, there are two different operational areas for PL. One is the direct local therapeutic application, e.g., for treatment of osteoarthritis in humans (31, 32) and horses (33), for tendon lesions in humans (34), to promote healing of corneal ulcers (35–37) or for wound healing (38, 39). All beforementioned publications

TABLE 3 Correlations between the different cytokines in the respective blood products.

	IL-1β	IL-4	IL-6	IL-10	TNF-α
Serum					
IFN-γ	$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.917$	$p = 0$ $r = 1.000$	$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.965$
IL-1β		$p < 0.001$ $r = 0.917$	$p = 0$ $r = 1.000$	$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.965$
IL-4			$p < 0.001$ $r = 0.917$	$p < 0.001$ $r = 0.908$	$p < 0.001$ $r = 0.873$
IL-6				$p = 0$ $r = 1.000$	$p < 0.001$ $r = 0.965$
IL-10					$p < 0.001$ $r = 0.965$
Plasma					
IFN-γ	$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.901$	$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.948$
IL-1β		$p < 0.001$ $r = 0.862$	$p < 0.001$ $r = 0.902$	$p < 0.001$ $r = 0.902$	$p < 0.001$ $r = 0.902$
IL-4			$p < 0.001$ $r = 0.947$	$p < 0.001$ $r = 0.947$	$p < 0.001$ $r = 0.947$
IL-6				$p = 0$ $r = 1.000$	$p = 0$ $r = 1.000$
IL-10					$p = 0$ $r = 1.000$
Concentrate					
IFN-γ	$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.873$	$p < 0.001$ $r = 0.948$	$p < 0.001$ $r = 0.822$	$p = 0$ $r = 1.000$
IL-1β		$p < 0.001$ $r = 0.843$	$p < 0.001$ $r = 0.889$	$p < 0.001$ $r = 0.798$	$p < 0.001$ $r = 0.948$
IL-4			$p < 0.001$ $r = 0.843$	$p = 0.006$ $r = 0.609$	$p < 0.001$ $r = 0.873$
IL-6				$p < 0.001$ $r = 0.798$	$p < 0.001$ $r = 0.948$
IL-10					$p < 0.001$ $r = 0.822$
PL					
IFN-γ	$p < 0.001$ $r = 0.845$	$p = 0.010$ $r = 0.559$	$p < 0.001$ $r = 0.845$	$p < 0.001$ $r = 0.928$	$p < 0.001$ $r = 0.885$
IL-1β		$p = 0.023$ $r = 0.505$	$p = 0.002$ $r = 0.644$	$p < 0.001$ $r = 0.804$	$p < 0.001$ $r = 0.822$
IL-4			$p = 0.002$ $r = 0.646$	$p = 0.006$ $r = 0.594$	$p = 0.020$ $r = 0.517$
IL-6				$p < 0.001$ $r = 0.804$	$p < 0.001$ $r = 0.700$
IL-10					$p < 0.001$ $r = 0.834$

describe an improved outcome compared to control populations. The decisive factors for clinical efficacy are presumably the growth factors contained in the PL, as well as anti-inflammatory cytokines, which can locally influence the inflammatory process (40). On the other hand, PL is already successfully used as a medium additive for cell culture of MSC, which can promote the cellular potency (6, 19, 41, 42). While this has been largely attributed to the growth factors contained in the PL, e.g., VEGF (19), other mediators should be considered as well. As already described by Barrachina et al. (43), stimulation with IFN-y or TNF-α in culture improves the immunomodulatory potency of MSC. Therefore, local application of primed MSC can be expected to yield better therapeutic results. The appropriate amount of pro-inflammatory cytokines is important, because an excess of them impairs the

differentiation potential and proliferative capacity of MSC (44). At this point, cytokines contained in the PL may also play a crucial role. With specific levels of pro-inflammatory cytokines, PL alone could possibly serve as a medium additive for priming.

In this study, cytokine levels were measured by sandwich ELISA in different blood products, such as serum, plasma, concentrate and PL. It became apparent that this method has its shortcomings regarding the exact quantification of low cytokine concentrations, as matrix effects occurred in samples measured at low dilutions. We aimed to overcome this on the one hand by repeated measurements at different dilutions and using the highest possible dilution for further analysis, and on the other hand by grouping the cytokine concentrations into categories, the latter to circumvent statistical analysis with inaccurate numbers. Transforming the continuous results data into ordinal data, however, limited the opportunities of inductive statistical analysis. To account for possible confounding variables and interactions of different factors, a generalized linear mixed model would have been more suitable than the analysis approach presented. However, only a multinomial model considering sample type and health status was applicable based on the given data set. This model confirmed that there was no interaction between sample type and health status, but it strongly misestimated the probabilities for the different cytokine categories in different groups, due to their uneven distribution between groups and the relatively small sample sizes per group. Therefore, although this model overall revealed the same trends, namely a significant influence of the health status but no major influence of the sample type on cytokine concentrations, we choose to present a data analysis based on basic group comparisons. While the shortcomings of the ELISA assay and the resulting statistical simplification represent limitations of the current study, it is important to note that the measurements of high cytokine concentrations can still be considered as reliable, as these could be performed with high dilution factors, preventing matrix effects.

In conclusion, this study illustrates that blood products, including PL, are subject to wide variations in cytokine content, which makes careful consideration regarding their use important. It is possible that blood chemistry parameters may provide clues to cytokine content in blood products in individual horses. If this can be confirmed and specified in future research, it would provide a prerequisite to targeted use of PL in clinic and laboratory.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Regierungspräsidium Gießen. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JM: conception of the study and complete experimental design (together with JB), blood collection, sample and data analysis, data interpretation, and drafting of the manuscript. AH: substantial contribution to the experimental design, blood collection, and processing and analysis. SN: substantial contribution to the experimental design and data interpretation. KB: data analysis and interpretation (together with JB and JM). JB: conception of the study and complete experimental design (together with JM), data interpretation, and drafting of the manuscript (together with JM). All authors have critically revised the manuscript for important intellectual content and approved the publication of its content.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1117829/full#supplementary-material

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Isolation and cultivation as well as *in situ* identification of MSCs from equine dental pulp and periodontal ligament

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Introduction: The lifelong eruption places a great demand on the dental pulp and periodontal ligament (PDL) of horse teeth. Cells within the pulp and PDL seem to play a key role during this remodeling.

Methods: In this study, we isolated and cultivated MSCs (medicinal signaling cells) from dental pulp, PDL and retrobulbar fat of four horses. Subsequently, we analyzed them by flow cytometry and immunohistochemistry to determine and compare their characteristics. In addition, we localized these cells within the tissue structure *via* immunohistochemistry of histological sections. For these analyses, several surface markers were applied.

Results: The described method illustrates a feasible approach to isolate and cultivate MSCs from equine dental pulp and PDL. In the flow cytometry a vast majority of cultivated cells were positive for CD90 and CD40 and negative for CD11a/18, CD45, CD105 and MHCII suggesting that these cells feature characteristics of MSCs. Immunohistochemistry of histological pulp and PDL sections showed the localization of CD90 positive cells especially in the perivascular region and the subodontoblastic layer.

Discussion: Our findings indicate that the isolation and cultivation of MSCs from equine dental pulp and PDL is feasible although an elaborate and complicated harvesting protocol is required. MSCs isolated from dental pulp and PDL are regarded as candidates for new therapeutical approaches in equine dental medicine like regeneration of periodontal lesions, enhancement of periodontal re-attachment after dental replantation and stimulation of pulp-obliteration and apexification in combination with endodontic therapies.

KEYWORDS

hypsodont teeth, periodontium, endodontium, equine dentistry, regenerative medicine, MSCs

1. Introduction

Dental diseases are very common in horses. Due to lifelong eruption, despite permanent mechanical load, horse teeth have to ensure the structural integrity of the periodontal ligament (PDL) and dental pulp (pulpa dentis).

The PDL belongs, together with the alveolar bone, the dental cementum and the gingiva to the periodontium, which supports the tooth (1, 2). It forms the connection between two hard substances, namely the alveolar bone and the cementum of the continuously erupting tooth. Regarding the permanent wear of the occlusal surface by a fibrous diet, this eruption of the equine hypsodont tooth is essential (3).

Corresponding to this dynamic process in the PDL, it should not be neglected that the dental pulp is also subjected to permanent remodeling. During tooth eruption, there must be a continuous production of subocclusal dentin to prevent occlusal pulp exposure. This assumes a permanent high productivity of odontoblasts. Histomorphometrical analyses, especially in the subodontoblastic layer, indicate that the equine dental pulp, unlike the brachydont dental pulp, remains lifelong in an immature, highly productive status (4). Although odontoblasts are regarded as postmitotic cells which survive lifelong, new odontoblasts, specifically after injury of dentin or odontoblasts, are regenerated from the subodontoblastic layer (5, 6).

Interestingly, cells of the dental pulp and PDL have the same genesis since both consist of mesenchymal tissue, which originates from migrating neuronal crest cells (7). Consequently, the cellular components of these tissues are derived from ectomesenchymal cells (1, 7, 8). However, blood vessels of the pulp and PDL are supposed to develop later during odontogenesis from migrating mesodermal cells (7–9).

MSCs (medicinal signaling cells) are most widely known as "mesenchymal stromal cells" or "mesenchymal stem cells". However, we used the new term "medicinal signaling cells", as introduced by Caplan et al. (10). Due to their supportive characteristics, which are based on immune modulation, creating trophic conditions, and regeneration we consider this term as more appropriate (11). The isolation of medicinal signaling cells (MSCs) from equine dental pulp and PDL has been described by different authors. Ishikawa et al. 2017 (12) isolated and characterized equine dental pulp stem cells from thoroughbred wolf teeth, and -Staszyk and Gasse 2007 (13) described a primary culture of fibroblasts and cementoblasts of the equine periodontium. A characterization of cells isolated from PDL was implemented for the markers CD31, pan-cytokeratin, CD90, and CD105 by Mensing et al. (14). For cells isolated from dental pulp, the expression levels of CD44, CD90, CD11a/18, CD105, MHCI, MHCII, CD34 and CD45 were evaluated (12, 15). Thereby, it must be noted that even though the ISCT (International Society for Cellular Therapy) defined some minimal criteria to be met by human MSCs (16), equine MSCs vary widely concerning their expression of surface markers. There are large differences concerning various tissues as well as individuals described (17–21).

Although the presence of MSCs has been documented in equine pulp and periodontal tissue, it remains unclear in which niche, extent, and amount the cells are present within the initial tissue. Merely Mensing et al. (14) compared MSCs isolated from gingiva, PDL and subcutaneous fat in the masseteric area, although they neither investigated the specific niche of the cells inside the original tissue nor isolated MSCs from pulp and PDL in parallel. However, a comparison between MSCs obtained from equine pulp and PDL is attractive concerning the common origin and the specific challenges MSCs face inside and adjacent to the erupting hypsodont tooth.

In this study, we aimed to develop a sustainable method to simultaneously obtain and cultivate MSCs from equine dental pulp and PDL. To this end, the cells isolated from the incisivi of four donors were cultivated and analyzed for different surface markers by flow cytometry and immunocytochemistry.

TABLE 1 Details of donors used for isolation.

Donor No.	Age	Sex	Breed	Time period pre-isolation*
1	2.5 y	♂	Warmblood	Fresh 1–2 h
2	13 y	♀	Warmblood	Cooling overnight 16 h
3	21 y	♀	Warmblood	Cooling overnight 24 h
4	24 y	♂	Haflinger	Fresh 1–2 h

*Time interval from slaughter to sampling.

These results were compared with those found for MSCs, which were isolated from the retrobulbar fat body since MSCs obtained from fat are already established as a reliable source and used for regenerative therapies. Another aim of the study was to demonstrate the specific localization of the isolated cells *in situ* by immunohistochemistry of the surface marker CD90, which was applied on histological slices of dental pulp and PDL.

2. Materials and methods

2.1. Donors

The cells were obtained from heads of four horses between the ages of 2.5–24 years (Table 1). All horses were slaughtered by a commercial butcher *via* captive bolt, followed by bleeding, due to reasons unrelated to this study. Sampling was performed within 24 h.

2.2. Sampling

To obtain the required tissues, the heads had to be prepared in different steps (illustrated in Figure 1). After a cleaning step with tap water, a macroscopic examination was performed to select donors with clinical healthy incisors. Due to their good accessibility, only incisors were used for cell isolation. Teeth were identified based on a tooth numbering system according to Triadan (22) and Floyd (23). Most of the soft tissue was removed from nasal up to the caudal end of the margo interalveolaris with a disinfected knife. Subsequently, the incisors were cleaned with tap water and a brush. The heads were cut with an oscillating saw through the margo interalveolaris to separate the parts of the maxilla and mandibular which include the incisors. Following a cleaning step with tap water and a short rinse with 80% ethanol, the samples were wrapped in wipes (Kimtech Wettask, Kimberly-Clark, Dallas, TX, USA) drenched with 80% ethanol and stored in the fridge at 4°C until tissue preparation for max. One hour. Before further preparation the remaining soft tissue was removed with a sterile scalpel and raspatory. For the extraction of retrobulbar fat, the skin above the retrobulbar fat body was removed with sterile forceps and scalpel. After disinfecting the forceps, scalpel, and subjacent tissue with 80% ethanol, two pieces of fat (~1 × 1 × 6 cm) were extracted. The samples were transferred to 50-ml tubes (Sarstedt AG &

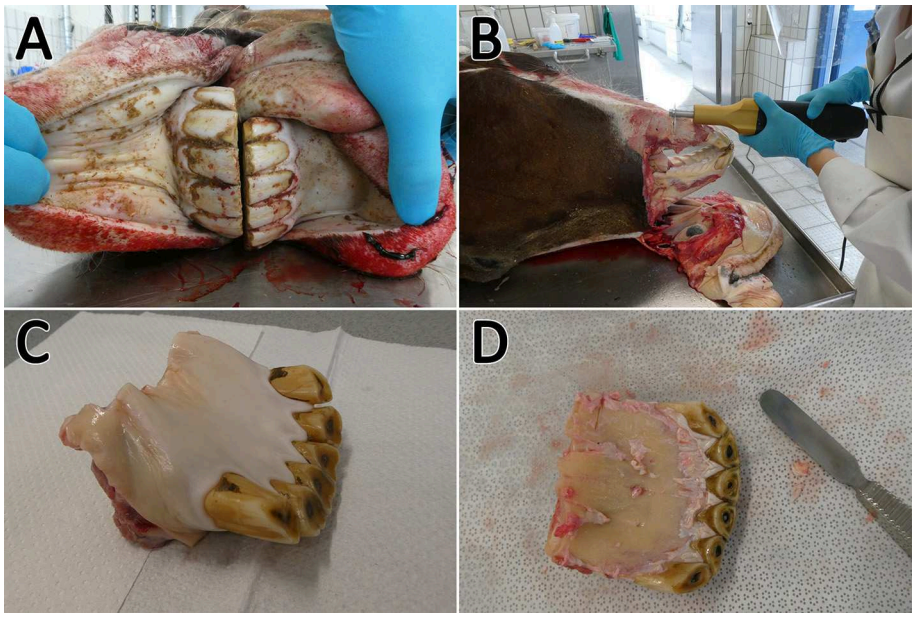

FIGURE 1
Sampling procedure. The incisors **(A)** were cleaned, the soft tissue was removed, and the arcades were dissected **(B)**. Afterwards the sample could be separated **(C)** and the remaining soft tissue was removed **(D)**. Subsequently, the sample was transferred to a microband saw.

TABLE 2 Composition of medium used for isolation and cultivation.

Medium	Ingredients	Manufacturer
Transport medium A	DMEM-HG[1] 100 U/ml Penicillin 0.1 mg/ml Streptomycin 1% Amphotericin 1% Tetracycline	Life Technologies GmbH, Darmstadt, Germany Life Technologies GmbH Life Technologies GmbH Capricorn Scientific GmbH, Ebsdorfergrund, Germany Carl Roth, Karlsruhe, Germany
Transport medium B	DMEM-LG[2] 100 U/ml Penicillin 1% Amphotericin 1% Tetracycline	Life Technologies GmbH Life Technologies GmbH Capricorn Scientific GmbH Carl Roth
Digestion medium A	Transport medium A 2 mg/ml Collagenase I 10 mg/ml BSA	Life Technologies GmbH Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany Capricorn Scientific GmbH
Digestion medium B	Transport medium B 1mg/ml Collagenase I 10 mg/ml BSA	Life Technologies GmbH Sigma-Aldrich Chemie GmbH Capricorn Scientific GmbH
Cultivation medium A	Transport medium A 10% FCS 1% MEM-NEAA[3]	Life Technologies GmbH Life Technologies GmbH Life Technologies GmbH
Cultivation medium B	Transport medium B 10% FCS	Life Technologies GmbH Life Technologies GmbH

[1]DMEM-High Glucose.
[2]DMEM-Low Glucose.
[3]MEM-Non Essential Amino Acids.

Co. KG, Nuembrecht, Germany) with 25 ml of transport medium B (Table 2) and stored in the fridge at 4°C for max 4 h until cell isolation.

2.3. Tissue preparation

To isolate the dental pulp and PDL, the extracted parts of the dental arch containing the incisors had to be further dissected. After a cleaning step with tap water, a midline cut with a diamond-coated, water-cooled micro-band saw (MBS 240/E, Proxxon S.A., Wecker, Luxembourg) was performed to separate each quadrant containing teeth 01 to 03. The clinical crowns were dissected and removed before further processing. In the following, the specimens were cut into horizontal slices with a height of ∼0.8 mm to isolate the dental pulp (Figure 2). For the isolation of the PDL, the sections were additionally cut through the transverse plane. After sectioning, the specimens were dipped in 70 % ethanol, washed with PBS (Life Technologies GmbH) for ∼15 s, and transferred into transport medium A (Table 2). The samples were stored in the fridge at 4°C for max. Two hour until cell isolation and cultivation.

2.4. Cell isolation and cultivation

To avoid contamination, tissue and cell isolation occurred under sterile bench conditions. In a first step, the dental pulp and PDL had to be extracted out of the previously prepared and sectioned samples.

For pulp isolation, the specimen was fixed with a forceps, and the tissue was pulled out of the pulp cavity with Hedstrom files (Figure 3A). If necessary, a further dissection with a scalpel was performed. After isolation, the tissue was directly transferred in a drop of cultivation medium A (Table 2) in a petri dish.

For PDL isolation, the smaller additional transversal sectioned samples were used. The specimens were fixed with a pincer on the alveolar bone, and a rongeur forceps was used to rupture

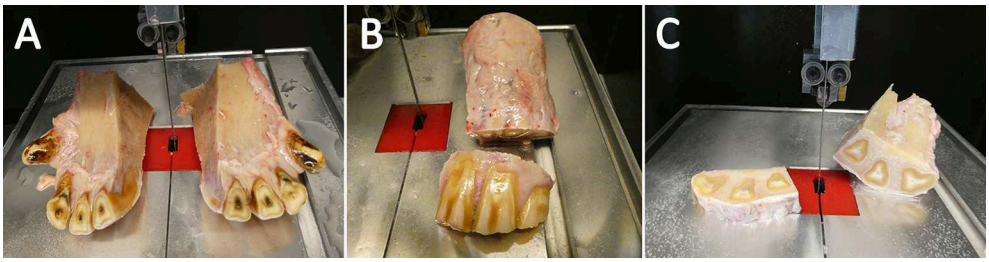

FIGURE 2
Tissue preparation. The samples were sliced through the sagittal plane **(A)**. Afterwards the clinical crone was segmented and rejected **(B)** Finally, horizontal sections were sliced **(C)**.

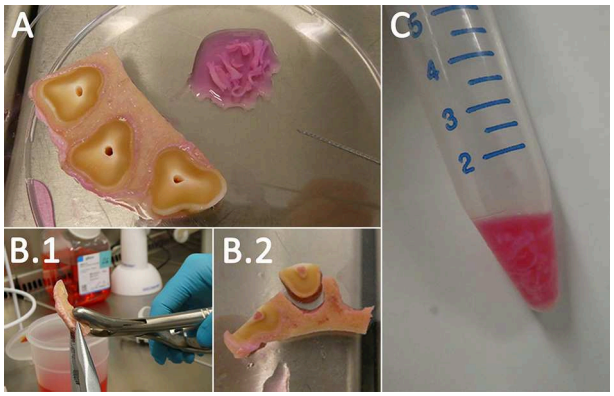

FIGURE 3
Cell isolation. The dental pulp was pulled out of its cavity *via* Hedstrom files **(A)**. To obtain the PDL the tooth and the alveolar bone were separated **(B.1, B.2)**. Afterwards, the PDL was scraped from the surfaces. Both samples were digested in 2 mg/ml Collagenase I **(C)**.

the tooth (Figure 3B). Subsequently, the exposed PDL could be scraped from the alveolar bone and cementum of the tooth with a rongeur forceps. If necessary, further dissection with a scalpel was performed. After isolation, the tissue was directly transferred in a drop of cultivation medium A (Table 2) in a petri dish. The extracted dental pulp and PDL were collected in 15-ml tubes (Sarstedt AG & Co. KG), and the same volume of digestion medium A (Table 2) was added. To digest the tissue, the tubes were transferred in a water bath at 37°C for ∼60 min while agitating the suspension occasionally. Following digestion, the suspension was centrifuged at 300 *g* for 5 min, and the detached pellet with some of the supernatant above (∼2 ml) was filtered through a 70-µm cell strainer (Sarstedt AG & Co. KG). The tissue remaining in the filter was directly seeded in 25-cm^2 (Sarstedt AG & Co. KG) cell culture flasks with cultivation medium A (Table 2). Additionally, the flow-through was centrifuged at 300 *g* for 5 min and, after discarding the supernatant, resuspended in 1 ml of cultivation medium A (Table 2) and seeded in 25-cm^2 cell culture flasks.

Afterwards, the MSCs from retrobulbar fat were isolated as previously described by Pascucci et al. (24) for MSCs isolated subcutaneous of the region above the dorsal gluteal muscles of horses. Briefly, the collected fat was cut into pea-sized pieces and the same volume of digestion medium 2 (Table 2) was added. Digestion was implemented for 40–50 min at 37°C while occasionally agitating the suspension. Following digestion, the suspension was centrifuged at 300 *g* for 5 min, and the detached cell pellet was filtered through a 70-µm cell strainer. The flow-through was again centrifuged at 300 *g* for 5 min and, after discarding the supernatant, resuspended in cultivation medium B (Table 2), and seeded in 75 cm^2 cell culture flasks (Sarstedt AG & Co. KG).

Cells from all sources were cultivated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. When they reached 80% confluence, they were passaged up to passage 3, which was used for further investigations. Only for immunohistochemistry, the cells were cultured up to passage 4. After one week of cultivation, supplementation of amphotericin and tetracylcine was stopped. The cell morphology of each passage was determined under phase contrast light microscope (Leica DM IL, Leica Microsystems GmbH, Wetzlar, Germany).

2.5. FACS-analysis

Fluorescence-activated cell sorting (FACS) analysis was performed to characterize the MSCs and compare MSCs isolated from different extraction points. Since there are no standardized criteria for equine MSCs, we decided to apply common markers as previously described by different authors for the horse [e.g., (25, 34, 39)]. Hence, CD11a/18, CD45, CD44, CD90, CD105, and MHCII were applied as markers. The FACS analysis was performed as described before by Leisengang et al. (26). We also tested the proportion of live cells by, staining with 7-amino-actinomycin D (7-AAD, Becton Dickinson, Heidelberg, Germany). For this, 7-AAD was added in a concentration of 1:50 to the cell suspension and incubated for 10 min in the dark. A list of applied antibodies can be found in Table 3. Finally, the measurement of the resuspended pellets was implemented with the FACS BD Accuri™ C6 (Becton Dickinson), and the evaluation was conducted using the BD Accuri™ C6 Software version 1.0.264.21.

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TABLE 3 Antibodies for FACS-anaysis.

Name	Manufacturer	Dilution
Primary antibodies		
Rat anti-mouse CD 44 Clone: IM7, Cat No.: 553131	BD Bioscience	1:400
Mouse anti-human CD 45 Clone: UCHL1, Cat No.: 304202	BioLegend GmbH, Koblenz, Germany	1:100
Mouse anti-human CD 90 Clone: 5E10, Cat No.: 5555593	BD Bioscience	1:400
Mouse anti-human CD 105 Clone: SN6, Cat No.: 14-1057-82	eBioscience™, Thermo Fisher Scientific	1:500
Mouse anti-horse MHC Class II Clone: CVS20, Cat No.: 1085ga	BioRad, Muenchen, Germany	1:200
Secondary polyclonal antibodies		
PE goat anti-mouse Ig Cat No.: 550589	BD Bioscience	1:800
APC goat anti-rat Ig Cat No.: 551019	BD Bioscience	1:600
Conjugated primary antibody		
FITC mouse anti-horse CD 11a/18 Clone: CVS9, Cat No.: MCA1081	BioRad	1:200
Isotype control		
normal mouse IgG-FITC Cat No.: sc-2339	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	1:100

2.6. Immunocytochemistry of isolated cells

Immunocytochemical analysis of the isolated and cultivated cells should show the expression of the markers CD90 and CD44, in addition to the histological sections, which should illustrate the localization of the cells inside the tissue structure. The MSCs in passage 4 were cultured on chamber slides (8-well, Sarstedt AG & Co. KG) until they reached 80% confluence. Subsequently, they were fixed with 1:1 methanol/acetone (1:1, Carl Roth), cooled to −20°C for 1 min and dried with a dryer. Afterwards, the samples were rehydrated three times for 5 min with PBS. This step was followed by blocking with a blocking solution (5% goat serum in PBS with 0.1% Tween) for 30 min and a washing step with PBS-Tween (0.1% Tween) for 3 min. The samples were incubated with the primary antibody (Table 4) overnight at 4°C in a humidity chamber, followed by three times washing for 5 min and incubation with the secondary antibody (Table 4) in the dark for 1 h. Subsequently, the antibody was washed out twice for 5 min, and the samples were incubated with 2-(4-amidinophenyl)-1H-indole-6-carboxyamidine (DAPI; Life Technologies GmbH; 1:20,000) for 2 min in the dark to stain the nuclei. After another washing step twice for 2 min, the slides were mounted with ibidi mounting medium (ibidi GmbH, Martinsried, Germany) and finally examined with the Zeiss Axio Observer Z.1 (Carl Zeiss, Göttingen, Germany). For negative controls, the samples were incubated with secondary antibody to exclude non-specific binding.

2.7. Immunohistochemistry of histological sections from dental pulp and PDL

To find the specific niche of the isolated MSCs inside the tissues, immunohistochemistry of CD90 was implemented on histological sections of dental pulp and PDL.

During tissue preparation for cell-isolation, additional section planes of the incisors were cut and fixed in 10% buffered formalin (pH 7). After fixation, the sections were watered, further trimmed with a diamond-coated, water-cooled micro-band saw, and decalcified on a platform shaker (Polymax 1040, Heidolph Instruments, Schwabach, Germany) in buffered EDTA (ethylenediaminetetraacetate) for 6 weeks as previously described by Roßgardt et al. (27). Following this decalcification process, the samples were trimmed using a scalpel to minimize section size but receive both PDL and pulp within one section. Afterwards, they were placed in embedding cassettes (Simport™ Acetal Macrosette, Fischer Scientific GmbH, Schwerte, Germany) and decalcified in EDTA for another 2 weeks. Subsequently, the samples were rinsed in tap water and stored in PBS overnight. Paraffin embedding, sectioning, and staining were implemented according to Roßgardt et al. (27). Toluidine blue staining was performed according to a standard protocol to evaluate the sections under a light microscope (Leica DM2500, Leica Microsystems GmbH). After the evaluation (example see Figure 4), paraffin sections which included both PDL and pulp were selected and incubated for 30 min at 60°C on a heating plate (MSH-20D, Witeg, Wertheim, Germany) to promote adhesion on the slides (SuperFrost Plus™, Fischer Scientific GmbH). This step was followed by de-waxing the samples in a descending alcohol series and a pre-treatment in heated citrate buffer (pH 6) at 70°C for 2 h. Afterwards, the slides were washed three times for 2 min in PBS-Tween and blocked for 30 min with a blocking solution (5% goat serum in PBS with 0.1% Tween). Incubation with the primary antibody (Table 4) was implemented overnight at 4°C in a humidity chamber, followed by washing three times for 2 min in PBS-Tween. Subsequently, the samples were incubated with the secondary antibody (Table 4) in the dark for 1 h and washed three times for 2 min with PBS-Tween. To stain the nuclei, the specimens were incubated with DAPI for 2 min in the dark and afterwards washed twice for 2 min. Finally, the slices were mounted with ibidi mounting medium and examined with the Zeiss Axio Observer Z.1 (Carl Zeiss, Göttingen, Germany). For negative controls, the samples were only incubated with secondary antibody to exclude non-specific binding.

2.8. Statistical analysis

The results of the FACS analysis were plotted using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and evaluated using SigmaStat 4.0 (Systat Softwares Inc., San José, CA, USA). A one-way analysis of variance with repeated measurements was applied to look for similarities in marker expression. The repetition of measurements was given by the different tissues of each donor. However, it must be considered that the number of donors was low, and thus, the power of the performed test was, in most instances, below the desired power. All data are expressed as mean ± SD.

TABLE 4 Antibodies for immunohistochemistry.

Name	Manufacturer	Dilution	Application
Primary antibodies			
Rat anti-mouse CD 44 Clone: IM7, Cat No.: 553131	BD Bioscience	1:100	On cells
Rabbit anti-mouse CD 90 Clone: D3V8A, Cat No.: 13801	Cell signaling, Danvers, MA, USA	1:700	On cells and on tissue slices
Secondary polyclonal antisera			
Rabbit F(ab')2 anti-rat IgG:FITC (Code: STAR17B)	BioRad	1:200	On cells
Cy3-conjugated Affinipure donkey anti-rabbit, polyclonal IgG (H+L) (Code: 711-165-152)	Jackson ImmunoResearch, West Grove, PA, USA	1:400	On cells and on tissue slices

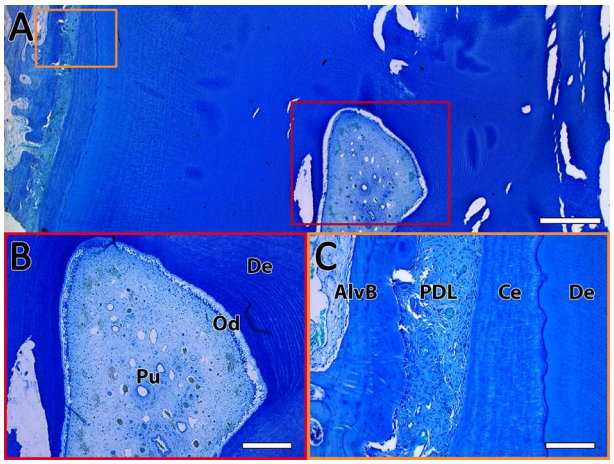

FIGURE 4
Exemplary toluidine blue staining of a horizontal sectioned incisor. **(A)** Overview of the dental pulp (red rectangle) and the PDL (orange rectangle). The scale bar represents 1,000 µm. **(B)** Enlarged view of the dental pulp (Pu) with the odontoblastic layer (Od) surrounded by dentin (De). The scale bar represents 400 µm. **(C)** Enlarged view of the PDL between the alveolar bone (AlvB) and the cementum (Ce) followed by the dentin (De). The scale bar represents 200 µm.

3. Results

3.1. Cell isolation and cultivation

The applied method was suitable to isolate viable and proliferating MSCs simultaneously from the equine dental pulp and PDL of the same tooth. Vital cells from all different tissues of the four donors were successfully isolated and cultivated (Figure 5). Donors and tissues varied in the time the cells needed to attach and become confluent. If the supernatant which remained in the cell strainer was directly seeded, cells attached (~2 d) and became confluent (~5 d) earlier, as if the flow-through was seeded. Due to their low quantity, it was not possible to obtain cells from the flow-through of donors 2 and 4. In Table 5, the different time periods of attachment and first passaging are listed for the different donors and tissues. Only the earliest time points are presented, which was constantly the case when seeding the remaining tissue inside the cell strainer. In general, most cells were gained from the retrobulbar fat and the fewest from the dental pulp. The youngest donor was attaching and proliferating much faster as a considerably higher amount of material was obtained. The MSCs needed 1–6 days to attach, and the first passaging was possible after 1–16 days. Thereby, cells gained from retrobulbar fat mostly attached early and therefore were passaged first, in contrast to cells isolated from the dental pulp, which generally needed more time. However, no difference in cell growth was detected when the time from slaughter to sampling was expanded up to 24 h. Furthermore, the donors aged between 13 and 24 years showed no obvious increased proliferation or decreased cell numbers.

3.2. Morphology of MSCs

To prove if the cells had features of MSCs, initially, the morphology was evaluated. The cells grew plastic-adherend and showed an MSC-like spindle-shaped morphology, except for some isolates from the PDL of the youngest donor (donor 1), which had polygonal, large, flattened cell bodies. When growing denser, these cells formed some cobblestone-like clusters (Figure 6).

3.3. FACS-analysis

In addition to the evaluation of cell morphology, the surface marker content was analyzed by flow cytometry. Most cells were positive for CD44 (95.53% ± 7.17) and CD90 (83.96% ± 11.11) and negative for CD11a/18, CD45, CD105, and MHCII (Figure 7C). Referring to the mainly negative surface marker, no marker was expressed by more than 2.8% (CD105, donor 2, PDL) of cells. In Figures 7A, B, the marker expression of one donor is shown exemplary. Between the extraction points, no statistical differences were detected. Almost all cells isolated from pulp tissue (97.8% ± 0.76), PDL (92% ± 12.21), and fat (96.55% ± 5.55) were positive for CD44. However, for CD90, less cells showed a positive expression. For cells extracted from pulp, on average, 81.8% ± 4.32, from PDL, 77% ± 16.67, and from fat tissue, 93.15% ± 3.61 were positive for CD90. The MSCS isolated from the PDL of donor 2 showed a lower CD44 and CD90 expression than others, an early detachment from the plastic surface, and high rates of cell death after passage 3. However, it was conspicuous that MSCs extracted from dental pulp

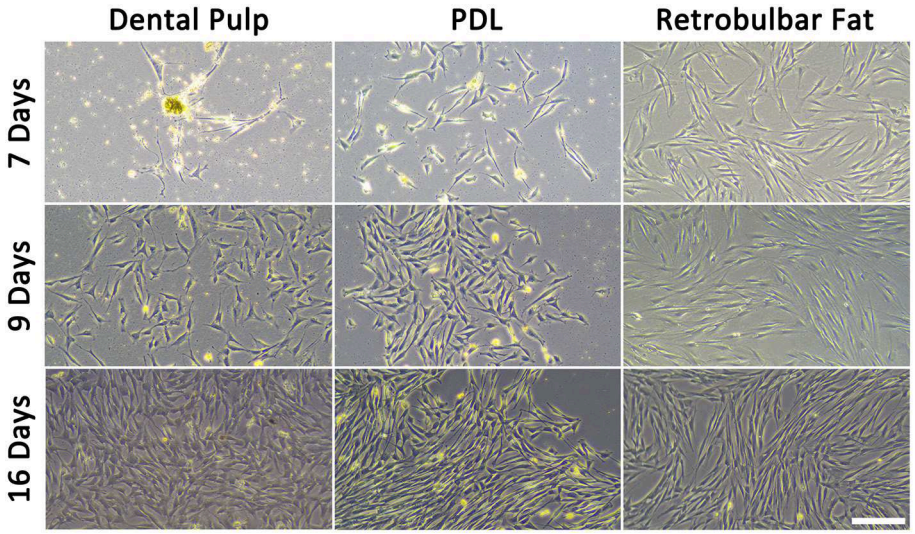

FIGURE 5
Isolated MSCs after different time points. The scale bar represents 200 μm.

TABLE 5 Time periods MSCs needed to attach and get confluent, when seeding the remaining tissue inside the cell strainer.

Donor No.	Age	tissue	attaching	First passaging (cells obtained)	Time period pre-isolation*
1	2.5	PU PDL Fat	1 d 1 d 2 d	1 d (sep.)[1] - 10 d P1[2] (3.15 Mio) 1 d (sep.) - 10 d P1 (9.4 Mio) 10 d (8.2 Mio)	Fresh 1–2 h
2	13	PU PDL Fat	6 d 6 d 6 d	16 d (1.6 Mio) 16 d (0.3 Mio) 13 d (5 Mio)	Cooling overnight 16 h
3	21	PU PDL Fat	3 d 3 d 2 d	15 d (0.7 Mio) 10 d (1 Mio) 8 d (4 Mio)	Cooling overnight 24 h
4	24	PU PDL Fat	4 d 4 d 3 d	16 d (0.9 Mio) 12 d (3.3 Mio) 12 d (3.8Mio)	Fresh 1–2 h

*Time interval from slaughter to sampling.
[1]Separation of cells since they were growing too dense.
[2]Passage 1 after 10 days.

and retrobulbar fat showed a tendency to be more homogenous than those isolated from the PDL (Figure 7C).

The ratio of living cells decreased from retrobulbar fat (98.23% ± 1.19) to PDL (95.78 ± 4.11) to pulp (85% ± 8.07), without a statistical significance (for detailed data, see Supplementary Figure 1).

3.4. Immunocytochemistry of isolated cells

Immunocytochemistry of the MSCs was performed to evaluate the morphology of cells positive for CD44 or CD90 (Figure 8). As shown in Figure 8, the MSCs of all three tissues showed a strong positive signal for CD44, which was evenly distributed throughout the cell. The cells showed a more flattened polygonal morphology compared to routine cell cultures, due to their low density in the chamber slides.

The signal for CD90 was missing in some cells, which matches the results of the flow cytometry, where a subpopulation of cells was negative for CD90.

3.5. Immunohistochemistry

The immunohistochemistry of tissue slices demonstrated the location of CD90-positive cells inside the tissue structure (Figure 9). In the dental pulp positive cells which showed a weak signal could be found diffuse inside the tissue. Cells with a high-intensity signal were localized peripheral in the area of the subodontoblastic layer. Also, the odontoblasts inside this area showed a strong signal for CD90. This was especially seen inside longitudinally broached dentine tubuli, where the odontoblastic extensions are located. Inside the PDL, perivascular localized cells with an intense signal were found. However, cells inside the PDL

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tissue showed a weaker diffuse signal, which was similar to those inside the dental pulp. No signal was detected in cells inside the alveolar bone and cementum.

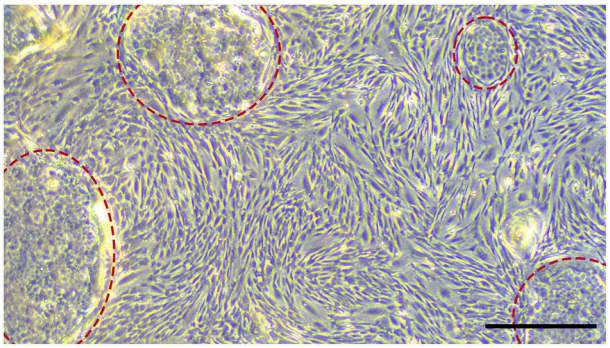

FIGURE 6
MSCs isolated from the youngest donor (2.5 years) mixed with a subpopulation of cells, which formed cobblestone-like clusters (highlighted with red circles). Scale bar represents 500 µm.

4. Discussion

We established a potential method to isolate MSCs from equine dental pulp and PDL simultaneously out of the same tooth. Furthermore, we demonstrate the localization of these cells *in situ*. The obtained cells of all donors were viable and showed important characteristics of MSCs, such as a spindle-shaped morphology and a characteristic content of surface markers, e.g., CD90 and CD44. In addition, we demonstrate the diffuse particular perivascular location of CD90-positive cells inside the PDL and their diffuse, more specifically subodontoblastic location, inside the dental pulp.

4.1. Cell isolation and cultivation

During cell cultivation, the variations in time the cells needed to attach and become confluent were conspicuous. Above all, cells attached and proliferated faster when the supernatant in the cell strainer was seeded instead of the flow-through. It can be assumed that the cells in the supernatant are partially still integrated in the tissue structure, and hence, attachment and proliferation might

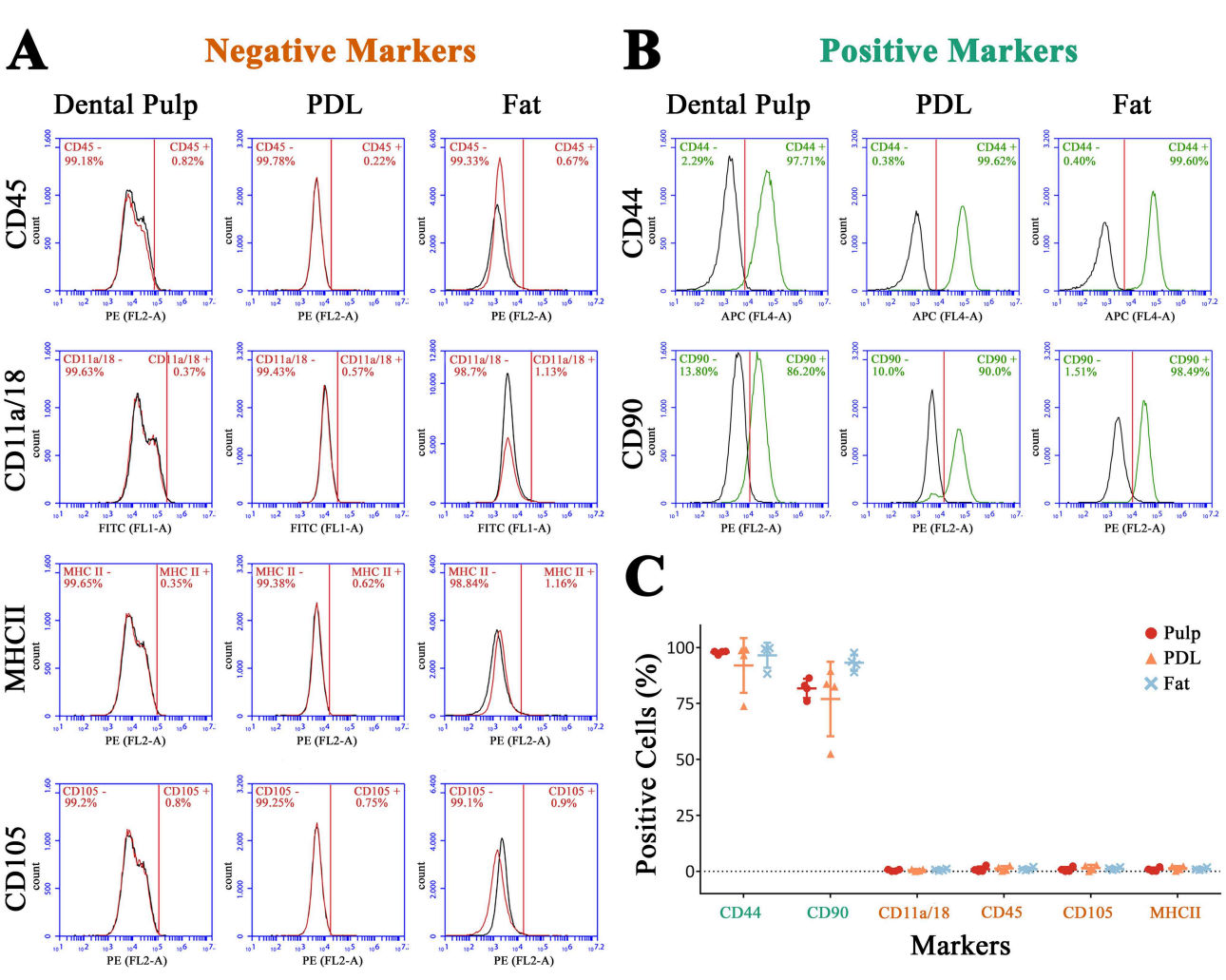

FIGURE 7
Flow cytometry for negative markers **(A)** and positive markers **(B)** of one donor exemplary. In **(C)** the ratio of positive cells for all donors is shown.

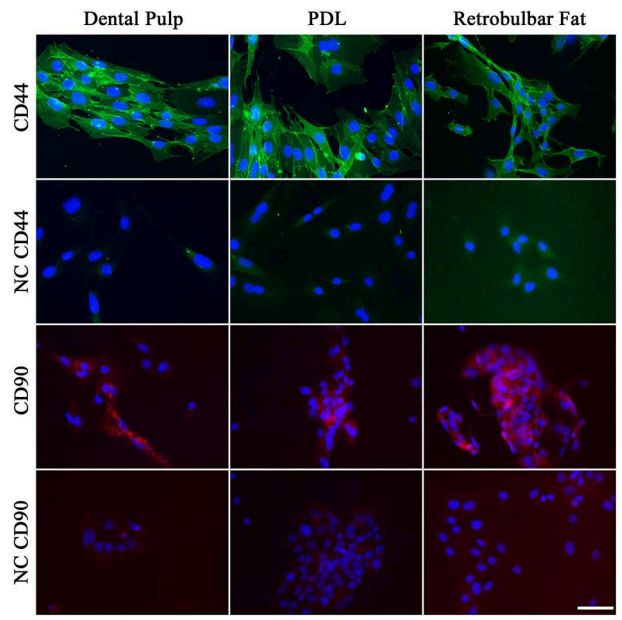

FIGURE 8
Immunohistochemistry of MSCs for CD44 (green) and CD90 (red). The nuclei were stained with DAPI (blue). Negative controls (NC) are shown beneath the sample. Scale bar represents 50 μm.

be easier, compared to when the cells are separated in the flow-through. Furthermore, it is possible that more cells remain in the cell strainer than are filtered. Most authors describe the isolation of dental MSCs for other species after filtration through a 70-μm^2 cell strainer to reduce extracellular components [e.g., (28, 29)]. Possibly, after filtration, the purity of the cell population is higher. However, during the isolation of equine dental MSCs, commonly, no filtering step is included (12, 14, 15), and our results indicate that a suitable cell population can be easily gained without filtering.

Most cells were gained from the retrobulbar fat and fewest from the dental pulp. This might be due to the high amount of fat tissue, which was easily obtained, instead of the small portion of PDL and dental pulp (Figure 4). From the youngest donor (2.5 years), more PDL and pulp tissue could be gained than from the donors aged 13–24 years. Subsequently, less cells were isolated from aged donors, and the cells needed more time to attach to the surface and grow confluent. Because of the eruption of the tooth and therewith reduction of especially pulpal tissue, fewer material might be obtained from older donors (30, 31). In addition, the pulpal cavity is steadily filled up with new layers of dentine and is therefore constantly becoming smaller (32). Schrock et al. (31) describe a starting reduction of incisor length at an age of 13–15 years. Since we found no obvious difference in cell growth in horses aged 13–24 years, a key factor for the reduction of obtained pulpal tissue in aged horses might be their smaller pulp cavity (27, 32). Even the conditions of the teeth and donor seemed to play a role since an infected tooth or a sepsis due to colic caused bacterial contamination during cultivation. Due to their resistance, it was important to avoid any contamination with yeasts, which are sometimes located in the oral cavity (33). Furthermore, the surrounding hard substances hamper the isolation of the soft tissue of teeth. Nevertheless, even using donors older than 20 years, it was possible to isolate viable cells, despite the reduction in pulpal tissue with increasing age (32).

4.2. Morphology of MSCs

Some cells isolated from the PDL of the youngest donor formed cobblestone-like clusters. This effect might be due to the impurity of cementoblasts, which are adjacent to the PDL and might easily get access to the culture during cell isolation. Because of the different condition in younger teeth, were the PDL detaches very easily together with cementoblasts from the teeth it is quite possible that even in younger donors cementoblasts are transferred into the culture. Staszyk and Gasse (13) describe equine cementoblasts as solitary flattened wide cells which build cobblestone-like clusters with increasing density. The authors identified cementoblasts by a missing expression of alkaline phosphatase, smooth muscle actin, and pro-collagen. As we obtained high amounts of similar cells in cultures of the youngest donor, we also cannot exclude the presence of epithelial cells derived from the enamel organ. Further investigations should, for example, include the detection of pan-cytokeratin to identify epithelial cells. Nevertheless, it is more likely that during the extraction of PDL, parts of the cementum remained adherend to the PDL and afterwards were displaced to the culture. Thus, for younger donors an adjustment of the isolation method is required. One ought to make sure that the scraping with the bone rongeur forceps is only implemented on the side of the PDL which is adjacent to the alveolar bone.

4.3. Cell surface marker

CD44 is largely applied and described as a positive marker for MSCs (12, 14, 15, 34), whereas CD11a/18 and CD45 are classified as negative markers (14, 15). This fits our finding as an average of 97.79% ± 0.59 of MSCs isolated from dental pulp and 92% ± 9.46 isolated from PDL expressed CD44. CD11a/18 was expressed by < 1% of cells from dental pulp and PDL and CD45 by <2.6%. For CD90, a subpopulation of cells was negative, which supports the heterogeneity of this marker. Barberini et al. (19) reported that 67.7% of MSCs isolated from the umbilical cord of horses expressed CD90, in contrast to other studies where the level was higher. According to Paebst et al. (21), MSCs isolated from adipose tissue showed the highest expression of CD90. This is consistent with our finding that MSCs from the retrobulbar fat body showed the highest expression of CD90. Nevertheless, in their study, only 24.4 ± 14.43% of cells isolated from fat tissue were positive for CD90. Our findings showed a substantially higher expression of 93.15 ± 2.8% of cells from the retrobulbar fat body. Unfortunately, in the literature, there are no quantities given for the expression of CD90 by MSCs isolated from equine dental pulp and periodontium; it is only reported that the expression is high (12, 14). Some authors describe CD105 as stemness marker (12) and some do not (20). In general, the expression of surface-markers is varies depending on the source (21) and even the individuum. In particular, MHCII expression is described as being

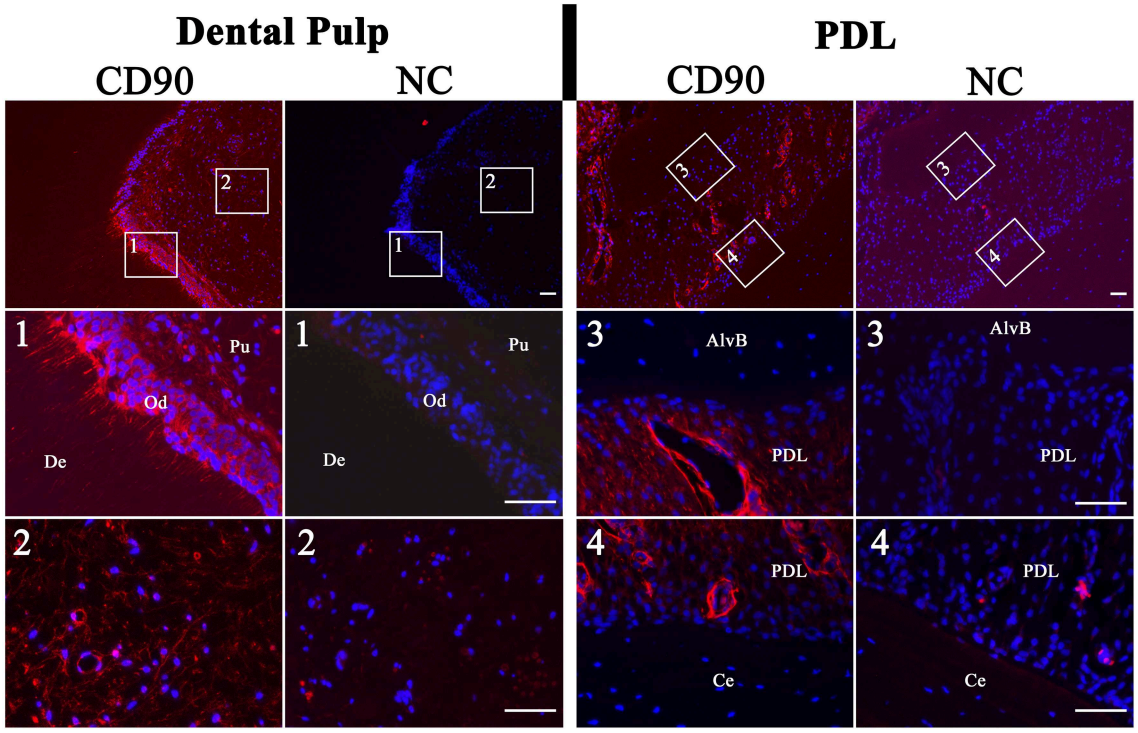

FIGURE 9
Immunohistochemistry of dental pulp and PDL for CD90 (red). The nuclei were stained with DAPI (blue). Associated negative controls (NC) are pictured besides the samples. The dental pulp showed a high-intensity signal of the odontoblastic layer (Od). Even inside the longitudinally broached dentine tubuli (De) and within the dental pulp (Pu) a signal was detected. The PDL showed a diffuse signal and a more intense perivascular signal. Cells inside the alveolar bone (AlvB) and cementum (Ce) were negative. Scale bar represents 50 μm.

inhomogeneous among different breeds and individuals (35, 36). In this study, the expression of all donors was <2.5%, which might later promote an allogenic application (36). The MSCs isolated from PDL appeared to be most inhomogeneous, which might be due to the proximity to the cementum, which promotes the impurity with cementoblasts. Nevertheless, only one donor showed a heterogeneous cell morphology and it should be taken into account that the surface marker expression of MSCs isolated from different equine tissues is quite various (21).

The descending rate of living cells from retrobulbar fat to PDL to pulp might be due to the greater senescence of MSCs isolated from PDL and dental pulp. The smaller number of cells obtained particularly from the dental pulp might lead to a faster senescence, on account of the required higher proliferation to become confluent. However, this tendency was not significant, and more donors ought to be analyzed.

4.4. Immunohistochemistry

The immunocytochemistry of the cells largely fits the flow cytometry. Most of the cells were positive for CD44. Although the signal for CD90 was missing in some cells, most cells were positive. This matches the results of the flow cytometry, where a subpopulation of cells was negative for CD90.

In addition, the immunohistochemistry of dental pulp and PDL clearly showed CD90-positive cells.

Inside the dental pulp, these cells were mainly found in the subodontoblastic layer, which is also described for rat by Hosoya et al. (6). This might support the theory that undifferentiated cells, which can differentiate toward odontoblasts, are provided by the subodontoblastic layer (37, 38). There was a remarkably strong signal of odontoblasts, even inside their processes, which has not been reported yet. Sano et al. (38) describe no immunoreactivity for CD90 in odontoblasts adjacent to the predentin in brachydont teeth of rats. However, after the authors had performed a cavity preparation, odontoblasts and subodontoblastic cells were disarranged and vanished, and the CD90 expression decreased after 1 day of preparation and increased again after 5 days. This result indicates that CD90-expressing cells play a role in the regeneration of subodontoblastic cells and odontoblasts (38). In addition, Hosoya et al. (6) describe an absent immunoreactivity for CD90 in odontoblasts of rat molars and incisors. Furthermore, it seems that CD90 is not expressed in the early phase of odontoblast development and thus appears later, when differentiation is proceeding. Until now, there is no description of CD90 expression inside the equine dental pulp. However, in the odontoblasts of rat brachydont molar and hypselodont incisors, CD90 expression seems to be missing (6). This is in contrast to our findings for the equine hypsodont tooth. In the study of Sano et al. (38), some CD90-positive cells in the superficial odontoblastic layer were found 3 days after cavity preparation. This might lead to the assumption of a steady process of lifelong odontoblast regeneration in unaffected

equine teeth, which resembles the process in brachydont teeth after cavity preparation. Furthermore, the results support the findings of Roßgardt et al. (27) that the equine dental pulp remains lifelong in an immature highly productive status and contains a subodontoblastic supportive zone to ensure the continuous production of dentin.

In the PDL CD90-positive cells were localized perivascular, showing a strong signal and diffuse inside the tissue with a weaker signal. Esteves et al. (39) reported that MSCs might originate from pericytes and retain some of their features in culture. This promotes our finding of perivascular cells with a strong positive CD90 expression, which might reach the cell culture. Zhao et al. (40) describe perivascular-associated CD90-positive cells inside the PDL of mice. They experimentally induced periodontitis of the upper second molars and found out that CD90-positive cells recover their ability to form cementoblasts under these conditions. Additionally, an increase in mechanical force seems to reactivate the C90-positive cell population to differentiate to cementoblasts. This process might also be found in the PDL of unaffected equine teeth permanently exposed to mechanical force by eruption and dental wear. Thus, we detected large amounts of perivascular CD90-positive cells inside the PDL of the hypsodont equine tooth in contrast to the condition in brachydont molars of adult mice (40).

As expected, the cells inside the cementum and alveolar bone showed no immunoreactivity for CD90 since they were differentiated cementoblasts and osteoblasts.

4.5. Conclusion

We developed a feasible method to isolate and cultivate cells from equine dental pulp and PDL. Our evaluation of morphology and surface marker content indicated that the obtained cells possessed features of MSCs. Although standardized criteria for equine MSCs are missing, not least because of their inhomogeneity, the odontoblastic and subodontoblastic localization of CD90-positive cells inside the dental pulp is an indicator for the lifelong remodeling since in brachydont teeth, CD90 is missing in the odontoblastic layer (6). The perivascular localization of CD90-positive cells inside the PDL is a hint that the MSCs originated from pericytes (11). Furthermore, there are larger amounts of these perivascular cells than described for molars of adult mice (40). Both the equine dental pulp and PDL show adjustments to the permanent dental wear and eruption by the alteration of CD90-positive cells, which play an important role during cell differentiation. These findings suggest that equine MSCs inside dental pulp and PDL are promising for further approaches to understand the processes in horse teeth during eruption, providing an opportunity for future starting points in equine dentistry.

4.6. Limitations of the study

One of the most important limitations of this study is the small sample size. To make clearer statements regarding the impacts of donor age and tissue source on the MSCs, a larger sample size is required. Nevertheless, the results show that the described method can be used to isolate viable MSCs from equine dental pulp and PDL of the same teeth, even if the donor is aged.

Especially when regarding its high expression rate, CD44 seems to be a better immunohistochemical marker for the localization of MSCs inside the tissues. Unfortunately, we found no well-performing CD44 antibody for immunohistochemistry. However, many authors describe parallels of pericytes *in vivo* with MSCs *in situ* [e.g., (11, 41–43)]. This fits our findings of strong CD90-positive cells inside the perivascular region of the PDL. In dental pulp, it seems that the odontoblastic and subodontoblastic layers are a source for MSCs. This finding is in line with the high remodeling rate inside this area (44).

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the animal study because all horses included in the study were slaughtered on ground unrelated to the study. Due to this no ethical approval was required. The associated kTV number given by the regional council is 19 c 20 15 h 02 Gi 18/17 kTV 5/2021.

Author contributions

LH, JR, and CS were responsible for conceptualization and study design. LH, JR, JD-W, JV, and CS performed the experiments. Original draft preparation and revision was conducted by LH and CS for visualization LH and for supervision CS was responsible. All authors have read and agreed to the published version of the manuscript.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1116671/full#supplementary-material

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Basal and inducible Osterix expression reflect equine mesenchymal progenitor cell osteogenic capacity

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Introduction: Mesenchymal stem cells are characterized by their capacities for extensive proliferation through multiple passages and, classically, tri-lineage differentiation along osteogenic, chondrogenic and adipogenic lineages. This study was carried out to compare osteogenesis in equine bone marrow-, synovium- and adipose-derived cells, and to determine whether osteogenic capacity is reflected in the basal expression of the critical osteogenic transcription factors Runx2 and Osterix.

Methods: Bone marrow, synovium and adipose tissue was collected from six healthy 2-year-old horses. Cells were isolated from these sources and expanded through two passages. Basal expression of Runx2 and Osterix was assessed in undifferentiated third passage cells, along with their response to osteogenic culture conditions.

Results: Bone marrow-derived cells had significantly higher basal expression of Osterix, but not Runx2. In osteogenic medium, bone-marrow cells rapidly developed dense, multicellular aggregates that stained strongly for mineral and alkaline phosphatase activity. Synovial and adipose cell cultures showed far less matrix mineralization. Bone marrow cells significantly up-regulated alkaline phosphatase mRNA expression and enzymatic activity at 7 and 14 days. Alkaline phosphatase expression and activity were increased in adipose cultures after 14 days, although these values were less than in bone marrow cultures. There was no change in alkaline phosphatase in synovial cultures. In osteogenic medium, bone marrow cultures increased both Runx2 and Osterix mRNA expression significantly at 7 and 14 days. Expression of both transcription factors did not change in synovial or adipose cultures.

Discussion: These results demonstrate that basal Osterix expression differs significantly in progenitor cells derived from different tissue sources and reflects the osteogenic potential of the cell populations.

KEYWORDS

osteogenesis, mesenchymal stem cells, Runx2, Osterix, bone formation

Introduction

Mesenchymal stem cells (MSC) are characterized by their capacities for extensive proliferation through multiple passages and multi-lineage differentiation; classically, along osteogenic, chondrogenic and adipogenic lineages (1, 2). Accepting these common features, the specific phenotypic and functional capacities of MSCs derived from different sources vary considerably, despite very similar isolation, *in vitro* expansion and differentiation protocols (3–9). The underlying

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TABLE 1 PCR primer sequences.

Gene (size)	Sense primer / Antisense primer	Annealing temperature
Runx2 (177 bp)	5′CAGACCAGCAGCACTCCATA (1,315)	57.7°C
	5′CAGCGTCAACACCATCATTC (1,492)	
Osterix (207 bp)	5′GGCTATGCCAATGACTACCC	57.7°C
	5′GGTGAGATGCCTGCATGGA	
ALP (221 bp)	5′TGGGGTGAAGGCTAATGAGG (357)	57.7°C
	5′GGCATCTCGTTGTCCGAGTA (578)	
EF1-α (328 bp)	5′CCCGGACACAGAGACTTCAT (48)	57.7°C
	5′AGCATGTTGTCACCATTCCA (376)	

mechanisms responsible for these lineage predispositions are not well understood. Therapeutic MSC applications derive from their ability to differentiate and contribute directly to tissue repair, regulate the activities of adjacent cells through trophic effects and/or immunomodulate host responses (10–16). During skeletal repair, osteo-progenitors from the periosteum and marrow cavity contribute directly to bone regeneration (17, 18). Strategies designed to stimulate endogenous osteoprogenitor activities or deliver exogenous stem cells to fracture sites have considerable potential to improve fracture repair by accelerating the time to skeletal stabilization. Self-evidently, therapeutic cells need to be capable of robust and rapid osteogenic differentiation for clinical efficacy.

Osteogenesis is one of the primary differentiation pathways used to characterize MSCs. The *in vitro* requirements for this process and informative phenotypic indices have been clearly defined, and transcriptional regulation of osteogenesis in developmental contexts has been well-characterized. Two transcription factors, Runx2 and Sp7/Osterix (OSX), are mandatory for this pathway, as clearly demonstrated in murine gene deletion models (19–21). Developmentally, Runx2 induces OSX expression and, collectively, these transcription factors drive expression of genes required for skeletogenesis (22).

This study was carried out to compare the osteogenic capabilities of three equine putative MSC populations, derived from bone marrow (BM), synovium (SYN) and adipose tissue (ADI), and to determine whether any osteogenic lineage predisposition is reflected in the expression of core osteogenic transcription factors under basal (non-induced) culture conditions. Both bone marrow- and adipose-derived MSCs are used in a wide range of clinical applications (23–26), while synovium-derived MSCs are representative of progenitors particularly predisposed to chondrogenic differentiation and have been applied experimentally for intra-articular therapy and articular cartilage repair (27, 28). The experiments were designed to test the hypothesis that basal and inducible Runx2 and OSX expression reflects the osteogenic capacity of equine progenitor populations.

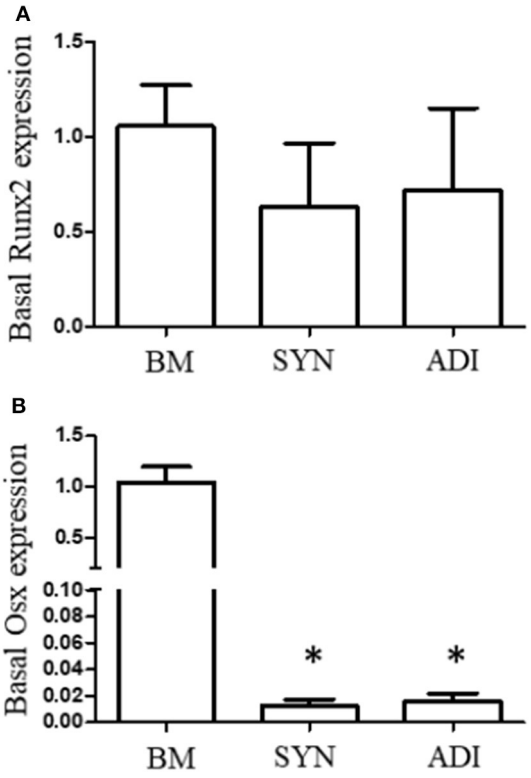

FIGURE 1
Basal expression of Runx2 **(A)** and Osterix **(B)** mRNAs in bone marrow- (BM), synovial- (SYN) and adipose- (ADI) third passage cells in control medium. Mean expression levels in BM samples were set at "1" in each analysis. Asterisks indicate mean + SE values significantly different from BM levels of expression (ANOVA $n = 6$; $P < 0.05$).

Materials and methods

Bone marrow aspirate, synovium and adipose tissue collection

Bone marrow (BM), synovium (SYN) and adipose (ADI) tissue were collected from six healthy 2-year-old horses that were being euthanized at the termination of an unrelated study. The use of these horses for this study was approved by the Institutional Animal Care and Use Committee. Horses were sedated with 1.0 mg of xylazine/kg IV. Anesthesia was induced with 2.2 mg of ketamine/kg and 0.1 mg of diazepam/kg and maintained with 5% guaifenesin solution containing 1 mg of ketamine/L and 1 gm of xylazine/L.

To collect bone marrow, the skin over the tuber coxae was clipped and aseptically prepared. A stab incision was made through the skin with a #11 scalpel blade and 10–15 ml of bone marrow was aspirated through a Jamshidi biopsy needle into a syringe containing 1,000 IU of heparin. Following collection of bone marrow aspirates, all horses were euthanized with an intravenous injection of 104 mg of sodium pentobarbital/kg. Adipose tissue and synovium were collected immediately following euthanasia.

Adipose tissue was collected from the subcutaneous depot lateral to the tail head. The skin was clipped and disinfected,

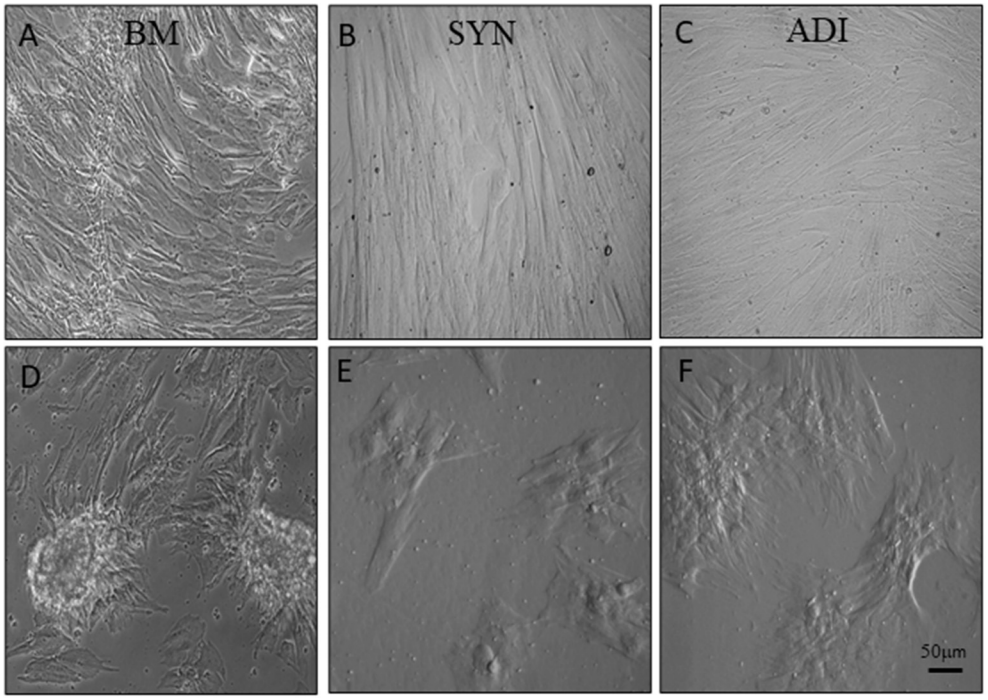

FIGURE 2
Representative microscopic images of bone marrow- [BM: **(A, D)**], synovial- [SYN: **(B, E)**] and adipose- [ADI: **(C, F)**] third passage cells in control **(A–C)** and osteogenic **(D–F)** medium after seven days.

8–10 g of adipose tissue was collected through a 10–15 cm skin incision and placed in a 50 ml polypropylene tube containing sterile phosphate buffered saline (PBS) solution. For synovium collection, the skin over the right radiocarpal joint was clipped, aseptically prepared and then reflected by sharp dissection to expose the dorsal aspect of the carpus. A transverse incision was made through the dorsoproximal aspect of the radiocarpal joint capsule and the synovial membrane was exposed by inverting the capsule. Approximately 2–3 g of synovium was excised from the inner surface of the capsule and placed into a 50 ml polypropylene tube containing sterile PBS solution.

Cell isolation and monolayer expansion

Bone marrow aspirates were diluted with 10 ml of PBS and centrifuged at 300 g for 15 mins. The cell pellet was washed with PBS and re-centrifuged. The supernatant was removed, and the cell pellet was re-suspended with 0.8% ammonium chloride to lyse red blood cells. The remaining nucleated cells were pelleted by centrifugation, as above, re-suspended and cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, Corning, NY), supplemented with 10% fetal bovine serum (FBS; GeminiBio, West Sacramento, CA) and 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD; growth medium) until the primary monolayers reached 80% confluence.

Synovial tissue was digested in 0.25% trypsin/EDTA (Corning) at 37°C for 30 mins. Subsequently, the tissue was transferred to 0.1% collagenase (type II; Worthington Biochemical Corporation, Lakewood, NJ) in DMEM (10 ml of medium/gram of tissue) supplemented with 10% FBS and 2% penicillin/streptomycin for 2 h at 37°C in a shaking incubator. Adipose tissue was diced into small pieces and digested for 3 h at 37°C in 0.2 % collagenase (type II; Worthington) in DMEM (Gibco-ThermoFisher Scientific, Waltham, MA: 10 ml of medium/gram of tissue) and 2% penicillin/streptomycin (Gibco). After digestion, SYN and ADI cells were filtered through 40 µm mesh filters (Corning) and collected by centrifugation at 300 g for 10 mins. The numbers of primary ADI and SYN cells were determined with a hematocytometer and cellular viability was assessed by trypan blue exclusion. Primary SYN and ADI cells were seeded at 5×10^3 cells/cm^2 in 100 mm culture plates (Corning) and maintained in DMEM/10% FBS at 37°C in 5% CO_2. The medium was changed three times per week.

When the primary BM, SYN and ADI cultures reached 80% confluence, the monolayers were lifted by brief 0.05% trypsin-EDTA (Gibco) digestion. The primary cell isolates were passaged twice, at initial seeding densities of 5×10^3 cells/cm^2, to enrich for highly and persistently proliferative progenitor cells and generate sufficient numbers of third passage cells for subsequent differentiation experiments.

Osteogenic cultures

Cells were seeded at 2×10^4 cells/cm^2 in DMEM/10% FBS (control medium) and maintained until the monolayers were 70–80% confluent. Cultures for Alizarin Red, von Kossa and ALP

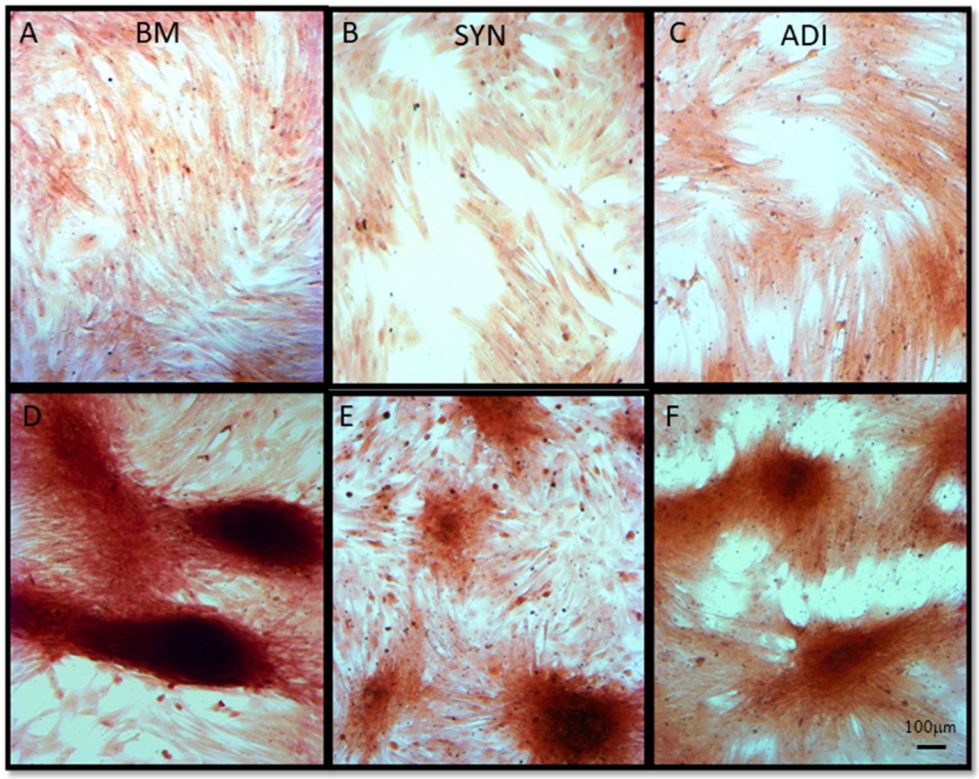

FIGURE 3
Representative microscopic images of bone marrow- [BM: **(A, D)**], synovial- [SYN: **(B, E)**] and adipose- [ADI: **(C, F)**] third passage cells in control **(A–C)** and osteogenic **(D–F)** medium after 14 days, stained with Alizarin Red solution to demonstrate the presence of ionized calcium deposition in basal **(A–C)** and osteogenic **(D–F)** cultures.

staining, and for RNA isolation were seeded in six well plates, while cultures designated for ALP activity assays were seeded in 12 well plates (Corning). Control cultures remained in DMEM/10% FBS, while osteogenic cultures were transferred to control medium supplemented with 100 nM Dexamethasone (Sigma-Aldrich, St. Louis, MO), 10 mM β-Glycerophosphate (Sigma-Aldrich) and 50 µg/ml ascorbic acid (Wako Pure Chemical Industries, Japan). The responses of BM, SYN and ADI cells to osteogenic medium were monitored daily *via* light microscopy, representative images were recorded (Leica Microsystems, Leica Application Suite—LAS—version 2.6.R1) and phenotypic transition was assessed after 7 and 14 days, as detailed below.

Alizarin Red staining

After 7 and 14 days, the monolayers were rinsed with PBS, fixed in 10% formalin for 30 mins, then washed three times with distilled water. One ml of fresh 2% Alizarin Red (Sigma-Aldrich) solution (pH 4.1) was added to each well. Following incubation at room temperature for 20 mins on a shaking platform, the stain was removed, and the cells were washed with distilled water until the rinse solution was clear. Mineral deposits within the cell layers were stained bright red. Representative pictures of stained monolayers were obtained, as above.

Von Kossa staining

Von Kossa stain (American MasterTech, Lodi, CA) was used to identify ionized phosphate in basal and osteogenic cultures. Following 30 mins fixation with 10% formalin, the cell layers were washed 2–3 times with distilled water. One ml of 5% silver nitrate solution was added to each well and exposed to a strong light for 30 mins. The cell layers were washed 2–3 times with distilled water and 1 ml of 5% sodium thiosulfate was added for 5 mins to remove excess silver salts. The cell layers were washed 2–3 times with distilled water. Finally, a neutral red solution was added for 5 mins as a counterstain. Calcium deposits in the extracellular matrices were evident as dark brown or black deposits within the cell aggregates. Representative images of stained monolayers were obtained, as above.

Alkaline phosphatase staining

After 7 and 14 days, cell layers were fixed with citrate-acetone-formaldehyde fixative solution for 1 min followed by three washes with distilled water. An alkaline dye consisting of a diazonium salt solution and naphthol AS-BI alkaline solution (Procedure No. 86, AP, leukocyte; Sigma Aldrich) was added to the cell layer and incubated in reduced light conditions at

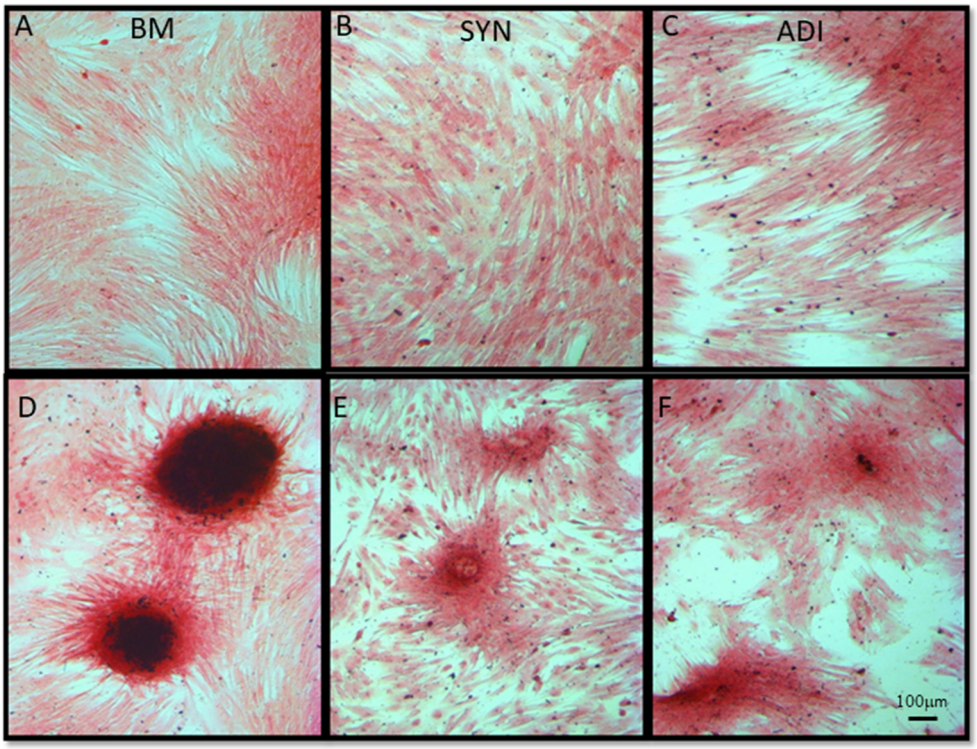

FIGURE 4
Representative microscopic images of bone marrow- [BM: **(A, D)**], synovial- [SYN: **(B, E)**] and adipose- [ADI: **(C, F)**] third passage cells in control **(A–C)** and osteogenic **(D–F)** medium after 14 days, stained with von Kossa stain to demonstrate the presence of ionized phosphate deposition in basal **(A–C)** and osteogenic **(D–F)** cultures.

room temperature for 15 min. The monolayers were washed again with distilled water, and the cell layers were then counterstained with neutral red solution for 5 min. Cells exhibiting ALP activity were marked by blue staining. Representative pictures of the stained monolayers were obtained by microscopy and digital photography.

Alkaline phosphatase enzymatic activity

ALP activity was assessed in triplicate samples of control and osteogenic cultures from each donor. At days 7 and 14, the cells were harvested in 1 ml of lysis buffer containing 20 mM Tris HCl, 150 mM NaCl and 1% Triton X-100 (Sigma-Aldrich). Each sample was homogenized using an IKA Labortechnik T 25 basic homogenizer (Janke and Kunkel GmbH and Co, Staufen, Germany), centrifuged at 2,500 rpm for 15 mins at 4°C and kept on ice for 30 mins. Two 100 µl aliquots of the lysates were collected for DNA measurements (see below). The supernatants were assayed for ALP activity using an AP assay kit (Wako), following the manufacturer's instructions. The concentration of *p*-Nitrophenol was measured at 405 nm wavelength (FLUOstar OPTIMA, BMG, Lab Technologies, Cary, NC). The relative activity in each lysate was normalized to DNA content (see below).

DNA measurement

DNA content was used as a surrogate indicator of cell number to normalize ALP activity data. DNA was measured using the Pico Green DNA kit (Quanti-iT^{TM} PicoGreen dsDNA, Invitrogen). Serial dilutions of calf thymus DNA were used to generate a standard curve. Duplicate 100 µl aliquots of each lysate were diluted 1:5 in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and transferred to black 96-well microplates (Corning). One microliter of Pico Green reagent diluted in 200 µl of TE buffer was added to each sample. Following 5 mins incubation in a lightproof container, fluorescence was measured at 485 nm wavelength (FLUOstar OPTIMA).

RNA isolation and quantitative PCR analyses

Total RNA was isolated using TRIzol® (Invitrogen Corporation), according to the manufacturer's recommended protocol. The lysates were homogenized prior to chloroform addition. One microgram of total RNA was reverse-transcribed, using oligo dT to prime the reactions (Superscript^{TM} First-Strand Synthesis System for RT-PCR, Invitrogen Life technologies, Carlsbad, CA).

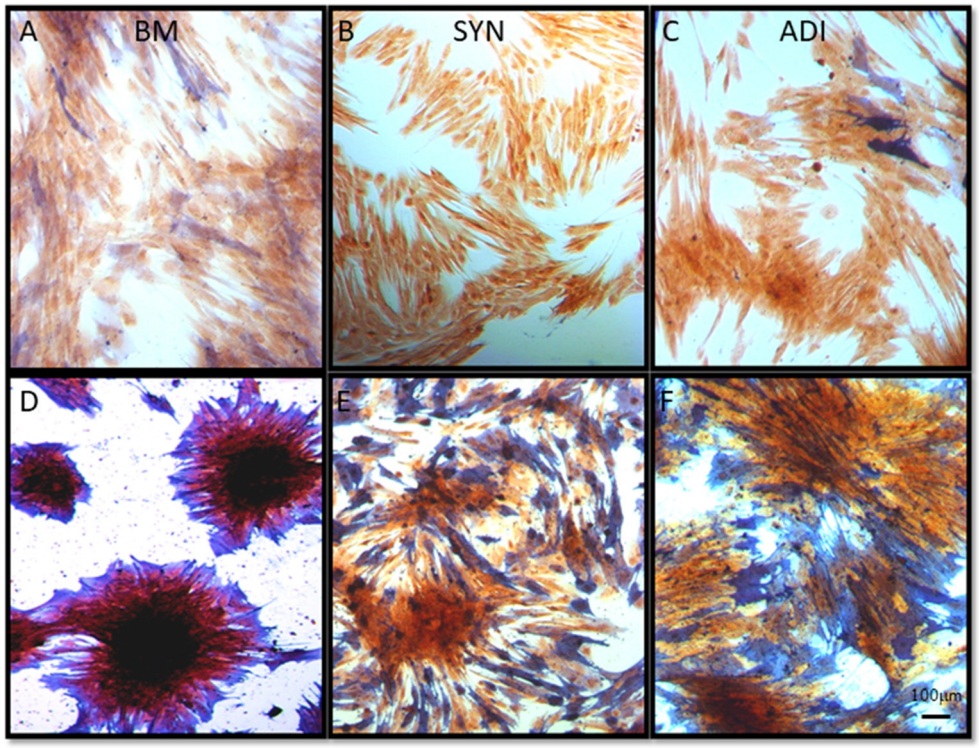

FIGURE 5
Representative microscopic images of bone marrow- [BM: **(A, D)**], synovial- [SYN: **(B, E)**] and adipose- [ADI: **(C, F)**] third passage cells in control **(A–C)** and osteogenic **(D–F)** medium after 14 days, stained with ALP solution to demonstrate the presence of cell-associated ALP activity.

ALP, Runx2 and OSX transcript levels was measured by quantitative real-time PCR (qPCR), normalized to expression of the reference gene, elongation factor-1 alpha (EF1-α). The primers used for qPCR analysis are listed in Table 1. For all primers, the optimum annealing temperature was determined to be 57.7°C. During initial primer optimization trials, the amplicons were sequenced to ensure that the correct transcript was being amplified. Quantitative PCR was performed using 5 µl of diluted cDNA template (1:10 dilution) combined with 20 µl of a mixture composed of 12.5 µl 1 × SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 1 µl each of the 10 µM forward and reverse primer stocks and 5.5 µl DNase/RNase-free water in a 96-well microplate. Each sample was run in triplicate. The PCR reactions were run in a BioRad iCycler iQ (BioRad) using the following conditions: initial denaturation for 3 mins at 95°C, 40 cycles of denaturation at 95°C for 10 s, annealing temperature of 57.7°C for 30 s and polymerase extension at 72°C for 20 s. The presence of a single amplicon was monitored by melting curve analyses. The relative expression for each target gene was calculated using the comparative ΔCt method (29). For analyses of basal Runx2 and OSX expression in third passage cells, the mean level of BM expression in six donor samples was assigned a value of "1." Within each data set from the control vs. osteogenic cultures, the "BM Day 7 Control" threshold cycle value in each horse was designated as "1" and the data from other times, cell sources and culture medium groups were adjusted accordingly.

Statistical analyses

Mean ± SE values were calculated for each quantitative outcome measure. Differences in the basal expression of Runx2, OSX and ALP transcripts and ALP activities in undifferentiated third passage BM, SYN and ADI cells were assessed by one-way repeated measures ANOVA. The response of each cell type to osteogenic medium (at days 7 and 14), the comparative expression of ALP, Runx 2 and OSX mRNAs under basal (control) and osteogenic conditions were assessed by two-way repeated measures ANOVA. As required, Bonferroni's *post hoc* tests were applied to identify specific significant pair-wise differences. In all analyses, P values < 0.05 were considered significant. Statistical analyses were carried out using GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA).

Results

Basal expression of Runx2 and OSX transcripts

Under basal conditions, Runx2 expression was not different between the three cell types. In contrast, basal OSX mRNA expression was significantly higher, ∼100-fold, in BM cells than in SYN and ADI cells (Figure 1).

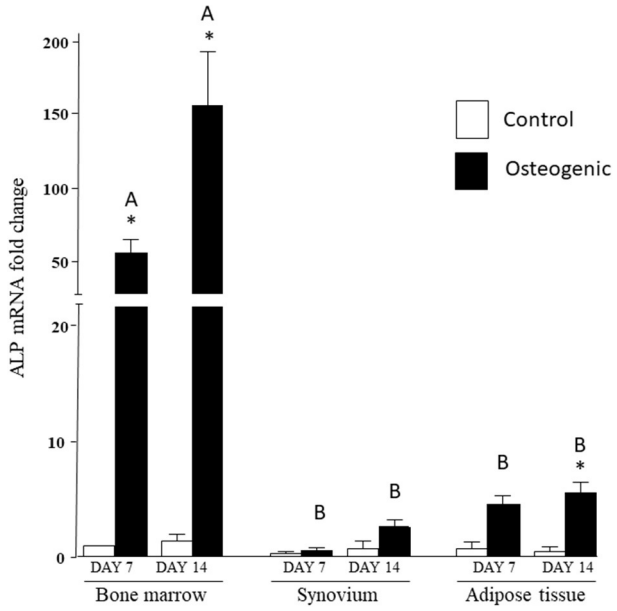

FIGURE 6
ALP mRNA expression in bone marrow-, synovium- and adipose tissue-derived cells in control (white columns) or osteogenic (black columns) medium after 7 or 14 days. Within each cell type, asterisks indicate mean + SE values significantly different from control levels of expression. Columns designated with different upper-case letters are significantly different in osteogenic cultures (two-way ANOVA $n = 6$; $P < 0.05$).

Monolayer culture responses to osteogenic medium

Cultures in control medium maintained a flattened morphology throughout the 14-day time frame of the experiments (Figures 2A–C). When BM cells were transferred to osteogenic medium, the cells rapidly aggregated to form highly refractile, multicellular clusters that were clearly evident within 7 days. In contrast, cell aggregation in SYN and ADI cultures was noticeably slower in onset and did not form multilayered aggregates characteristic of BM cultures (Figures 2D–F).

Matrix mineralization

Alizarin Red staining of the control cultures was minimal at both time points (Figures 3A–C). The cellular aggregates in the osteogenic BM cultures showed intense stain uptake at both day 7 and 14 (Figure 3D). Alizarin Red staining of osteogenic SYN and ADI cultures was restricted to the cell aggregates and was less intense than seen in BM cultures (Figures 3E, F). This was also evident in von Kossa-stained cultures. There was little or no stain uptake in control cultures (Figures 4A–C). In osteogenic BM cultures, the multicellular aggregates stained strongly for ionized phosphate by day 14 (Figure 4D), whereas phosphate deposition in osteogenic ADI and SYN cultures was minimal (Figures 4E, F).

Alkaline phosphatase induction and activity

Staining of control cultures for ALP activity demonstrated very faint and diffuse ALP signal in both the BM and ADI cultures. No stain was detectable in the SYN cultures (Figures 5A–C). Under osteogenic conditions, intense ALP activity was present within and immediately around the cell aggregates in BM cultures, whereas staining in the SYN and ADI monolayers was less intense and more diffusely distributed (Figures 5D–F).

In control medium, ALP mRNA levels were statistically similar in the three cell groups at both time points. In osteogenic SYN cultures, ALP induction did not reach statistical significance, while ALP induction in ADI cultures was significant at day 14. ALP induction in BM osteogenic cultures was significant at both time points and significantly greater (100+ fold increase) than SYN and ADI culture values (Figure 6).

Comparative ALP enzymatic activities are presented in Figure 7. The differential activities were less substantive than the transcriptional analyses but followed similar profiles. In control medium, basal ALP activity was significantly higher in BM cultures than in SYN (both time points) and ADI (day 14) cultures. In osteogenic medium, ALP activity increased significantly (~10-fold) by day 14 in BM cultures. ALP activity was also significantly increased in osteogenic ADI cultures, although activity in these cells was still significantly lower than in the corresponding BM cultures. There was no increase in ALP activity in SYN cultures maintained in osteogenic medium.

Comparative expression of Runx2 and OSX transcripts

In control medium, Runx2 mRNA expression was stable over the 14 days of culture and remained similar across the three cell types (Figure 8). In osteogenic medium, Runx2 mRNA expression increased significantly in BM cells (~5-fold) at both time points. The slight increases in Runx2 transcript levels detected in SYN and ADI cultures were not statistically significant. Runx2 up-regulation in osteogenic BM cultures was significantly higher than Runx2 expression in osteogenic SYN or ADI cultures at both time points (Figure 8).

The significant differences in basal OSX mRNA expression were maintained in control cultures throughout the 14 days of the experiments (Figure 9). Osterix expression increased significantly in osteogenic BM cultures. In contrast, there were negligible changes in OSX expression in SYN and ADI cell osteogenic cultures (Figure 9). By day 14, mean OSX mRNA levels in osteogenic cultures were ~100-fold higher in BM cells than in SYN or ADI cells.

Discussion

This study was carried out to assess the comparative osteogenic capabilities of progenitor populations derived from equine bone marrow, synovium and adipose tissue, and to determine whether any "pre-differentiation" lineage predisposition exists, as reflected in the expression of core osteogenic transcription factors.

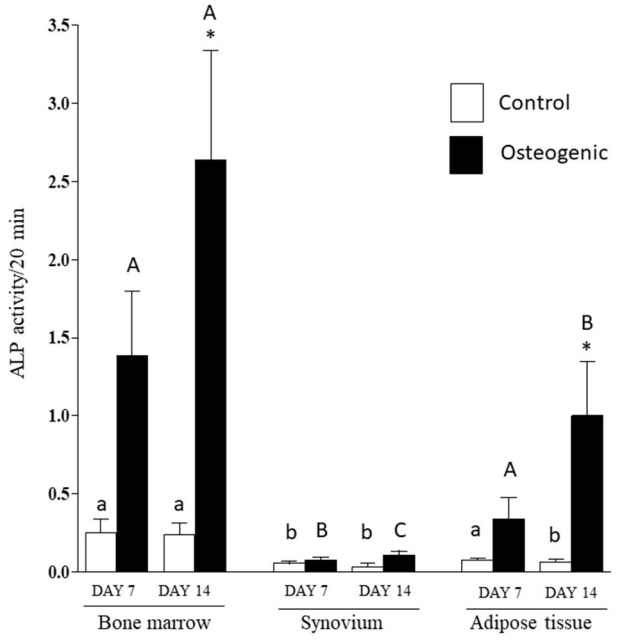

FIGURE 7
ALP enzymatic activities in bone marrow-, synovium- and adipose tissue-derived cells in control (white columns) or osteogenic (black columns) medium after 7 or 14 days. Within each cell type, asterisks indicate mean + SE values significantly different from control levels of expression Columns designated with different lower-case letters are significantly different in control cultures. Columns designated with different upper-case letters are significantly different in osteogenic cultures (two-way ANOVA $n = 6$; $P < 0.05$).

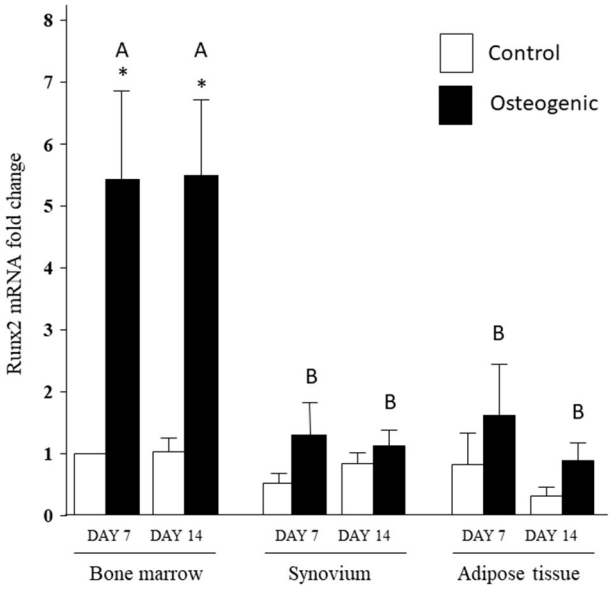

FIGURE 8
Runx2 mRNA expression in bone marrow-, synovium- and adipose tissue- derived cells in control (white columns) or osteogenic (black columns) medium after 7 or 14 days. Within each cell type, asterisks indicate mean + SE values significantly different from control levels of expression. Columns designated with different upper-case letters are significantly different in osteogenic cultures (two-way ANOVA $n = 6$; $P < 0.05$).

The study addressed the hypothesis that basal Runx2 and OSX expression reflects the osteogenic capacity of MSC populations. Not surprisingly, given the proximity of bone marrow-derived progenitors to sites of bone homeostasis, BM-MSCs were far more capable of osteogenic differentiation than cells isolated from synovium or adipose tissue within the 14-day time frame of the differentiation experiments, as indicated by significant differences matrix mineralization, ALP induction and activity. These observations do not preclude the possibility that osteogenic ADI and SYN cultures might have expressed osteogenic phenotypes more robustly, with longer times in culture.

Surprisingly, Runx2 expression under basal conditions did not differ between the cell groups at any time point. In this respect, our hypothesis was disproved. In marked contrast, basal OSX expression was substantially higher in BM cells and this differential expression profile was maintained in control cultures during the subsequent osteogenesis phase of the analyses. Further, the significant up-regulation of OSX mRNA levels in BM cells exposed to osteogenic medium did not occur in the other cell groups. These differential OSX expression profiles do support the hypothesis and suggest that screening for basal OSX expression could be used to identify cell populations with high osteogenic potentials. It also supports the use of OSX induction or expression strategies to increase or induce the osteogenic differentiation in cell populations for cell-based therapeutics, as has been experimentally demonstrated in bone marrow stromal cells (30).

Our experimental protocol did not include surface marker-based cell sorting or immunophenotyping to isolate or enrich for MSCs. We did apply prolonged, multi-passage *in vitro* expansion, using initially low seeding densities, to enrich our experimental populations for cells capable of sustained proliferation over many population doublings. Ranera et al. used isolation and expansion protocols almost identical to those used in the current study to demonstrate very similar immunophenotypes in third passage equine adipose- and bone marrow-derived cell populations, characteristic of MSCs (31). Heo et al. performed similar analyses in human progenitor cell populations, also using very similar isolation and expansion protocols, with the same finding (32). Unquestionably, our initial isolates from all three sources contained heterologous cell populations, however, the outcomes of the above-mentioned studies indicate that progenitor enrichment is achieved through multi-passage proliferative expansion of primary isolates.

Developmentally, OSX transcriptional expression is directly controlled by Runx2 trans-activity (19, 21, 22). Our results suggest that this regulatory pathway is not dominant in postnatal stem cell populations, and that factors distinct from Runx2 regulate OSX expression in adult progenitor populations. Both BMP and TGF-β signaling pathways upregulate OSX *via* a Runx2-independent transcriptional pathway, and Wnt and FGF signaling also impact OSX expression (33–36). It is plausible that differences in intrinsic signaling activities through one or more of these pathways determine the intrinsic osteogenic potentials of stem cell populations derived from different tissues, reflected in the levels of basal and inducible OSX expression (37).

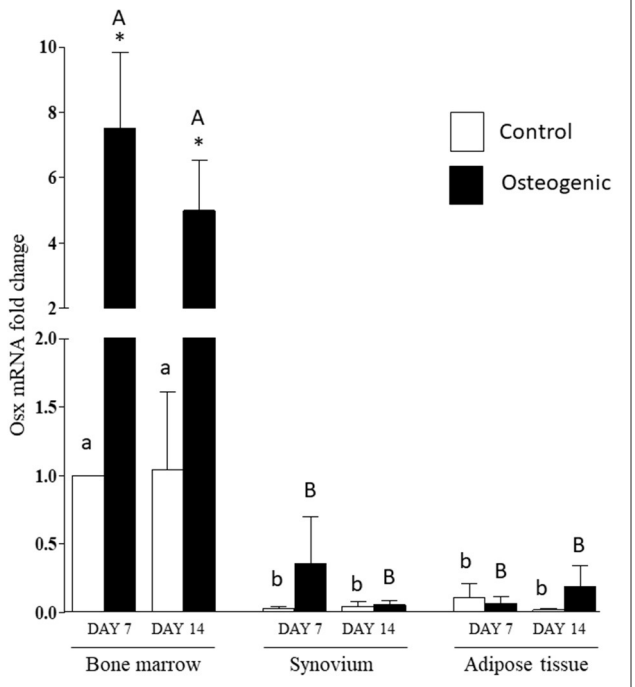

FIGURE 9
OSX mRNA expression in bone marrow-, synovium- and adipose tissue- derived cells in control (white columns) or osteogenic (black columns) medium after 7 or 14 days. Within each cell type, asterisks indicate mean + SE values significantly different from control levels of expression columns designated with different lower-case letters are significantly different in control cultures. Columns designated with different upper-case letters are significantly different in osteogenic cultures (two-way ANOVA $n = 6$; $P < 0.05$).

The results of this study highlight the somewhat qualitative aspect of MSC phenotypic assessment through monolayer culture staining, as is commonly performed to demonstrate tri-lineage potential of putative MSC isolates. We assessed the ability of each cell type to undergo osteogenesis, using commonly used matrix staining protocols and statistically significant increases in Runx2, OSX and ALP expression or activity as criteria for differentiation. As expected, BM-MSCs were capable of robust and consistent osteogenic differentiation by all criteria used within the 14-day period of the experiments. In fact, these responses were clearly evident in BM cultures by day 7. ADI cell cultures maintained under osteogenic conditions for 14 days also exhibited some focal calcium deposition and ALP localization in the monolayer assays and significantly up-regulated ALP activity, but these responses were markedly less than were seen in BM cultures. More telling, von Kossa staining was negligible in ADI osteogenic cultures, and Runx2 and OSX expression did not increase significantly. SYN cell cultures also showed some matrix mineralization at day 14, along with a diffuse increase in ALP localization; however, by von Kossa staining and all quantitative assays, the SYN cells did not undergo significant osteogenesis within the 14-day time frame of the experiments. These outcomes are consistent with previous comparisons of the relative osteogenic capacities of putative stem cell populations (3–9). The collective outcomes of these analyses emphasize the need for multi-assay panels for rigorous assessments of MSC lineage commitment. Although culture staining and ALP assays are straightforward and inexpensive protocols, monolayer stain uptake and ALP induction can occur in the absence of other, more lineage-specific osteogenic transitions.

Regardless of the specific mechanism(s) that distinguishes the osteogenic potentials of bone marrow, synovial and adipose progenitor populations, these results emphasize that stem cell populations from specific tissue and fluid sources retain source-specific lineage potentials and predispositions. This observation needs to be taken into account with any anticipated clinical utilization of MSCs.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by IACUC, University of Illinois at Urbana-Champaign.

Author contributions

AA, SD, BN, and MS contributed to tissue collection, cell isolation, and experimental protocol optimization. AA and BN were primarily responsible for cell culture maintenance, sample collection, outcome assays, and data collection. SD and MS were primarily responsible for data analyses. AA and MS were primarily responsible for manuscript development. All authors contributed to the article and approved the submitted version.

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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.

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Induced pluripotent stem cells in companion animals: how can we move the field forward?

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Following a one medicine approach, the development of regenerative therapies for human patients leads to innovative treatments for animals, while pre-clinical studies on animals provide knowledge to advance human medicine. Among many different biological products under investigation, stem cells are among the most prominent. Mesenchymal stromal cells (MSCs) are extensively investigated, but they present challenges such as senescence and limited differentiation ability. Embryonic stem cells (ESCs) are pluripotent cells with a virtually unlimited capacity for self-renewal and differentiation, but the use of embryos carries ethical concerns. Induced pluripotent stem cells (iPSCs) can overcome all of these limitations, as they closely resemble ESCs but are derived from adult cells by reprogramming in the laboratory using pluripotency-associated transcription factors. iPSCs hold great potential for applications in therapy, disease modeling, drug screening, and even species preservation strategies. However, iPSC technology is less developed in veterinary species compared to human. This review attempts to address the specific challenges associated with generating and applying iPSCs from companion animals. Firstly, we discuss strategies for the preparation of iPSCs in veterinary species and secondly, we address the potential for different applications of iPSCs in companion animals. Our aim is to provide an overview on the state of the art of iPSCs in companion animals, focusing on equine, canine, and feline species, as well as to identify which aspects need further optimization and, where possible, to provide guidance on future advancements. Following a "step-by-step" approach, we cover the generation of iPSCs in companion animals from the selection of somatic cells and the reprogramming strategies, to the expansion and characterization of iPSCs. Subsequently, we revise the current applications of iPSCs in companion animals, identify the main hurdles, and propose future paths to move the field forward. Transferring the knowledge gained from human iPSCs can increase our understanding in the biology of pluripotent cells in animals, but it is critical to further investigate the differences among species to develop specific approaches for animal iPSCs. This is key for significantly advancing iPSC application in veterinary medicine, which at the same time will also allow gaining pre-clinical knowledge transferable to human medicine.

KEYWORDS

veterinary, regenerative medicine, IPSC, one medicine, horse, dog, cat

1. Introduction

Veterinary regenerative medicine is a multidisciplinary field with a focus on developing innovative treatments for animal patients. Structural and functional healing of injured tissues and organs can be achieved by using either cells alone, cells combined with tissue engineered constructs or by delivery of the secretome without cells. These strategies involve different therapeutic mechanisms of action with the ultimate objective of enhanced treatment for diseases of veterinary interest (1–5). The advancement of the field is strongly influenced by the One Health—One Medicine approach. By adopting a panoramic view of the challenges, and exploiting the emerging positive outcomes in human and veterinary regenerative medicine, both may advance synergistically. Simply put, valuable insights can be translated in both directions to accelerate translation (6). For example, clinical experience with veterinary species can provide pre-clinical knowledge on safety and efficacy for human application, potentially reducing or eliminating the need for laboratory animals (7, 8). Therefore, a robust and inclusive One Health approach has exceptional value in human and veterinary medicine.

Among different cell types currently being explored for regenerative purposes, mesenchymal stromal cells (MSCs) isolated from multiple species and tissue sources are extensively studied for a wide array of veterinary applications, mostly due to their cell regulatory abilities (9–11). The relative ease in tissue collection, processing and *in vitro* culture has made MSCs an attractive therapeutic option. However, *in vitro* expansion of these cells is limited and extensive passaging may lead to cell senescence. In addition, the differentiation potential of MSCs is limited to mesodermal cell lineages (12–14). Embryonic stem cells (ESCs) can overcome these obstacles as they are pluripotent cells with a virtually unlimited capacity for self-renewal, and can provide a constant source of cells in terms of number and types. However, obtaining ESCs requires the use of embryos, with associated ethical concerns (15). In 2006, Takahashi and Yamanaka reported for the first time an alternative type of stem cell that could overcome the limitations of both adult and embryonic stem cells: the induced pluripotent stem cells (iPSCs). These cells are not naturally occurring but produced in the laboratory: adult somatic cells, such as dermal fibroblasts, can be reprogrammed into pluripotent cells by inducing the expression of four pluripotency-associated transcription factors (Oct4, Sox2, c-Myc, and Klf4, also known as Yamanaka factors). Theoretically, any cells of the body can be transformed into pluripotent cells without the disadvantages associated with ESCs (16). Therefore, the discovery of iPSCs has revolutionized regenerative medicine, not only because of their therapeutic potential but also because of their usefulness for disease modeling, drug screening, and even species preservation strategies (17).

Reprogramming somatic cells into iPSCs involves a global reset of the mature epigenome of the somatic cell, in order to go from its differentiated state back to a pluripotent one. To do that, the endogenous pluripotency-associated genes have to be re-activated while the somatic genes, associated with the specialized function of the cell, need to be repressed. These changes are initiated by inducing the ectopic expression of transcription factors Oct4, Sox2, c-Myc, and Klf4, which are proteins able to interact with the DNA to control gene expression (16, 18). These factors can be delivered to the cell by different methods that may involve or not viral vectors, and may result or not in the integration of transgenes in the genome of the cells (19). Even though the process of delivering the Yamanaka factors may seem relatively simple, complex epigenomic remodeling needs to take place (18). In order to succeed in this process, it is important to consider all the stages of reprogramming, starting from the selection of the somatic cells (20), the choice of the factors and the method to deliver them (19, 21), as well as the signaling pathways that can be regulated by adding growth factors or small molecules to the media (22, 23). Once the cells are reprogrammed, culture conditions need to be optimized to expand iPSCs while maintaining a pluripotent state. Of note, the complexity of the process may result in a mixture of cells at different stages of the reprogramming, thus making it critical a throughout characterization to confirm their identity as iPSCs (24). Therefore, there are many factors that can impact the resulting iPSCs and their subsequent applications. Therapeutic application of iPSCs is a major goal to pursue, however, the complexity of these cells and the genetic changes that they undergo during reprogramming have raised concerns that need to be addressed to move this application forward. Tumorigenicity and immunogenicity are among the main concerns, for which a number of strategies are being developed, from differentiation of iPSCs into specialized cells to the creation of haplobanks (25, 26). While the development of therapeutic applications moves forward, a number of other applications for iPSCs have emerged, being disease modeling one of the most relevant. Generating patient-specific iPSCs allows the subsequent derivation of specialized cells with specific genetic signatures or alterations that otherwise would be extremely complex to obtain primarily from tissues (27). *In vitro* disease modeling can help reducing the need of *in vivo* models and allows involving the species of interest since the pre-clinical phase of drug development. Basic research to unveil mechanisms of disease and physiology, as well as to conduct toxicological studies and drug screening can also be greatly facilitated by iPSCs (28).

Although iPSC technology is still a young field needing intensive work, nonetheless important advancement has been made in the human side (29). However, the veterinary iPSC field is significantly less developed and with much fewer publications. In fact, the first reports on canine iPSCs emerged in 2010 (30), equine in 2011 (31), and felids in 2012 (32). The majority of studies in veterinary species have focused on the generation of iPSCs and/or on their *in vitro* use, mostly to derive cell types relevant for clinical or disease modeling applications, while only very few works have pursued an *in vivo* application. The knowledge on human iPSCs can greatly contribute to advance the veterinary side, as most of the interests and challenges are shared between human and veterinary medicine. However, we also need to increase our understanding on the differences among species (33). Pluripotency networks, epigenomic landscape and identity of the iPSCs, and their derivatives need to be addressed from a comparative perspective rather than directly extrapolating from the human side. Advancing the field of veterinary iPSCs is important to improve the standard point-of-care of companion animals as patients, but also because of their potential as translational models. For instance, dogs suffer several spontaneous diseases with similar pathophysiology and incidence than in humans, like diabetes, epilepsy, or various types of cancers (34). Furthermore, dogs and humans also share the genetic basis of some diseases affecting the cardiovascular, neuromuscular, or immunological systems, thus creating a unique landscape for

iPSC-based research (35). The domestic cat can serve as a natural animal model of Alzheimer's disease (36) or hypertrophic cardiomyopathy (37), and also suffers genetic diseases that affect people too, like retinal blindness or polycystic kidney disease (38). Horses have long been considered as one of the most suitable animal models to study musculoskeletal pathologies like tendon injuries or joint pathologies (39) and, more recently, they are also acknowledged as models for immune-mediated diseases and to study immune responses (40–42). Therefore, joining efforts would revert in mutual benefit for human and veterinary patients, but additionally, there are some animal-specific applications like species conservation for which iPSCs can be of great importance.

The aim of this review is to provide an assessment of the current state of the art of iPSCs in companion animals, focusing on the equine, canine, and feline species because of their relevance as veterinary patients and their potential as animal models. Following a "step-by-step" approach, we firstly discuss the different stages in the generation of iPSCs from veterinary species (Figure 1) and secondly the development of different applications of iPSCs in companion animals (Figure 2). Our intention is to identify those technical aspects that need further optimization and to provide helpful guidance on future advancements.

2. "Step-by-step" approach for generating iPSCs in companion animals

2.1. Selecting the tissue source

To obtain iPSCs, the first step is to consider the type of somatic cells to be reprogrammed. Even though theoretically any cell can be induced into a pluripotent state, there is evidence that some cells are more easily reprogrammed than others. In addition, the invasiveness of the cell harvest procedure and the ease of culture should be taken in to account. Human iPSCs have been established from a wide range of tissue sources including dermal fibroblasts (43), peripheral blood mononuclear cells (PBMCs) (44), bone marrow derived MSCs (BM-MSCs) (45), and adipose derived stromal cells (ADSCs) (46). PBMCs are easily accessible while the

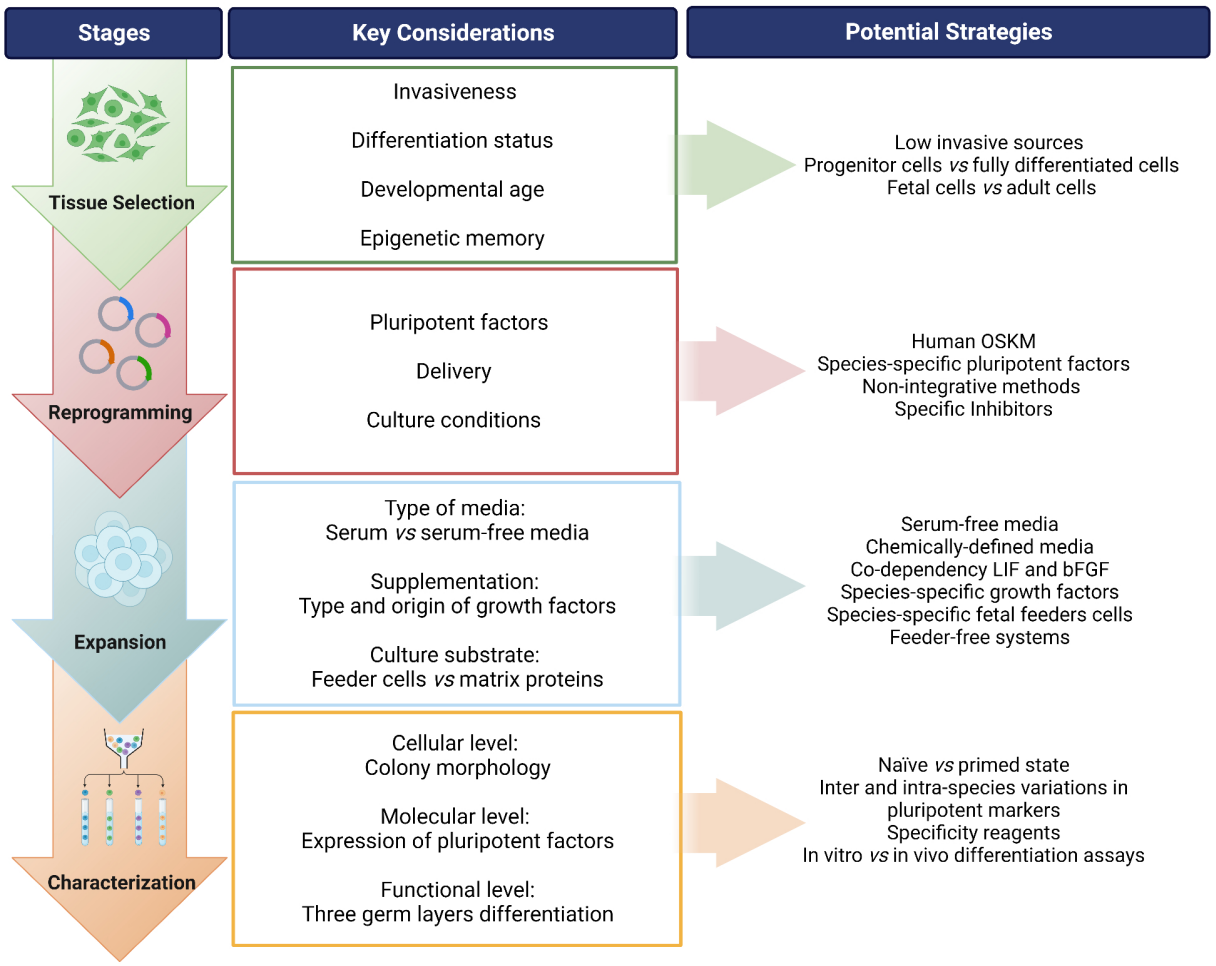

FIGURE 1
Overview of the process for generating induced pluripotent stem cells (iPSCs) from companion animals. A "step-by-step" flow is presented identifying the stages of the process, along with the key considerations in each step and suggested potential strategies to address each point. OSKM: Oct-4, Sox-2, Klf-4, c-MYC; i.e., Yamanaka factors. LIF, leukemia inhibitory factor; bFGF, basic fibroblast growth factor. Created with Biorender.com.

other cell types require more invasive intervention. Indeed, waste or discarded tissues, such as foreskin fibroblasts (47), periodontal tissue (48), or renal cells from urine (49) have also been taken used. Umbilical cord blood banks are also a useful resource for iPSC reprogramming (50, 51).

The differentiation status of the cell can influence the reprogramming efficiency, as the epigenomic state needs to be reset to a pluripotent state. For instance, hematopoietic stem and progenitor cells can be reprogrammed into iPSCs much more efficiently than terminally differentiated lymphocytes (52). In addition, the developmental age of the cells can affect their capacity to revert to an earlier state of pluripotency, as shown by the fact that embryonic and fetal tissues may be reprogrammed more efficiently than adult tissues (53). Furthermore, iPSCs are reported to retain epigenetic memory of the parent cell type (54, 55), which may influence the differentiation potential of iPSCs toward a desired lineage. For example, iPSCs derived from human cardiac-derived mesenchymal progenitor cells and pancreatic islet beta cells demonstrated enhanced differentiation toward the parent lineages compared to cells from other tissues (56, 57).

In companion animals, adult and fetal fibroblasts have been the most commonly used to obtain iPSCs, but other cell types have been explored such as keratinocytes (58), MSCs from different tissue sources (adipose tissue, bone marrow, umbilical cord tissue, and peripheral blood) (59–61), myogenic mesoangioblasts (MAB) (60), tenocytes (62, 63), and PBMCs (64). Very few studies have directly compared the generation of iPSCs from different cell types in these species.

In horses, Pessôa et al. (59) reported that the tissue of origin of the cell may significantly influence the capacity for reprogramming. Adult fibroblasts, umbilical cord tissue (UC)-MSCs, and adipose tissue (AT)-MSCs were successfully reprogrammed, with AT-MSCs showing the highest colony formation potential, whereas BM-MSCs did not produce iPSCs. In the same study, authors observed differential miRNA expression profile among iPSC lines, which may be the result of different responses to reprogramming. Direct comparison of iPSC generation from cells of adult and fetal origin has not been performed in horses. However, when comparing fibroblasts from young and old individuals, it was suggested that the derivation of equine iPSCs is not impaired by aging (65).

Regarding canine iPSCs, it seems that adult cells are more "resistant" to reprogramming compared to fetal cells. Questa et al. (66) hypothesized that chromatin remodeling and accessibility were behind this resistance to reprogram since chromatin remodeling is required for the inactivation of somatic loci and activation of pluripotent ones. Therefore, these authors explored the implications of chromatin accessibility for canine somatic cell reprogramming by comparing different embryonic and adult cell types. The transduction efficiency was similar between adult and embryonic cells, however, only iPSCs from embryonic origin met pluripotent criteria whereas adult reprogrammed cells did not form stable colonies. Authors identified global patterns of chromatin openness, finding that iPSCs and embryonic fibroblasts shared substantially more features than iPSCs with adult cells. Actually, adult canine cells showed a region of closed chromatin that was open in embryonic cells and in which pluripotency associated genes are located. Findings were aligned with that expected during reprogramming and may explain why adult cells are more 'resistant' to reprogramming, which may help enhancing the process by targeting reprogramming barriers.

The influence of epigenetic memory in the differentiation potential of iPSCs has not been deeply studied in companion animals, but it has been suggested that equine iPSCs retain some lineage commitment since those originated from MAB formed a higher quantity of muscle patches in teratomas, while iPSCs from PB-MSCs produced larger chondrogenic patches (60). The same group similarly showed that canine iPSCs derived from MAB had enhanced propensity to differentiate into skeletal muscle lineage compared to iPSCs derived from fibroblasts, which could be attributed to the DNA methylation pattern of MAB (67). Furthermore, authors also differentiated canine iPSCs from MAB and from fibroblasts into mesodermal progenitors (MiPS) and administered them in dystrophic mice, showing that the engraftment in the skeletal muscle was higher when MAB-MiPS were delivered compared to fibroblast-MiPS.

Evidence in companion animals is still limited to suggest superior cell sources for iPSC reprogramming, but collectively with human evidence points at carefully considering this choice. Not all cell sources are equally suitable for reprogramming and iPSC properties can be impacted by their origin, so identifying the most suitable source for a particular application is of utmost importance. To do that, it is also important to unveil and understand the differences among distinct cell types in each species, particularly at the epigenomic level.

2.2. Reprogramming

2.2.1. Inducing the expression of pluripotent factors

Once the tissue source is selected, the choice of the pluripotent transcription factors and the method to deliver them to the cells should be carefully considered. Studies on companion animal iPSCs have mostly used human or murine factors for reprogramming, typically the four Yamanaka factors (OSKM) (16, 43). Of course, the mRNA and protein sequence homology of these transcription factors should be as high as possible. For example, in the horse, the homology is higher with human sequences than with mice (68) and, even though some works have generated equine iPSCs using murine factors (31, 69, 70), the studies that have directly compared both of them reported success only with human factors (59, 68). Studies comparing human and murine factors to generate canine iPSCs found that both were able to reprogram canine cells, but only human transgenes were silenced (71). To the best of our knowledge, only one early canine iPSC study was carried out using species-specific factors (30). Later studies used factors of human or murine origin. Based on what we understand to date, it is unclear whether species-specificity of reprograming factors is important for iPSC generation in companion animals (72). Interestingly, while the four OSKM factors are sufficient to reprogram equine and canine cells, it seems that the addition of NANOG is key in felids (32), including the domestic cat (73). Based on these considerations, it seems especially prudent to pay attention to the species origin of the factors, as well as the specific combination of factors used.

Methods used to induce expression of the selected pluripotent factors may be classified as integrative/non-integrative and viral/non-viral. Human and murine iPSCs were originally generated using

integrating retrovirus and lentivirus vectors to introduce the OSKM reprogramming factors (16, 43, 74). Genome-integrating methods may result in heterogeneous iPSC lines that are not suitable for clinical applications because the transgenes may become reactivated in iPSC derived cells, leading to a risk of tumor formation (21, 75). This is a limitation that will be further discussed in the Application section. Transgene free, non-integrating methods have been utilized to overcome these safety concerns, including Sendai-virus (76), episomal vectors (77, 78), and RNA based methods (79, 80). Furthermore, clinical-grade human iPSCs have been developed using modified mRNAs and non-integrating episomal vectors (45, 81). However, while significant advancement has been accomplished in the generation of human iPSCs by non-integrative methods, these strategies have been rarely applied in companion animals, with the majority of applications involving integrative viral methods (35, 82).

In the generation of equine iPSCs, retroviral vectors were mainly used in early efforts and lentiviral vectors more recently. Specifically, the use of a STEMCCA cassette can increase the efficiency of reprogramming by delivering the four factors together and thus also reducing the number of integrations in the genome (83). Only a few studies in equine iPSCs report the use of non-viral methods. The Piggy-back transposon technique was indeed used in the first report on equine iPSCs (31), and the lines obtained were also used in later studies (84, 85). However, Moro et al. (86) compared the lentiviral and transposon systems and found that only the former was efficient at generating equine iPSCs from fetal fibroblasts. Transposon reprogramming allows more control of transgene expression by using excisable or inducible systems, but it is still an integrative method. Transgenes can also be excised if delivered by lentivirus, as done for example by Chauveau et al. to generate canine iPSCs (87). A relatively simpler way of controlling the expression of transgenes is by using an inducible promoter, however the transgene remains integrated (31, 70, 88). In general terms, these strategies can improve the safety profile of integrative methodologies, but integration still takes place with the potential risk associated with activation of unwanted genes such as those related to tumorigenicity.

Exploration of transgene-free strategies has been more extensive in canine iPSCs, however direct extrapolation of the conditions used for human iPSCs does not seem to be straightforward. Baird et al. (61) used both retroviral and Sendai-virus based delivery in the same cells, but iPSC colonies appeared only with the former (61). Chow et al. also used the Sendai-virus system and reported that only a single colony was viable upon further passaging after colony picking (89). However, this was sufficient to establish a line that was used in this and subsequent studies (90). Similarly, Tobias et al. could not maintain stable canine iPSCs for longer than 26–30 days after reprogramming when using Sendai-virus (91). Tsukamoto et al. generated canine iPSCs with Sendai-virus that could be maintained for multiple passages, but only one line was obtained which failed to produce all three germ layers in teratoma assays in mice, suggesting that it might be a heterogeneous population (64). Later efforts by the same group showed that canine iPSC generation with Sendai-virus is possible but requires very specific conditions, including supplementation with a cocktail of small molecules. With this improved protocol, these authors were able to culture the generated canine iPSCs over 40 passages (92). Other non-integrative virus strategies have been tested for canine iPSC generation, such as the use of a vector based on the Venezuelan equine encephalitis RNA virus, which overall was not successful (93).

Non-viral and non-integrative methods for pluripotent factors delivery have also been explored for canine iPSCs. Yoshimatsu et al. (94) reported on the use of episomal vectors delivered by electroporation. While colonies could be obtained with this method it was only when highly defined media was used, and still the reprogramming efficiency was very low (94). Chandrasekaran et al. also used episomal reprogramming with electroporation, but even though morphological changes were observed in the cells, complete reprograming was not achieved and lentivirus was used subsequently, resulting in generation of canine iPSCs from the same somatic cells (95).

Although iPSCs generated with integrative methods can be effectively used for *in vitro* applications (basic research, disease modeling, drug screening, etc.) (96), the optimization of non-integrative methods is needed in the veterinary field to develop safer therapeutic applications. In spite of the efforts of several groups, we currently do not have a robust and widely used tool for transgene-free obtainment of iPSCs in companion animals. One possible reason may be that in companion animals iPSCs, the continuous expression of the exogenous transgenes may be required to maintain the pluripotency, as the endogenous networks might not be fully activated (33) thus significantly dampening the derivation of stable transgene-free lines. Therefore it is key to better understand such pluripotency networks in animals to provide the required conditions for iPSC generation.

2.2.2. Culture conditions during reprogramming

Following delivery of the pluripotent factors, appropriate culture conditions are needed to facilitate the changes in gene expression that allow the cell to alter its fate from somatic to pluripotent. This process can be facilitated by inhibitors of certain protein kinases, like glycogen synthase kinase 3 (GSK3), mitogen-activated protein kinase (MAPK), MAPK/extracellular signal-regulated kinase (MEK), Rho-associated kinase (ROCK), or activin-like kinase (22). Furthermore, histone acetylation facilitates the binding of transcription factors to DNA, so chemical inhibitors of histone deacetylase (HDAC) such as valproic acid, sodium butyrate, or ascorbic acid can increase chromatin accessibility and thus potentially improve reprogramming efficiency (23).

Only a few equine iPSC studies have reported the use of kinase inhibitors (31, 97) or HDAC inhibitors (65) during reprogramming. However, these studies have not compared different combinations and neither have explored in detail the specific changes elicited by the inhibitors. In feline iPSCs, in spite of the limited number of studies published, the use of inhibitors has been reported (98). Optimization of iPSC generation has been further pursued in dogs. For instance, Moshref et al. (99) hypothesized that HDAC inhibitors would increase chromatin accessibility and facilitate reprogramming of adult canine cells. These authors found that neither valproic acid nor sodium butyrate effectively inhibited canine HDAC. On the other hand, panobinostat, another HDAC inhibitor, significantly increased histone acetylation and improved chromatin accessibility but without evidence of increased efficiency of generating iPSCs (99). Furthermore, Kimura et al. found that a cocktail of small molecules including some of the inhibitors mentioned (ROCK inhibitor, MEK inhibitor, GSK3b inhibitor, TGFβ antagonist, forskolin, and ascorbic acid) contributed to efficient generation of canine iPSCs (92).

These findings certainly appear to indicate that each species has a unique epigenomic landscape that requires specific approaches, and

this might help to explain the relatively limited success in obtaining iPSCs in companion animals. While human studies can provide a basis of knowledge, directly extrapolating the same protocols into other species would not be an optimal strategy. Thus, more studies in this direction are needed to understand the conditions required for reprogramming in each species.

2.3. Expansion of iPSCs in companion animals

Once the cells are reprogrammed, the next stage is to maintain them in a pluripotent state and this generally requires the use of specific media containing selected growth factors and chemical components, as well as layers of feeder cells or matrix proteins (100, 101). Media composition for expansion of veterinary iPSCs has been reviewed elsewhere (35, 82), so this review will only focus on two aspects directly related to species-specific aspects and transferability.

One of these is the use of either serum-containing or serum-free media, the latter being more suitable for therapeutic applications as it avoids potential xeno-contaminants and/or infectious diseases, as well as reduces batch-to-batch variation. Nevertheless, it should be noted that some serum-free media may still contain components of human or animal origin that are potential xeno-contaminants for veterinary species. Furthermore, it has been shown for animal MSCs that serum-free media developed for human cells may not work as well in veterinary species [reviewed by (102)]. For iPSCs, it seems that serum-free conditions may work better in equine species. Some papers have reported success in using fetal bovine serum (FBS), but studies directly comparing FBS vs. knockout serum replacement (KOSR) reported better results with the latter (69). In canine iPSCs, a majority of works have also used serum-free media [reviewed by (35)], however other studies suggest that media containing FBS result in higher colony formation compared to KOSR during reprogramming (91). On the other hand, reports in felid iPSC suggest that FBS-containing media are more advantageous (32, 73, 98, 103).

A second important aspect for veterinary iPSC media composition relates to growth factor requirements. The dependence of iPSCs on either basic fibroblast growth factor (bFGF) or leukemia inhibitory factor (LIF) is related to the stage of the embryonic development that is mimicked by pluripotency induction. ESCs from the inner cell mass present a more naïve phenotype, with mounded colonies that are dependent on LIF. When ESCs are derived from the epiblast, a structure formed later during the development, these cells seem to be already primed, possibly representing a more restricted state of pluripotency, and are dependent on bFGF with colonies presenting a flat morphology (33). The majority of human iPSC lines resemble the primed phenotype (104), however there are mixed reports on the naïve/primed nature of iPSCs from companion animals (24). The scarce literature in felid iPSCs points at LIF-dependency (32, 73, 98, 103) while only bFGF-dependent (59, 60, 68, 71) and only LIF-dependent (70, 97) iPSC lines have been reported in both equine and canine species; however, the evidence so far points at a co-dependency on both factors in these two species (69, 105, 106). The reason for this co-dependency is not well understood. A study in canine iPSCs found that bFGF would act by inhibiting spontaneous differentiation toward ectoderm and mesoderm, while LIF activated the JAK-STAT3 pathway involved in pluripotency maintenance, but in a different manner than described in mouse ESCs (107). These studies collectively show that iPSCs from different species may present unique mechanisms for maintenance of pluripotency. Understanding such mechanisms is critical to provide the optimal conditions for expansion of iPSCs for different applications in the veterinary field. Moreover, LIF and bFGF used in animal studies are usually from human or murine origin. The use of species-specific factors has been suggested (108) but scarcely reported. Interestingly, only feline LIF, but not murine LIF, can maintain the pluripotent features of iPSCs in the domestic cat (73). Species-specific reagents usually present more limited availability (102), but may represent an important strategy to enhance pluripotency maintenance in these species.

In addition to specific media composition, iPSCs require to grow onto layers of feeder cells, for which inactivated mouse embryonic fibroblasts (iMEF) are commonly used. This possess another concern when the application of interest is therapeutic: the presence of xeno-contaminants in the cell products, or even the risk of disease-transmission. A possible strategy to avoid xeno-contamination at this point is the use of feeder cells from the same species. For example, human iPSCs can be cultured onto neonatal foreskin fibroblasts with good results (109). Similarly, Nagy et al. used 1:1 iMEF and equine fetal fibroblasts (31), and Zhou et al. used cat fetal fibroblasts (98) to prepare feeder layers. As discussed above, fetal cells can be more easily reprogrammed, and the same cells could be used as feeders after inactivation. This strategy would be more time-consuming and less standardized than the purchase of batch-tested, ready-to-use iMEF, but it might be interesting to explore whether using species-specific feeder cells could better support iPSCs, in addition to avoid xeno-contamination.

For the development of clinical grade hiPSCs, standardized and quality-controlled xeno-free and feeder-free culture products are commercially available including media such as Essential E8 (Gibco), mTeSR plus (Stemcell Technologies), StemFit (Ajinomoto) and NutriStem (Sartorius) (110–113), and matrix substrates that are more defined with less batch-to-batch variability, such as vitronectin (114), laminin-521 (115) or-511 (116), CellStart (117), and synthetic materials (118). The use of such systems is much more rapidly evolving for human iPSCs (100, 101), while most reports in companion animals rely on feeder cells and mostly on iMEF (24, 35, 82). Some attempts have been done to adapt the use of commercially available iPSC media and feeder-free substrates formulated for human iPSCs into companion animal cells. Such systems present the advantage of having a defined composition, being more stable and homogeneous, and providing serum-free, cell-free, or even xeno-free conditions. In horses, there are only brief mentions to the use of the StemFlex system (Thermofisher) to maintain equine iPSCs once the lines were established (62, 63). In cats, StemFlex media was used for reprogramming and expansion of feline iPSCs but supplementation with LIF and protein kinase inhibitors was needed (98). In dogs, Kimura et al. compared the suitability of different commercial media and feeder-free substrates and found that StemFit media (Ajinomoto) and iMatrix-511 (Nippi) provided the most suitable conditions for canine iPSC maintenance and large-scale expansion, and even LIF could be removed (108).

In summary, similarly to that discussed for previous steps, there are not standardized culture systems for iPSCs from companion animals, being of great importance to develop serum-free and feeder-free options for clinical application. This would require fine-tuning of

the conditions and a more in-depth understanding of animal iPSC requirements, along with increasing availability of species-specific reagents to truly avoid xeno-contamination.

2.4. iPSC characterization in companion animals

Once iPSC putative lines are established, it is critical to confirm that these really are pluripotent cells. iPSCs are characterized at three levels: cellular (morphologically), molecular and functional. The cells should have a large nucleus and form compact colonies. They should endogenously express pluripotency markers at both gene and protein levels, and have the potential to spontaneously differentiate into the three embryonic germ layers [either *in vitro* via embryoid body (EB) formation assays, and/or *in vivo* via teratoma assay in immunocompromised mice]. Finally, iPSCs must have a stable karyotype as they can acquire chromosomal abnormalities after genetic reprogramming and long-term culture (43, 119, 120). Reports on the characteristics of iPSCs from companion animals at these three levels have been reviewed and compared by other authors (24, 35, 82). A detailed description is out of the scope of this review, but instead we are highlighting the main aspects to consider at each level of characterization.

First, and as discussed in the previous section, at the cellular level it is not clear which type of colony morphology features each species of companion animals. Both naïve and primed-like morphology have been described, which also relates to the dependency of these cells on different growth factors. Thus, so far we do not have a strict criteria at this level to consider the cells as iPSCs in each species. This also adds complexity to the selection of colonies when these start emerging from reprogrammed cells. Colonies are picked individually mostly based on their morphology and ideally should be monoclonal, i.e., starting from a single reprogrammed cell. This process is challenging and labor intensive, and can lead to a heterogeneous selection of lines that are apparently similar but hold subtle phenotypic differences. Such differences may be very difficult to appreciate at the morphological level but could eventually result in different characterization profiles and varying differentiation capacity (58).

Second, at the molecular level, different works have reported the expression at the gene and/or protein level of several pluripotent factors. It is important to note two points in this regard: the lack of a standardized panel of markers, and the relevance of ensuring the specificity of antibodies and primers used for characterization. Human and murine pluripotent cells have shown differential expression of certain markers (121), and this variability is also expected across veterinary species. To determine which pluripotent markers are expected in each species we could look at the expression pattern in ESCs. However, intra-species variability has also been noted for ESCs. For instance, in horses and dogs, the same marker has been reported as both positive and negative in ESCs of the same species [reviewed by (24)]. Furthermore, the limited number of ESC lines derived from companion animals makes it difficult to elucidate whether ESCs and iPSCs are truly equivalent and what developmental stage reflect in these species (24). An additional obstacle for the analysis of pluripotent markers in veterinary species is the complexity of finding antibodies which are either species-specific or presenting good cross-reactivity with the species of interest. Whereas the availability of suitable antibodies for veterinary species has substantially improved in the last years, it still can be difficult to find reliable antibodies for some specific markers. Moreover, the use of different clones for the same marker among different studies might contribute to the heterogeneity observed intra-species (24). The analysis of gene expression is also an important tool and designing primers specific for the species of interest is easier than developing antibodies. However, because of the high homology in the mRNA sequences between the human exogenous factors used for reprogramming and the endogenous genes activated in the cell (68), it is critical to ensure that the primers are only amplifying the target of interest.

Finally, variable outcomes have been reported in the different species when it comes to the functional pluripotency of iPSCs, i.e., their ability to differentiate into cells of the three germ layers. The *in vitro* EB formation assay has provided more consistent results, which have shown successful differentiation of iPSCs. However, *in vivo* formation of teratomas has not been observed in all the reports in companion animals, or only partial differentiation has been recorded (24, 82). A potential explanation for this would be an incomplete reprogramming of the cells into the pluripotent state, even though the other criteria are met.

As aforementioned, different studies have used different methodologies and conditions for reprogramming and culture, and this lack of standardization could probably influence differences observed in the characterization of iPSCs in companion animals (24). Thus, it is imperative to advance in determining the pluripotent features representative of each species and in developing suitable methods to analyze them with confidence. Standardization of iPSC characterization in companion animals is key to develop robust applications, and is tightly influenced by a previous proper establishment of reprogramming strategies and maintenance conditions.

3. Applications of iPSCs in companion animals

The use of iPSCs finds multiple applications allowing development of novel treatments in human and veterinary medicine. These applications range through various biomedical disciplines, including development of cell therapies, disease modeling and drug testing, and clinical application for untreatable diseases in both people and companion animals (35, 122, 123). In addition, as a characteristic application in animal species and important for the maintenance of biodiversity, iPSCs have been generated from critically endangered mammalian (32, 103, 124, 125) and avian species (126). These are important for the study of developmental and physiological species-specific processes, and as wildlife preservation efforts they are important for the conservation of genetic resources and maintenance of a healthy eco-system.

When it comes to the applicability of iPSCs in companion animals, reports are scarcer than for their generation and mainly focus on therapeutics or disease modeling. Therapeutic use of iPSCs has been mainly proposed for musculoskeletal conditions in horses (60–63, 88, 127–129) and for neurological and cardiovascular conditions in dogs (67, 95, 130–133). In terms of disease modeling, equine iPSCs can also be used for neurological conditions (58, 134). In dogs and humans, there is an interest in modeling genetic diseases by deriving

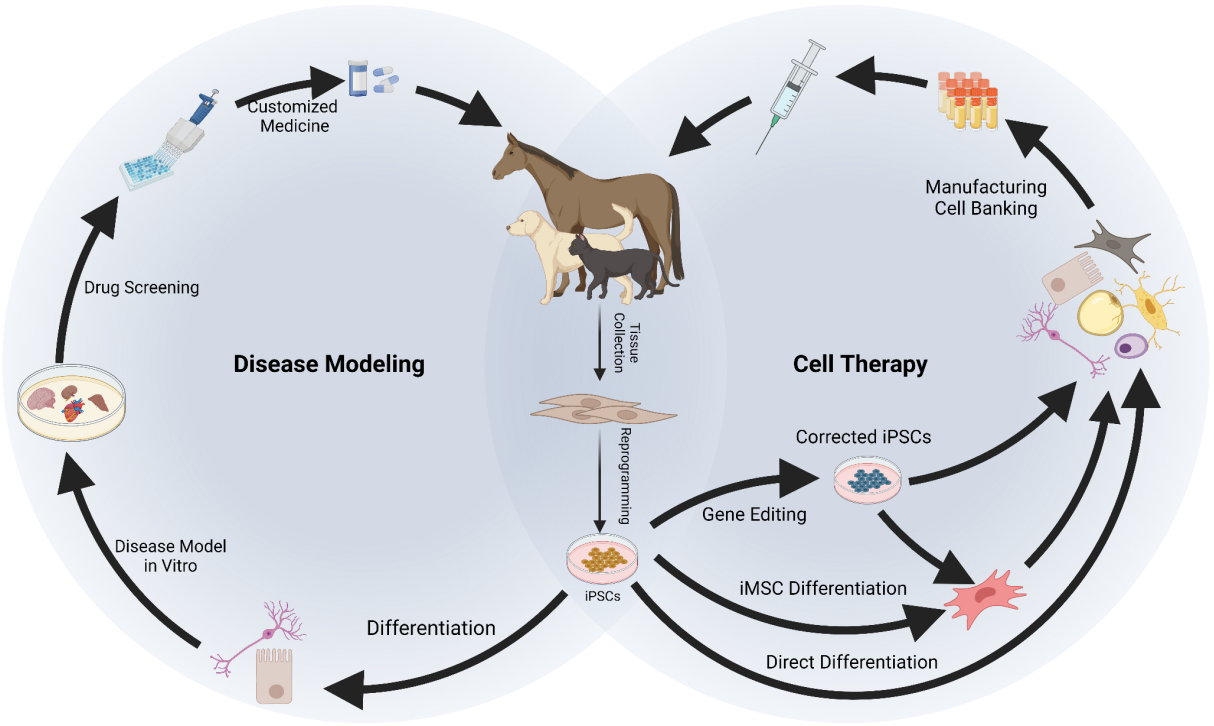

FIGURE 2
Applications of induced pluripotent stem cells (iPSCs) in companion animals for disease modeling and therapy. After reprogramming, iPSCs can be differentiated into specialized cells to recreate a "disease in a dish." *In vitro* disease modeling can be used to better understand mechanisms of disease, physiology of the cells and to conduct drug screening, resulting in a more customized approach for therapy. Therapeutic application of iPSCs requires their differentiation into specialized cells or into intermediate progenitors like mesenchymal stromal cells (iMSCs). Gene editing can be used at this stage to obtain healthy cells from patients carrying genetic disorders. The iPSCs, iMSCs, and/or their derivatives can be manufactured and banked for therapeutic use. Created with Biorender.com.

patient-specific iPSCs with genetic abnormalities, which poses a remarkable scenario for translational medicine (35). In cats, to the best of our knowledge, there are no reports on therapeutic or disease modeling approaches, being the bibliography in this species the most limited. However, interestingly, iPSCs have been proposed in wild feline species as strategy to preserve biodiversity (32, 103).

The different applications of veterinary iPSCs clearly remain less developed than in human research, but hold a similarly high potential yet to be explored. Nevertheless, the challenges to accomplish safe therapeutic application are essentially the same in all species. Some of these limitations arise during the generation process, as has been exposed in the first part of this review, or are inherent to the nature of these cells. In this section, we will review the current state of the art of iPSC applications in companion animals and their main limitations (the present), and we will discuss potential strategies to address such challenges and to keep moving the field forward (the future).

3.1. Where are we: present of iPSC applications in companion animals

3.1.1. iPSC-based cell therapy in companion animals

The current clinical investigation of iPSC-derived cell therapies in companion animals is still limited as the field is in its initial stage. The rationale behind the interest on these cells is that they could act as direct replacement of diseased cells with healthy and functional cells able to re-establish the tissue homeostasis, which results in an approach closer to actual tissue regeneration than with adult stem cells. The published studies in veterinary species so far offer insights on proof of concepts and initial clinical evidence in low number of clinical cases. In equines, based on the interest in treating recurrent sport injuries, the majority of studies focused on generating iPSC-derived cell types clinically relevant for musculoskeletal and wound healing injuries. In this regard, equine iPSCs have been differentiated into several cell types including osteoblasts (129), chondrocytes (60), tenocytes (62, 63, 127), myocytes (128), and keratinocytes (85). In spite of the *in vitro* evidence of iPSC differentiation potential, the functionality of the obtained cells has only been demonstrated to certain extents and would need to be further tested in the clinical setting. Similarly, the transplantation of functional neuronal cells derived from iPSCs would be of great benefit in the specific context of the nervous system, characterized by very limited regenerative abilities and accompanied by the incidence of neuropathies and traumatic spinal cord injuries in canine and equine patients. Based on this, equine functional motor neurons have been generated (58) and canine iPSC-derived neuronal progenitors have been tested for the treatment of traumatic spinal cord injury in three canine patients. While adverse effects were not noted, neither clinical improvement nor tissue remodeling were observed up to a 1-year follow-up (131).

Thus, besides *in vitro* evidence of iPSC differentiation, optimal clinical use of iPSC-derived cells requires demonstration of cell

engraftment and functionality *in vivo*. A proof of concept study using autologous canine iPSCs showed successful myocardial delivery in healthy dogs as monitored by non-invasive imaging techniques. Additionally, these canine iPSCs were differentiated into endothelial cells and administered *in vivo* into two murine models: hind limb ischemia and myocardial injury, in both of which these cells suggested efficient functionality (130). Similarly, canine iPSC-derived mesodermal progenitors showed engraftment and functional improvement in a murine model of cardiac and skeletal muscle, with no off-target tissue formation (67). A murine model of muscle injury was also used to test equine iPSC-derived myofibers, which showed engraftment and histological improvement, but the regeneration was not complete (70).

An alternative option for delivering therapeutic cells is by generating MSCs from iPSCs (iMSCs) as an intermediate cell type that can be further used for cell therapy development with a major interest in musculoskeletal applications in companion animals. Canine iMSCs have been successfully differentiated into osteogenic and chondrogeneic lineage (90, 135). Furthermore, from a functional perspective, canine iMSCs show an immunomodulatory capacity similar to primary MSCs derived from adipose tissue and bone marrow, with similar gene expression profiles, effects on the proliferation of T cells, maturation of dendritic cells and response to priming with pro-inflammatory cytokines (89, 136). Furthermore, canine iMSCs IV injected in three healthy dogs did not produce adverse events in the short term, neither tumor formation was observed up to 15 months of monitoring (89). Equine iMSCs have also been tested *in vivo* for the treatment of a variety of naturally-occurring musculoskeletal injuries in equine patients, showing overall positive effects with absence of serious side effects (88). The heterogeneity of conditions included in this study and the lack of a control group hamper extracting definitive conclusions, but the results are valuable as a proof of concept for the therapeutic potential of iMSCs in horses.

3.1.2. iPSC-based disease modeling and drug screening in companion animals

Efficient prevention and treatment of diseases requires advanced knowledge of the altered genes and pathways responsible for the diseased phenotype of interest. To better understand the molecular background and establish the appropriate treatment, iPSCs can be exploited as *in vitro* models of disease. Furthermore, the defect of interest can be induced and subsequently multiple compounds of interest can be tested to identify a candidate drug with higher therapeutic efficiency (24, 27). In addition, when it comes to genetic disorders, genome editing tools like CRISPR-Cas9 technology can be used to correct the mutation and generate isogenic iPSC lines as controls. This allows accounting for the influence of the genetic background, since the isogenic line only differs from the original one in the disease-causing mutation (137).

Compared to current advances in the use of human iPSCs in disease modeling and drug screening, there is scarce published literature in companion animals and is mostly limited to neurological disorders. In canines, an iPSCs line was generated from a West Highland White Terrier affected by mild cognitive impairment, showing an important proof of concept on successfully generating iPSCs from a geriatric patient (95), while on the equine side iPSC-based *in vitro* models have been generated to study the process of neurotropic viral infections (134). Although limited, these studies constitute relevant milestones for generating efficient *in vitro* modeling systems that will not depend on limitations of adult somatic cells and can eventually lead to a personalized/customized medicine approach where the most efficient treatment will be made available in a patient-centered approach.

3.1.3. Limitations for iPSC applications in companion animals

The iPSC field comes with as many promises as challenges, the latter being even more present in veterinary medicine. Potential applications of iPSCs are almost endless for therapy and research, however unleashing all of this potential requires overcoming several limitations, owed to the complexity and particularities of these cells. The process of generating iPSCs in companion animals faces several challenges itself, as detailed in the first part of this review. Furthermore, once iPSCs are generated, their posterior use for *in vitro* or *in vivo* applications does not come without limitations. Some of these limitations directly arise from the generation stage, such as transgene expression or xenogeneic contamination already discussed, while other handicaps derive from the inherent characteristics of these cells.

One of the key challenges, particularly for *in vivo* application, is the pluripotent nature of these cells. As aforementioned as part of the functional iPSC characterization, these cells have the potential to form benign tumors composed of multiple cell types, known as teratomas, if they are administered undifferentiated in immune-compromised recipients (138). This can pose significant health risks for the recipient and limit the use of iPSCs in medical or veterinary applications. Because of this, and as it will be discussed later, *in vivo* applications aim at using differentiated cells derived from iPSCs that have lost their pluripotency. Furthermore, iPSCs also possess a risk for malignant tumorigenesis. This risk is particularly concerning if integrative methods are used for reprogramming, which are so far the most commonly reported in veterinary species. The random integration of the transgenes into the genome of the cell can activate tumorigenic genes and, even if transgene expression is silenced, they are still present and can reactivate even after differentiation. This is particularly concerning for *in vivo* applications, but can also impact the outcome of *in vitro* research and applications if iPSCs become tumorigenic (25).

Another consideration concerning the therapeutic use of the iPSCs is their immunogenicity. As it will be discussed later in the Banking section, the use of allogeneic cells presents several advantages, particularly in the case of iPSCs which obtainment is highly demanding. However, the immune system of the recipient may recognize and target allogeneic cells, thus affecting the effectiveness and safety of the therapy (139). Importantly, even if the iPSCs are autologous, i.e., derived from the own patient, they can still be rejected by the immune system. This autologous rejection may be related to different factors. iPSC-derived cells are often immature and thus express low levels of the major histocompatibility complex (MHC), which make them targets of natural killer cells. In addition, the genomic and epigenomic changes that the cell undergoes during reprogramming and subsequent *in vitro* expansion and differentiation may result in immunogenic triggers (26).

Another hurdle for the therapeutic application of iPSC derivatives is the lack of proper function, where differentiated iPSCs may not function properly *in vivo*, especially if they are not fully matured or are not adequately integrated into the recipient tissue (140). This issue will be covered in the next section along with strategies to enhance

applicability of iPSCs in companion animals. Finally, the use of iPSCs in medical applications is also highly regulated and requires regulatory approval, which can significantly slow the development and commercialization of iPSC-based therapies. Furthermore, the development and production of iPSCs is still a relatively new, complex and expensive field, limiting the accessibility of iPSC-based therapies for many patients (96, 141, 142).

3.2. Where are we going: future of iPSC applications in companion animals

3.2.1. Differentiation of iPSCs into specific cell types

Most iPSC applications require their differentiation into the desired cell type, either if they are used for therapy, for disease modeling or for drug screening. Differentiating the iPSCs substantially reduces the potential risks associated with teratoma formation and facilitates the regulatory approval process. Additionally, these differentiated cells are more mature and functional, which can increase their effectiveness and reduce their immunogenicity in therapeutic applications. Directed differentiation methods have made significant progress in recent years in the human side, allowing for the efficient and specific differentiation of iPSCs into a variety of cell types, including neurons (143), cardiomyocytes (144), and hematopoietic cells (145), among others. Therefore, direct differentiation of iPSCs represents a promising alternative for the development of new therapies and *in vitro* applications, holding the potential to significantly impact the field of regenerative medicine. However, directed iPSC differentiation presents several obstacles. To begin with, the process of inducing iPSCs to differentiate into specific cell types can be inefficient, with high variability, low specificity and poor reproducibility, as well as constituting a time-consuming and costly process (146). Furthermore, the differentiated cells may be heterogeneous and result in a population with varying degrees of differentiation and functional activity, and the presence of residual undifferentiated iPSCs can compromise the purity of the differentiated cell population (147). Overall, the lack of control over the differentiation process and the variability of iPSC lines pose significant challenges to their practical use.

To overcome the challenges in inducing differentiation of iPSCs, researchers are actively pursuing several strategies to improve the efficiency, specificity, and reproducibility of the process. The optimization of differentiation protocols is a crucial aspect of this research, as it involves refining the methods and conditions used to induce iPSC differentiation. This can involve adjusting the presence of specific growth factors that can influence the differentiation process or applying engineering-derived approaches to promote iPSC differentiation by mimicking the extracellular matrix (148). Directed differentiation is a strategy that involves directing iPSCs toward specific cell types using signaling pathways and small molecule inhibitors. This can help to increase the specificity of the differentiation process and reduce the formation of unwanted cell types, but requires deep knowledge on the embryonic development of the target cells to mimic the corresponding pathways (149), which is often complex and particularly in veterinary species. Reporter lines and cell sorting methods to identify and purify the cell population of interest is frequently used in human iPSC differentiation (150, 151). Including this approach into the strategies for veterinary iPSC differentiation would require further characterization of animal markers and species-specific antibodies to correctly identify the cells of interest. Another strategy is choosing iPSC lines that have a high propensity for differentiation into specific cell types, as has been described in the first part of the review (55). Finally, quality control measures are also essential for ensuring the purity of differentiated cell populations and minimizing contamination with residual undifferentiated iPSCs. This can be accomplished using molecular markers and other techniques that can help to distinguish between different cell types (119). Importantly, checking the identity of the obtained cells possesses its own challenges, as not all cell types exhibit a well-defined and stable pattern of markers, so several tests may be needed possibly including functional ones. Therefore, combining different strategies in a multi-faceted approach can help to address the challenges in the iPSC differentiation process from multiple angles, each one constituting a unique opportunity for research and development.

3.2.1.1. iPSC-derived cells in companion animals

As commented above, different cell types have been derived from iPSCs in the equine and canine species, but not all of them have shown functional properties *in vitro* or *in vivo*. For instance, equine iPSCs have been differentiated *in vitro* into neurons, keratinocytes, myocytes, tenocytes, osteoblasts and chondrocytes. However, only neurons and myocytes have shown functional properties such as depolarization and contraction (128, 134). Equine tenocytes are apparently challenging to obtain (127) but mechanical loading can improve differentiation (63), and osteoblasts derivation can be promoted in 3D scaffolds that would also facilitate clinical application (129). Obtainment of chondrocytes from equine iPSCs has been limited and non-conclusive. Equine chondrocytes were obtained during spontaneous differentiation of iPSCs (60), but its derivation using an intermediate MSC stage has shown mixed results (60, 88, 152). More progress has been reported in the derivation of functional specific cells from canine iPSCs, including mature megakaryocytes able to release functional platelets (153).

Regarding all the considerations that have been discussed for iPSC differentiation, in companion animals it should be emphasized the need of further research into their embryological development to fine-tune differentiation protocols adapted to the particularities of each species, as well as on characterization of the obtained cells thus requiring species-specific reagents. Furthermore, the approach to generate iPSCs can later influence their differentiation potential, not only because of the epigenetic memory of the cell, but also because the permanent expression of transgenes may interfere with the differentiation process, which needs pluripotency silencing (154).

3.2.1.2. Derivation of MSCs from iPSCs

Provided the complexity of deriving specific types of cells from iPSCs and the limitations of directly using undifferentiated iPSCs, an intermediate approach has been proposed: the derivation of MSCs from iPSCs, known as iMSCs. While this might look as a considerable round about, the use of iMSCs has several notable advantages over primary MSCs. In contrast to primary MSCs that are commonly obtained from more invasive sources like bone marrow or adipose tissue and require large quantities of tissue for isolation, iPSCs can be generated from small numbers of cells obtained from less invasive sources such as skin or blood. Furthermore, while primary MSCs are

subjected to considerable variability among tissue sources, iMSCs can be derived from iPSC lines coming from single cell colonies, thus substantially increasing the homogeneity of the cell population and facilitating standardization of the cell product. In addition, primary MSCs do not possess a limitless self-renewal potential and enter senescence after some time expanding *ex vivo*. This requires collecting tissue again for MSC isolation and results in a new population of cells, even if the same donor is used. On the other hand, iMSCs can be derived from the same iPSC clonal line multiple times, and both iPSCs and iMSCs can be easily cryobanked for later use. In summary, iMSCs could be used for large scale production of homogeneous population of cells leading to phenotypical, molecular and biological stability that ultimately is needed for an ideal off-the-shelf product for therapeutic use (155, 156).

Despite of the advantages, the development of efficient and scalable methods for generating high-quality iMSCs remains a challenge in need of further investigation. Of note, MSCs found in adult tissues do not have all the same embryological origin. Most MSCs derive from the mesoderm, but some of them come from the paraxial mesoderm while others generate in the lateral plate mesoderm, or even in the extraembryonic mesoderm (157). Furthermore, the origin of some MSC populations has been traced back to neural crest cells generated in the ectoderm (158). These observations are important to understand the natural heterogeneity of MSCs, as well as to define strategies to derive them *in vitro* from iPSCs. General approaches for iMSC generation have been reviewed elsewhere (159) and are beyond the scope of this review, however it is worth highlighting that the derivation methodology can influence the properties of the resulting iMSCs. While main characterization features seem to be preserved, the functional properties of iMSCs may differ from their natural counterparts and among derivation strategies (160). This could constitute a limitation, but also an opportunity to direct iMSCs toward specific characteristics depending on the intended use.

Mesenchymal stromal cells from iPSCs have been generated in horses and dogs to obtain multipotent progenitor cells readily available for therapeutic use. Lepage et al. (152) used equine fetal fibroblasts to generate equine iPSCs lines that were subsequently differentiated into iMSCs. These displayed a typical fibroblast morphology, testing positive for CD29, CD44, and CD90 surface markers, and when tested for tri-lineage differentiation were able to differentiate into osteogenic and adipogenic lineage while failing to achieve chondrogenesis in 3D pellet culture (152). Similarly, Chung et al. (88) generated equine iMSCs by serial passaging of iPSCs in MSC-defined media which were characterized as CD29 and CD44 positive and were able to differentiate successfully into the chondrogeneic, osteogenic, and myogenic lineages (88). On the canine side, iMSCs have been generated by inhibiting the TGF-β/Activin pathway in MSC-defined media following serial passaging. Once generated, the canine iMSCs expressed CD73, CD105, STRO1+ and CD24 (135), with variable expression of CD90 and CD44, and did not express the pluripotency marker Oct3/4 as well as the negative surface markers CD45 and CD34 (89). All the canine iMSC lines generated in these studies showed tri-lineage differentiation *in vitro* and, interestingly, when canine iMSCs were compared with BM-MSCs, there was evidence of different time and concentration-dependent effect of dexamethasone and BMP-2 on the onset of osteogenesis, which needs to be taken into consideration for the generation of clinically relevant cells (90). The risk of uncontrolled *in vivo* differentiation would not be an issue since injected iMSCs locally in immunocompromised mice and systemically in healthy Beagle dogs did not form any teratomas or abnormal tissues showing potential for therapeutic safety (89). Although limited, the number of published studies shows the feasibility to generate successfully iMSCs in companion animals.

In addition to the interest on iMSCs for therapy, in human medicine patient-specific iMSCs have been exploited significantly as platforms for drug screening and toxicity for multiple conditions affecting mesenchymal lineages such as osteogenesis imperfecta (161), Fanconi anemia (162), fibrodysplasia ossificans progressiva (163), and Hutchinson-Gilford progeria syndrome (164). Based on this, future studies are needed in companion animals to assess efficiently the quality and optimize the large-scale production of iMSCs-based therapeutics and research platforms.

3.2.2. Master banks of iPSCs for veterinary applications

Genetic mutations that cause the disease can be present in the starting cells, which could be transmitted to the newly generated iPSCs. While this is valuable for disease modeling applications by generating patient-specific iPSCs, it turns out to be a barrier for therapeutic use of autologous iPSCs. Furthermore, the quality of somatic cells to generate autologous iPSCs can be diminished by such genetic diseases or by aging, leading to a reduced yield of functional iPSCs and increasing the risk of rejection. This, in turn, can prolong treatment timelines and negatively affect therapeutic outcomes (165, 166). Moreover, the complexity and cost of generating iPSCs make it currently unpractical to produce these cells from the own patient, added to the prolonged time required to obtain, expand and characterize the iPSCs significantly delaying the treatment (167).

To overcome these limitations, allogeneic therapy has been proposed as a more feasible alternative. iPSCs can be generated from a healthy donor and then used to treat multiple patients. This eliminates the need for individualized cell sourcing, reducing the time and costs associated with autologous therapies. Additionally, allogeneic iPSCs can be characterized in detail prior to banking them to ensure their identity and quality, which otherwise would add significant further delay to the autologous treatment (167). Stem cell banks increase the availability and ensures the quality of the cell products, while reducing the time to administer the therapy (168). Not only iPSCs can be banked, but also their derivatives including iMSCs (169) and some types of differentiated cells (170), as well as their secretomes (171). However, it is important to note that allogeneic therapy also has its own limitations, highlighting the risk of immunological rejection (167). Even though the immune responses generated against allogeneic iPSCs and their derivatives requires further investigation, various strategies are being developed to overcome this potential hurdle. The use of immunosuppressive drugs and the genetic engineering of cells to reduce their immunogenic potential have been suggested. These approaches may be effective but also raise several concerns, such as drug side effects or further manipulation of the cell's genome (172). Therefore, the focus could be put on the selection of donors.

Haplobanks have been proposed as a solution to provide a more widely available source of allogeneic iPSCs. The underlying idea is to select donors who are homozygous for the most common haplotypes for the MHC. While the genetic diversity of MHC haplotypes is high,

some haplotypes are more common within a population. Identifying which haplotypes are more prevalent and banking iPSCs from healthy donors carrying such MHC types can allow providing MHC-matched cell products to a considerable part of the population (173, 174). This way, by using a limited number of selected donors, haplobanks can reduce the genetic diversity of the iPSCs and limit the risk of immunological rejection. Furthermore, the iPSCs stored in haplobanks can be differentiated into various cell types, which can then be used for transplantation or *in vitro* disease modeling (175). For instance, a clinical-grade iPSC haplobank in Japan has been established from seven donors and can provide HLA-matched iPSCs for approximately 40% of the Japanese population. This haplobank was released in 2015 and since then has provided iPSCs for over 10 clinical trials (176). This strategy could be transferred the veterinary field owed the growing knowledge on MHC haplotype diversity in different breeds of companion animals (177–180). To the best of our knowledge, iPSC haplobanks for companion animals are not yet a reality, but there are initiatives to create haplobanks for veterinary MSCs. The impact of MHC matching in MSC therapy in veterinary patients is being increasingly acknowledged (181–184), and the most common MHC haplotypes have been defined in several equine populations (177, 178). Following this path, the same concept could be implemented for animal iPSCs in the coming years.

3.2.3. iPSC-based cell-free therapy

Another alternative to the limitations posed by iPSCs for their clinical application is the utilization of extracellular vesicles (EVs) or the entire secretome obtained from iPSCs, or from their derivative cells such as iMSCs or other cell types (185). It has been proposed that cells mainly communicate through their secretome, which consists of either packed or free components. The packed secretome, also known as EVs, are nano-sized sacs produced by a wide range of cell types, including different stem cells with therapeutic potential. EV-based therapy has gained substantial attention in recent years due to their benefits over traditional cell therapy, as it allows a cell-free modality that overcomes concerns related to immunogenicity and cell survival, and increases product standardization. The EVs comprise a diverse range of biologically active substances, including proteins, lipids, and nucleic acids, that can be delivered directly to target cells, leading to a desired therapeutic outcome (186). As intermediaries in cell therapy, EVs transmit information similar to their cells of origin. In comparison to cell therapy, where cells need to survive, migrate and differentiate for a therapeutic effect to occur, EVs can be more easily delivered to the site of injury because of their smaller size, either through direct injection or intravenous administration, without being recognized by the host immune cells. In the case of iPSC-based therapy, using their secretome also prevents additional concerns related to this specific type of cells, like their tumorigenicity (187).

Induced pluripotent stem cell-derived EVs offer the possibility of personalized medicine by producing patient-specific EVs and can be engineered to contain specific drugs or components. Despite this is a relatively new field needing further work, iPSC-derived EVs have been investigated in human regenerative medicine for various diseases, including osteoarthritis, skin and auto-immune disorders (188–190), however their application in companion animals has yet to be fully explored. To date, only one study has been conducted in dogs, serving as canine model for using human iPSC-derived cell-free secretome to enhance post-pneumonectomy compensatory response. Human iPSC-derived secretome showed improved angiogenesis and alveolar remodeling leading to enhanced gas exchange after the pneumonectomy (191). This highlights the therapeutic potential of iPSC-derived secretome and EVs in human and veterinary patients, particularly due to their ease of administration. Nevertheless, further research is necessary to fully realize their potential and to scale-up and standardize the production.

3.2.4. Manufacturing of iPSCs for veterinary applications

The clinical adoption of robust and high quality iPSCs and their cell-derivatives requires a standardized and reliable cell Good Manufacturing Process (cGMP). Due to their ability to proliferate indefinitely, these cells can represent a true off-the-shelf product that can be manufactured in high number of identical doses from one cell line (192). To achieve this, the intrinsic challenges elaborated in details above for the generation of iPSCs need to be addressed in a large-scale-up context needed for cGMP. These aspects have not been assessed and published for companion animals, however, they have been implemented for human cGMP manufacturing and important considerations can be translated (112). For example, careful choice of the starting tissue source is fundamental and for large-scale manufacturing it would be ideal to use one less prone to chromosomal aberrations and epigenetic memory such as umbilical cord blood or peripheral blood due to sampling accessibility. The variability in cell reprogramming strategies represents a bottleneck and ideally, a process ensuring genomic and phenotypic stability would need to be implemented using non-integrating vectors or peptide-based delivery of transcription factors that can be easier to standardize for regulatory approvals. The laborious manual selection of iPSC colonies based on morphology would need to be automated in a high accuracy and robust process based potentially on micro-devices able to perform immunoselection for clinical grade sorting, and the *in vivo* teratoma assay could be replaced by a rapid qPCR throughput testing (193). The costs and standardization of cGMP manufacturing in general represent a limiting factor in veterinary medicine as also species-specific differences are a critical factor that needs to be taken into consideration. The differentiation toward an intermediate cell type such as iMSCs could be a beneficial step since guidelines for MSCs manufacturing in veterinary medicine have been published (102) and these can be easily translated into the process.

4. Discussion and conclusion

The application of iPSCs in the veterinary field is clearly less developed than in the human side. While this is not surprising because this is a complex and relatively new field, it seems that iPSC advancement in companion animals substantially faces bigger challenges. When thinking about the whole process of developing an application using iPSCs for companion animals, one can realize that there are additional hurdles since the very beginning. The generation of iPSCs in dogs, horses or cats has proved to be less effective than in humans. While transgene-free methodologies for reprogramming are already customarily used to obtain human iPSCs, in the veterinary side the integrating virus methods remain the most common.

Furthermore, even for this approach, there are no standardized protocols yet and different methodologies with variable outcomes are reported, plus the same approach does not always work depending on the cell type (59). Even when putative iPSCs are generated, their characterization can show mixed results suggesting that some lines may not be fully reprogrammed, as it is also pointed out by the permanent transgene expression detected in some works (24). Hurdles continue after iPSC lines are established and ready for application. The pluripotent nature of these cells carries higher risks of tumorigenesis than the use of adult stem cells, and immunogenicity can be a concern not only in the allogeneic scenario but also in autologous application (26), which may compromise the safety of the therapy. Furthermore, xeno-free systems need to be further developed in veterinary iPSCs to reduce the risk of xeno-antigens.

Some of these limitations can be overcome by using cells already differentiated, derived from iPSCs. While theoretically iPSCs can be differentiated into any cell of the body, highly specific protocols are needed to provide the exactly required conditions, being the development of such protocols another field of great importance (194). A relatively easier alternative can be the derivation of iMSCs, which can greatly increase the availability, standardization and homogeneity of these cells compared to primary sources (160). Furthermore, manufacturing and cell banking strategies could be transferred from primary MSCs to iMSCs, or even to iPSCs and/or their derivatives like EVs (187). Creation of cell banks would facilitate the availability of different cells and cell products for several applications while reducing the time for treatment by using allogeneic cells. In this regard, the creation of haplo-banks to match donors and patients by their MHC has been proposed in human iPSCs (173) and is gaining consideration for veterinary MSCs, so this could also be transferred to veterinary iPSCs.

Considering the gaps in the field of iPSCs in companion animals, we propose five main areas in which focus needs to be placed:

First, we need to acknowledge and understand the differences between animal species and human. While extrapolating methodologies from the human side is common in veterinary research and certainly useful at initial stages, it is important to unveil the differences and work toward them. Better understanding on companion animal embryology is of utmost importance both to understand the pluripotency networks of ESCs and to direct the differentiation of iPSCs toward the desired cell lineage. Therefore, we need more basic research on this area and more work to transfer that knowledge into application in the iPSC generation, characterization and differentiation.

Second, more basic research is also needed to characterize clinically relevant cell types in companion animals. Most iPSC applications are based on deriving cells, either for therapy or for *in vitro* research like disease modeling. We need not only to understand how to derive these cells, but also to develop the tools to ensure their identity and, importantly, their functionality.

Third, even though it is crucial to firstly laying the foundations, it is equally important to start developing tools to keep building the field in the future. In this sense, parallel efforts are needed to establish approaches allowing implementation of iPSC applications in companion animals. Optimization and standardization of protocols for veterinary iPSC generation and characterization are very much needed, but in a later stage we will also need the tools to scale up the production of these cells and their derivatives in xeno-free conditions, using cGMP manufacturing and banking.

Fourth and closely related to all of the above, another area in need of improvement is the production and validation of species-specific reagents, like growth factors, antibodies, or other molecules needed during the processes of generation, characterization, expansion, and differentiation of iPSCs in companion animals. While there are some commercially available products suitable for veterinary species, the increasing specialization requires further development of custom solutions. This constitutes an interesting opportunity of collaboration with industry, and is an integral part of the cell therapy field in veterinary medicine.

Last, but not the least, promoting collaboration among researchers working in the veterinary iPSC field is key. Provided the many challenges we face, the best way to advance is to do it together by sharing expertise and resources, as well as experiences and failures. Some actions in this direction could include establishing a network of researchers and creating task forces, as well as considering the creation of bio-resources like cell banks, while seeking funding to support these actions. As in the fourth point above, the involvement of industry could also bring interesting opportunities to develop such networking and collaborations by taking advantage of industrial management skills and resources.

In conclusion, we are in front of a field of great promise that can significantly contribute to developing new therapies for veterinary patients, but also to providing critical information for human medicine. The veterinary field can greatly benefit from the advancements in the human side, but we also need to appreciate the differences and conduct basic research, while getting ready for the application.

Author contributions

LB and FB contributed to the conception and structure of the manuscript. LB, TE, AO'B, and AI contributed to the literature revision and manuscript writing. TE designed the figures. LB, TE, AO'B, AI, and FB critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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.

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Characterization of canine adipose- and endometrium-derived Mesenchymal Stem/Stromal Cells and response to lipopolysaccharide

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Mesenchymal stem/stromal cells (MSCs) are used for regenerative therapy in companion animals. Their potential was initially attributed to multipotency, but subsequent studies in rodents, humans and veterinary species evidenced that MSCs produce factors that are key mediators of immune, anti-infective and angiogenic responses, which are essential in tissue repair. MSCs preparations have been classically obtained from bone marrow and adipose tissue (AT) in live animals, what requires the use of surgical procedures. In contrast, the uterus, which is naturally exposed to external insult and infection, can be accessed nonsurgically to obtain samples, or tissues can be taken after neutering. In this study, we explored the endometrium (EM) as an alternative source of MSCs, which we compared with AT obtained from canine paired samples. Canine AT- and EM-MSCs, formed CFUs when seeded at low density, underwent tri-lineage differentiation into adipocytes, osteocytes and chondrocytes, and expressed the CD markers CD73, CD90 and CD105, at equivalent levels. The immune genes IL8, CCL2 and CCL5 were equally expressed at basal levels by both cell types. However, in the presence of the inflammatory stimulus lipopolysaccharide (LPS), expression of IL8 was higher in EM- than in AT-MSCs ($p<0.04$) while the other genes were equally elevated in both cell types ($p<0.03$). This contrasted with the results for CD markers, where the expression was unaltered by exposing the MSCs to LPS. Overall, the results indicate that canine EM-MSCs could serve as an alternative cell source to AT-MSCs in therapeutic applications.

KEYWORDS

MSC, veterinary MSC, dog, canine, regenerative, repair, differentiation, LPS

Introduction

Mesenchymal stem/stromal cells (MSCs) are multipotent cells used in regenerative therapy in companion animals. MSCs have been classically obtained from adipose tissue (AT) and bone marrow (BM) (1–7) but other tissues, including endometrium (EM) (8–10), Wharton's jelly (11) and umbilical cord blood (12) have also been used as the source of these cell preparations. Previous studies in human and veterinary MSCs (8, 13–16), exploring different tissue sources (16, 17), showed that although MSCs share many similar properties, they also evidenced

individual features depending on the tissue of origin. Indeed, when compared to AT-MSCs, equine EM-derived MSCs (EM-MSCs) have distinct immune (17) and transcriptomic signatures (14), possibly consequence of being a tissue naturally exposed to infection and inflammation.

Traditionally, veterinary MSCs have been defined following the International Society for Cellular Therapy (ISCT) guidelines for human cells, namely on the expression of cell surface markers CD73, CD90 and CD105 and ability of these cells to undergo trilineage differentiation into adipocytes, chondrocytes and osteocytes (18, 19). Although valuable, some of the initial ISCT guidelines proved difficult to apply to veterinary species, commonly consequence of the natural absence of expression of particular CD markers or due to technical difficulties associated with the lack of appropriate antibodies (20, 21). Over the years, it also became clear that these criteria did not necessary contemplate or correlate with cell function. Therefore, toward a better definition and standardization of veterinary MSC preparations, different groups published guidelines, based essentially in equine and to a less extent in canine data, to help addressing this issue (22, 23).

In addition to cell differentiation capability, MSCs produce diverse angiogenic and immune factors (24) which are relevant during repair and anti-infective body responses. Altogether, these findings stimulated great interest in the use of MSCs for different therapeutic applications in companion animals, for example on joint disease, wound infection, chronic gingivostomatitis, atopic dermatitis, multidrug resistant infections, among others (25–29).

Whilst MSCs from humans and veterinary species, particularly from horses but also from dogs (23, 30), have been extensively studied toward their therapeutic use in inflammation-associated disease, namely in cartilage degeneration and osteoarthritis, considerably less work has been done on the anti-infective properties of MSCs, which is an area that has just recently started being explored. Relevant to both inflammatory-and infection-associated settings is the communication between MSCs and immune cells. Indeed, MSCs are highly responsive to inflammatory stimuli, for example when exposed to cytokines IL1β, TNFα, IL8 and IL6 (31, 32). Likewise, infectious products such as bacterial lipopolysaccharide (LPS), polyinosinic:polycytidylic acid or the peptidoglycan dipeptide iE-DAP (17, 31, 33) activate Toll-like and nucleotide-binding oligomerization domain (NOD)-like receptors (TLR, NLR), respectively, resulting in increased expression of immune modulators and antimicrobial factors (17, 31, 34, 35). Indeed, activation of human and equine MSCs with LPS, upregulates the expression of chemokines such as CCL2, CCL5, IL8 and IL6 (17, 34, 35), which are involved in recruitment and maturation of immune cells, neutrophils and monocytes (36–38). LPS is a toxin present in the outer membrane of Gram-negative bacteria such as *Escherichia coli*, which is frequently associated with canine infection, for example of the urinary tract. Testing canine MSC response to LPS will inform on the behavior of these cells in an infectious context, namely on their response upon LPS activation *in vitro*. Importantly, priming of MSCs with LPS was shown to be of benefit both *ex vivo*, and in *in vivo* studies involving rodent models of disease (39, 40), therefore supporting activation of MSCs as a way to enhance the properties of these cell preparations.

Considering what was described above, in this study we compared canine MSCs derived from two tissue sources, EM and AT. In addition to the standard MSC characterization, and to compare EM- and AT-MSCs further, we measured a selected group of immune factors (IL8, CCL2 and CCL5) that were expressed in these cells at basal levels and, as we have previously assessed in equine MSCs, were induced by LPS.

Materials and methods

Extraction of canine MSCs from endometrium and adipose tissue

Samples were obtained from spare tissues of the reproductive tract of female dogs ($n = 3$; Supplementary Table S1) undergoing sterilization at the Royal (Dick) School of Veterinary Studies, following approval by the Ethical Review Committee, University of Edinburgh. For each animal, ovaries, uterine horns and uterine body were removed as one piece and immediately transported (on ice) to the laboratory to be processed. A solution of cold phosphate buffered saline (PBS) with 1% of penicillin/streptomycin mix (P/S; Life Technologies), and 5 µg/ml amphotericin B (Gibco-Thermo Fisher Scientific) was used for washing the tissues. Then, the uterine body and horns were cut longitudinally with a scalpel to obtain the EM by scraping the tissue, and the AT surrounding the reproductive tract was also harvested for extraction, from each animal. The collected tissues were washed, minced and digested. EM was digested with collagenase I (5 mg/ml; Gibco-Thermo Fisher Scientific, 17100-017) and AT with collagenase II (1 mg/ml, Gibco-Thermo Fisher Scientific, 17101-015) for 45 min at 37°C, and under constant moderate agitation (70 rpm). Collagenase activity was stopped with Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Sigma-Aldrich) supplemented with 20% fetal bovine serum (FBS) (Gibco-Thermo Fisher Scientific). Cells were then filtered through a 100 µm strainer and cultured in DMEM containing 20% FBS, 1% of P/S at 37°C. Pictures were taken using a Nikon Eclipse TE2000U Microscope. All experiments were performed with MSCs grown between passages 3–5.

Clonogenicity

To obtain colony forming units (CFUs), 500 cells/well were seeded in 6-well plates. Cells were grown for 10 days in complete growth medium DMEM (Sigma-Aldrich) containing 20% FBS (Gibco-Thermo Fisher Scientific) and 1% of P/S (Life Technologies). After that, CFUs were washed with PBS, fixed with PFA (2%; 30 min) and stained with 0.5% crystal violet for 30 min.

Cell differentiation

For adipogenic differentiation (41), MSCs were seeded in triplicate in 24-well plates (50,000 cells/well) and expanded in growth medium until confluence. Adipogenesis was induced using the medium containing 10% FBS, 1 µM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 10 µg/ml insulin (Sigma-Aldrich), 100 µM indomethacin (Sigma-Aldrich), 1% P/S in DMEM. Cells were kept in differentiation

medium for 6 days and then changed to 10% FBS, 10 µg/ml insulin and 1% P/S for a total of 14 days. Cell growth medium was used for the control cell group. Both differentiated and control MSCs were fixed with 4% paraformaldehyde before visualization of lipid droplets by Oil Red O staining. Imaging was performed in a Zeiss Axiovert 25 Inverted Phase microscope using Zen 2 software (Advanced Micro Devices).

Osteogenesis was induced with a mixture of DMEM high glucose and DMEM low glucose (50:50 v/v; Sigma-Aldrich), supplemented with 10% FBS, 100 nM dexamethasone (Sigma-Aldrich), 10 mM sodium β-glycerophosphate (Sigma-Aldrich) and 0.1 mM stabilized ascorbic acid (Sigma-Aldrich). After 3 days, cells were changed to DMEM low glucose medium with the same supplements. Cells were cultured for 19 days and medium was changed every 3 days. At the end of the differentiation period cells were fixed with 4% paraformaldehyde, stained with Alizarin Red (2%; pH 4.2) and imaged in a Zeiss Axiovert 25 Inverted Phase microscope using Zen 2 software (Advanced Micro Devices).

For chondrogenesis, MSCs were suspended in a small volume of media to generate a concentrated cell solution of 1.6×10^7 cells/ml. Micromass cultures, 5 µl droplets of this cell suspension, were seeded in 96-well plates and incubated at 37°C for 2 h under high humidity conditions. STEMPRO® chondrogenesis differentiation media (Gibco, Fisher Scientific) was then added to the micromasses and refreshed every 2–3 days. Cell growth media was used for controls. After 16 days, cells were fixed with 4% formaldehyde for 30 min and stained with 1% alcian blue solution prepared in 0.1 N HCL.

LPS stimulation experiments

MSCs were plated in 12-well plates (75,000 cells/well) and kept for 48 h, prior to incubation for 16 h with 0.1 µg/ml lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma, L2630), alongside with unstimulated control cells. AT- and EM-MSCs were harvested with Trizol and immediately stored at −80°C prior to RNA extraction.

RNA extraction and cDNA synthesis

RNA was extracted from cells in Trizol following manufacture's protocol and reverse transcribed into cDNA using Superscript III (Invitrogen-Thermo Fisher Scientific). A NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, United States) was used to measure the quality and concentration of RNA. Negative controls were produced either without RNA sample or superscript enzyme.

Quantitative PCR analysis

Gene transcript levels were quantified by qPCR using the primers listed in Table 1 using SensiFAST SYBR Lo-ROX kit (Bioline) in a MX3005P thermocycler (Stratagene), using the conditions, Step 1: denaturation at 95°C for 2 min, followed by Step 2: 40 cycles of, 95°C for 5 s, 60°C for 11 s, 72°C for 5 min. Step 3: Final extension; 95°C for 1 min, 60°C for 30 s, 95°C for 30 s. Results were analyzed with MxPro software (Stratagene) relative to a standard curve obtained from a pool of cDNA samples. Two housekeeping genes (18S and GAPDH) were used to normalize the individual gene expression results.

TABLE 1 Primers used in qPCR.

Gene	Sequence (5′ to 3′)
CD45	F: TCGGCTTTGCCTTTCTGGAT
	R: TTCTGGGGAAACAGAACTGGA
CD73	F: TACACAGGTACTCCACCTTCCA
	R: AACCTTCCGCCCATCATCAG
CD90	F: AGGACGAGGGGACATACACA
	R: CTTGACCAGTTTGTCTCTGAGC
CD105	F: CCTGGAATCCTCAAGGGAGC
	R: ACTGAGGACCAGGAACACCT
IL8	F: TGTGAAGCTGCAGTTCTGTCAA
	R: TTGGGATGGAAAGGTGTGGAG
CCL2	F: AAGCTGTGATCTTCAAGACCGT
	R: CATGGAATCCTGGACCCACT
CCL5	F: CAGTCGTCTTTGTCACCCGA
	R: TGTACTCCCGCACCCATTTC
18S	F: GCTGGCACCAGACTTG
	R: GGGGAATCAGGGTTCG
GAPDH	F: GCCTGGAGAAAGCTGCCAAA
	R: TTTGAGGGGTCCCTCCGATG

F and R stands for forward and reverse primers, respectively.

Statistical analysis

Results were analyzed by Student's *t*-test, or two-way ANOVA followed by LSD *post hoc* test as appropriate, by using GraphPad Prism 9. Statistical significance was set at $p < 0.05$.

Results

Characterization of AT- and EM-derived MSCs

Both AT- and EM-MSCs growing in culture presented the typical spindle-like morphology of MSCs (Figure 1A), form CFUs (Figure 1B) and expressed CD markers CD73, CD90 and CD105 (Figure 1C) at similar levels, while CD45 was undetectable in both cell types. After incubation with adipogenic media, AT- and EM-MSCs gradually changed their morphology and lipid droplets accumulated as shown by oil red O staining (Figure 2A). In MSCs undergoing osteogenesis alizarin red staining evidenced the deposition of calcium in the differentiated cells (Figure 2C). For chondrogenesis, MSCs cultured at high cell density as micromasses acquired a round morphology, and differentiation was confirmed by alcian blue staining 16 days following the start of differentiation (Figure 2B). No differences were observed between EM- and AT-MSCs differentiated cells.

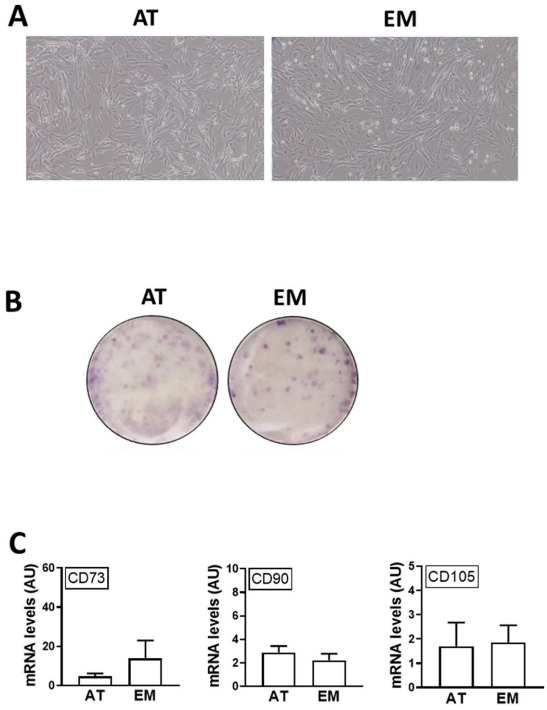

FIGURE 1
Characterization of AT- and EM-derived MSCs. **(A)** Micrographs of cells in culture taken at 10x magnification. **(B)** Cell colonies obtained from 500 cells/well and stained with crystal violet after 10days in culture. **(C)** Expression levels of CD markers CD73, CD90 and CD105 quantified by qPCR. All results are shown as mean±SEM; AU, arbitrary units.

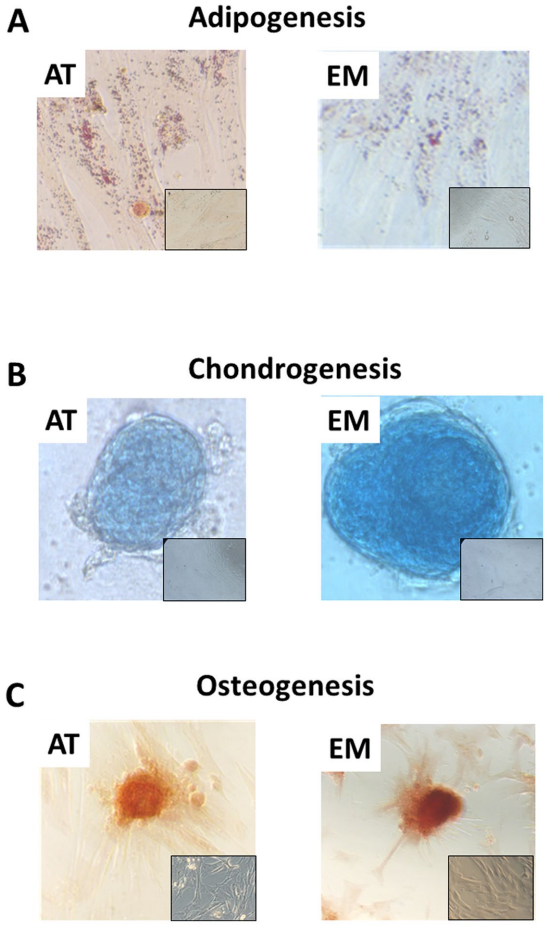

FIGURE 2
Tri-lineage differentiation of AT- and EM-MSCs. Micrographs showing **(A)** adipocytes, **(B)** chondrocytes and **(C)** osteocytes stained with Oil red O, Alcian blue and Alazarin Red, respectively. Insets show control non-differentiated cells. Micrographs were taken at 40× magnification.

Gene expression of MSC markers was unchanged by stimulation of AT- and EM-MSCs with LPS

In order to test if MSC markers were affected by cell activation, gene expression of CDs 73, 90 and 105 was measured by qPCR following incubation of cells with LPS for 16 h. LPS treatment did not affect the morphology of the cells, as shown in Figure 3A, and did not cause variation in gene expression levels of CDs 73, 90, and 105 in both AT- and EM-MSCs (Figures 3B–D). Likewise, no differences were observed between AT- and EM-MSCs CDs expression levels (Figures 3B–D).

Gene expression of immune mediators in MSCs was increased by LPS

Both cell types, AT- and EM-MSCs, expressed the cytokine IL8 and chemoattractants CCL2 and CCL5 at similar basal levels (Figures 4A–C). Those genes were significantly increased when cells were exposed to LPS ($p < 0.03$), except for CCL5 in EM-MSCs where gene expression was not significantly altered. In the presence of LPS, IL8 values were higher in EM-than AT-MSCs ($p < 0.04$), while no other differences were observed between the MSCs obtained from these two tissue sources.

Discussion

In this study, we compared MSCs from two different tissue origins, EM and AT, obtained from the reproductive tract of healthy female dogs undergoing sterilization. EM- and AT-MSCs in culture displayed the standard spindle-like shape, formed CFUs when seeded at low cell density, expressed CD markers (CD73, CD90 and CD105) and underwent tri-lineage differentiation at similar level. Of note, both EM- and AT-MSCs expressed the cytokines IL8, CCL2 and CCL5 at basal levels. Therefore, to further characterize and compare the canine MSCs, and based on previous results in equine MSCs showing differential expression of these cytokines (17), we measured the effect of LPS on the expression of IL8, CCL2, and CCL5, in EM- and AT-MSCs. This resulted in elevated values of IL8, CCL2 and CCL5 in LPS-induced MSCs, with levels of IL8 for EM-MSCs being significantly higher than for AT-MSCs. Contrary to these results, CD marker levels remained unchanged upon priming of the cells with LPS, and therefore CD marker expression did not correlate or reflect cell activation.

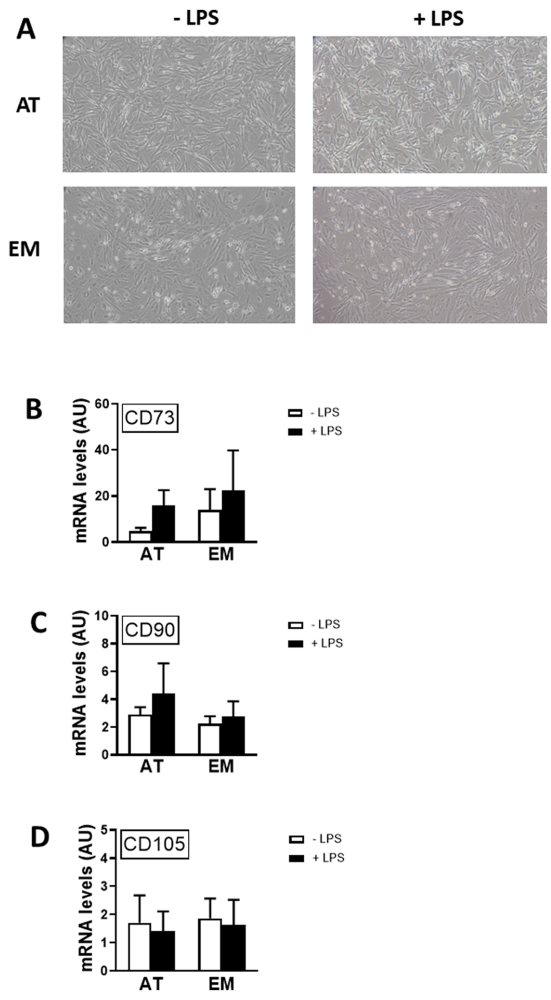

FIGURE 3
Effect of LPS on the expression of MSC markers in AT- and EM-MSCs. **(A)** Micrographs of control cells (– LPS) or stimulated with LPS (+ LPS). **(B–D)** Expression levels of the CD markers CD73, CD90 and CD105 quantified by qPCR, of controls (– LPS; white bars) versus LPS-stimulated cells (+ LPS; black bars). All results are shown as mean±SEM; AU, arbitrary units.

Canine AT- and EM-MSCs displayed comparable cell features as defined by the ISCT, in agreement to what was observed in equine and human MSCs (13, 17). Indeed, MSCs obtained from different tissue origins share a variety of common features (42), including expression of CD markers and tri-lineage differentiation (8). Expression of CD markers in MSCs is sustained in different conditions, for example when human BM-MSCs are maintained in culture for an extended period of time, although other features including cell morphology, doubling time and osteogenic differentiation are affected (43). Likewise, culturing of equine BM-MSCs in platelet lysate improved chondrogenesis compared to FBS, but did not change the MSC markers CD105 and CD44 (44), and canine BM-MSCs cultured in the presence of FBS had significant higher survival rate compared to cells cultured in serum-free conditions, while CD marker expression levels were unaffected (45). Similarly, in the present study we observed that both AT- and EM-MSCs expressed CD73, 90 and 105 at equivalent basal levels, which were unchanged in the presence of LPS, but immune mediator genes were upregulated upon MSC stimulation with LPS. These results support the idea that expression of CD markers cannot be used as reliable indicators of stem cell content or biological function of MSC preparations. Different surface markers, including Stro-1, SSEA-4, CD271, and the pericyte marker CD146 have been considered as candidates (46) for this purpose. CD146 is present both in MSCs and pericytes, including in the horse (1, 7, 47) where it has been used to isolate cells with superior angiogenic potential compared to the corresponding MSC preparations. Still, considerable more work needs to be done, especially in veterinary species, toward the establishment of proper guidelines for a better characterization and standardization of MSCs preparations, although attempts in this direction have already been made by different groups, especially for equine MSCs (20, 22, 23).

A diverse number of studies in humans, rodents and veterinary species (principally in horses but also in dogs) have tested the effect of priming MSCs with inflammatory and infectious stimuli (24, 48, 49) in order to assess MSC response to disease milieu and to enhance their therapeutic properties (50). Human and rodent MSCs (principally obtained from BM and AT, but also from other sources) primed with a variety of activators such as TNFα, IL1β, IFNγ, IL17A, LPS and Poly I:C showed increased MSC expression of immune modulators, antibacterial peptides, growth and angiogenic factors. TNFα, alone or combined with IL1β, increases the levels of IL6, VEGF, FGF2, IGF-1, and HGF (51, 52), while IFNγ was shown to increase CCL2, IDO, TGFβ, HGF (53, 54), and IL17A to elevate IL6 (55). Infectious stimuli, such as LPS increases pro-inflammatory molecules including CXCL1, IL8, IL6, CCL2 and LL37 (56) while activation of TLR3 results in upregulation of IDO and PGE-2 (57). These findings show the diversity of phenotypes that can be generated by MSC activation depending on the stimulus used.

It is evident from the literature that a diverse number of studies tested inflammatory stimuli with just a few assessing infectious- or bacterial-associated activation. This, together with our previous results in different equine MSCs types, prompted us to follow the same approach here, as the main objective of this study was to compare canine EM- and AT-MSCs properties, although the use of LPS as a single stimulant is a limitation in this study. Indeed, since MSCs are highly responsive to a variety of stimuli, not only immune- and infectious-related but also a diversity of others, additional inducers could be included in future work in order to cover a broader range of responses comparing canine EM- and AT-MSCs. In addition, aiming for a wider characterization, comparison between canine EM- and AT-MSCs could be further complemented by comprehensive gene expression and protein analysis of MSC responses by performing RNA sequencing, LC–MS-based proteomics, or multiplex immunoassays, and we are planning to perform these experiments in future studies.

Similarly to humans and rodents, preconditioning of equine BM-MSCs with IL1β resulted in increased expression of IL1β, IL6, IL8, but not of IL10 and TNFα, while the combine action of TNFα and IFNγ resulted in an anti-inflammatory phenotype, with

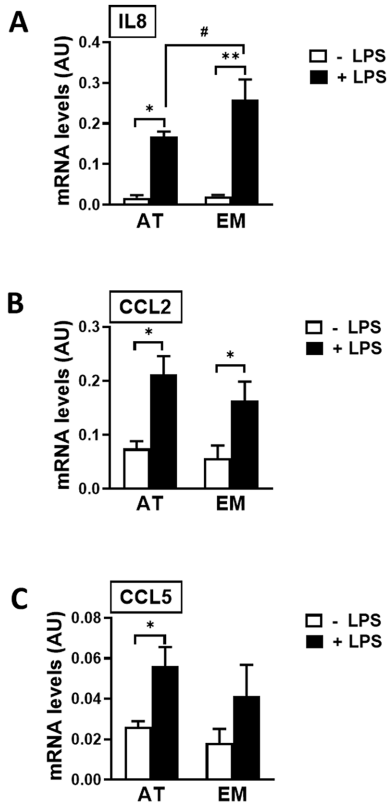

FIGURE 4
Expression of immune mediator genes in AT- and EM-MSCs activated with LPS. mRNA levels of **(A)** IL8, **(B)** CCL2 and **(C)** CCL5 measured by qPCR of control (– LPS; white bars) and LPS-stimulated MSCs (+ LPS; black bars). All results are shown as mean±SEM; AU, arbitrary units. * and ** $p<0.05$ and <0.001, respectively, of control versus LPS-treated cells. # $p<0.05$ of AT- versus EM-MSCs.

increased expression of COX-2, iNOS, IDO, IL6 (32), which effect has also been observed in human MSCs (58). CCL2 was also elevated by TLR3 activation when equine BM-MSC were stimulated with poly I:C (33), but priming with LPS increases CCL2, IL8 and IL6, showing that equine MSCs are highly responsive to bacterial wall components, part of the indirect but relevant role of these cells in modulating the immune response to combat infection.

Likewise, expression of immune genes is altered when canine MSCs are activated, for example COX-2 increases when AT- and BM-MSCs are stimulated with TNFα and IFNγ (59), and priming of AT-MSCs with deferoxamine, a hypoxia-mimetic agent, potentiates anti-inflammatory effects in RAW 264.7 macrophages (60). Also, stimulation of canine MSCs with TNFα elevates TSG-6 and PGE2 resulting in *in vivo* benefit by regulating colonic inflammatory cytokines such as IL1β, IL6, and IL10, and ameliorating induced colitis in mice (61). However, compared to other species, work performed on canine MSC activation is limited.

Here we showed that stimulation of canine MSCs with LPS increased the expression of immune genes IL8, CCL2 and CCL5, except CCL5 in EM-MSCs that was not upregulated. Of note was that in both equine (17) and canine EM-MSCs the expression of CCL2, but not CCL5, was significantly induced by LPS. This indicates that these chemoattractants are differentially induced by LPS in EM-MSCs, at least in these veterinary species. Also, EM-MSCs expressed IL8 at higher levels compared to AT-MSCs, following cell stimulation with LPS. Indeed, the immune properties of MSCs may vary depending on the tissue of origin (8, 42, 62, 63). Equine EM-MSCs activated with LPS express IL6 at higher levels than AT-MSCs (17) and human dental MSCs express INF-γ, PDGFA, VEGF and IL10 more elevated levels than BM-MSC (64), while human AT-MSCs produce higher levels of IL6 and TGF-β1 than BM-MSCs. In contrast, IFNγ treatment of bovine BM- and AT-MSCs increased IL6, PTGER2 and IDO gene expression at similar levels (65), indicating that species and type of stimulus play a role in the expression of immune factors in MSCs from different tissue origins.

In this study, comparison of EM- and AT-MSCs showed that their MSC properties were similar. This agrees with previous studies in equine MSCs and suggests that EM-MSCs could serve as a viable alternative to AT-MSCs, especially given the availability of tissues resulting from routine spays. Expression of CD markers was similar in both cell types, including upon cell incubation with LPS, while the expression of cytokines was upregulated. These findings corroborate with the general position in the field that CDs are not reliable markers defining stem cell content and biological function of MSC preparations. The results also showed that both canine EM- and AT-MSCs are highly responsive to LPS demonstrated by the upregulation of cytokine gene expression, similarly to what has been previously observed in other veterinary studies, mostly in the horse. However, compared to other species, work performed on canine MSC activation is scarce. Higher levels of IL8 expression were observed in LPS-activated EM-MSCs compared to AT-MSCs, but not for other cytokines. We have previously found differential expression of immune genes in EM-MSCs compared to AT-MSCs preparations. However, how these two cell types would perform in *in vivo* repairing settings is currently unknown and warrants further studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

HP and AA performed the experiments, analyzed the results and wrote the manuscript. KM provided the samples for analysis. CE designed the study, analyzed the results and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1180760/full#supplementary-material

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Influence of Rho/ROCK inhibitor Y-27632 on proliferation of equine mesenchymal stromal cells

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Mesenchymal stromal cells (MSC) isolated form bone marrow and adipose tissue are the most common cells used for cell therapy of orthopedic diseases. MSC derived from different tissues show differences in terms of their proliferation, differentiation potential and viability in prolonged cell culture. This suggests that there may be subtle differences in intracellular signaling pathways that modulate these cellular characteristics. The Rho/ROCK signaling pathway is essential for many cellular functions. Targeting of this pathway by the ROCK inhibitor Y-27632 has been shown to be beneficial for cell viability and proliferation of different cell types. The aim of this study was to investigate the effects of Rho/ROCK inhibition on equine MSC proliferation using bone marrow-derived MSC (BMSC) and adipose-derived MSC (ASC). Primary ASC and BMSC were stimulated with or without 10ng/mL TGF-β3 or 10μM Y-27632, as well as both in combination. Etoposide at 10μM was used as a positive control for inhibition of cell proliferation. After 48h of stimulation, cell morphology, proliferation activity and gene expression of cell senescence markers p53 and p21 were assessed. ASC showed a trend for higher basal proliferation than BMSC, which was sustained following stimulation with TGF-β3. This included a higher proliferation with TGF-β3 stimulation compared to Y-27632 stimulation ($p<0.01$), but not significantly different to the no treatment control when used in combination. Expression of p21 and p53 was not altered by stimulation with TGF-β3 and/or Y-27632 in either cell type. In summary, the Rho/ROCK inhibitor Y-27632 had no effect on proliferation activity and did not induce cell senescence in equine ASC and BMSC.

KEYWORDS

Y-27632, mesenchymal stem cell, equine, senescence, Rho/ROCK

Introduction

Mesenchymal stromal cells (MSC) are a promising therapeutic tool for the treatment of orthopedic diseases (1). MSC can be derived in relatively high numbers from various tissues, such as blood, fat, bone marrow or umbilical cord blood (2–6). The most commonly investigated and used are bone marrow-derived MSC (BMSC). However, since the collection and cultivation of BMSC is associated with some limitations, such as painful aspiration technique of bone marrow, low cell yield, and early cell aging (7), adipose-derived MSC (ASC) have appeared to be a good alternative. While ASC and BMSC are comparable in terms of their morphology and cell surface markers, they show several differences regarding their differentiation and proliferation ability (8–10). This suggests that there may exist subtle differences in intracellular pathways that modulate these cellular characteristics.

The Rho/Rho-associated protein kinase (Rho/ROCK) signaling pathway plays a critical role in the regulation of many cellular functions. One of the major targets of the Rho/ROCK signaling

pathway is the regulation of phosphorylation of myosin-light-chain phosphatase and a number of other phosphokinases and cytoskeleton-binding proteins (11). Through this, Rho/ROCK controls cytoskeletal contraction which is essential for many basic cellular processes, including apoptosis, migration, proliferation, and differentiation (11). Thus, the Rho/ROCK pathway is often targeted to influence cell proliferation and differentiation. For this purpose, there are several small molecule inhibitors of ROCK which affect cells differently depending on the cell type and combinations of growth factors. For example, the competitive ROCK inhibitor Y-27632 can inhibit differentiation triggered by extracellular matrix and mechanical stimuli (12–14) but can promote differentiation triggered by paracrine factors (15–17).

Inhibition of ROCK however has variable effects on other cellular functions. While complete silencing of ROCK protein via gene knockout or potent Rho/ROCK inhibitors promotes cellular senescence and limits proliferation (18), Y-27632 appears to be beneficial for cell viability and proliferation. It can promote long-term proliferation of human embryonic stem cell (ESC)-derived endothelial cells and primary keratinocytes and reduces cell senescence (19–21). Similarly, human ESC show improved viability, cell growth and regeneration ability after cryopreservation when supplemented with Y-27632 (22, 23).

Thus, the modulation of the Rho/ROCK signaling pathway with Y-27632 has proven to be a simple, efficient and versatile approach in embryonic stem cell applications for regenerative medicine research. Should these desirable properties of the Y-27632 inhibitor be applicable to MSC, with the potential positive influence on differentiation and proliferation, the inhibitor would represent a promising candidate for preconditioning of MSC during cell expansion. However, there are no studies to date on the effect of Y-27632 on equine MSC proliferation. Therefore, the aim of the study was to investigate the effects of Rho/ROCK inhibition on the proliferation of equine MSC, taking different tissues of origin into account by comparing ASC with BMSC.

Methods

Cell culture and treatment

Cell culture ingredients were purchased from ThermoFisher Scientific (Warrington, United Kingdom) unless stated otherwise. Adipose-derived MSC and bone marrow-derived MSC were collected from eight different donors (ASC $n = 4$, BMSC $n = 4$). The use of equine MSC was approved by the Royal Veterinary College Clinical Research Ethical Review Board (URN 2022 2127-2 and URN 2021 2035-2). The donors were warmbloods, warmblood crosses (BMSC, age 6–15 years) or welsh cob ponies (ASC, age 2–5 years).

MSC from bone marrow aspirates were prepared as previously described using a standardized protocol in our laboratory for use in the equine clinic (24). Briefly, 10 mL of bone marrow aspirate was diluted with an equal volume of Dulbecco's PBS and layered over 15 mL of Lymphoprep (Stem Cell Technologies, Cambridge, United Kingdom) and centrifuged at 1,200 RCF for 10 min. The buffy layer containing the mononuclear cell fraction was removed and the cells seeded in tissue culture flasks (T-75, Falcon) in cell culture media (DMEM, 1 g/L glucose; Gibco®, 0.11 mg/mL sodium pyruvate) supplemented with 10% FCS (Gibco®) and 1% Penicillin–Streptomycin (Gibco®). Plastic adherent MSC were expanded and passaged to passage number 2 or 3 with an estimated population doubling level of 11–12. Cells were resuspended in cell freezing medium (Cellbanker 2, AMS Biotech, United Kingdom) and stored frozen in liquid nitrogen until used for experiments. Further characterization of the BMSC was not performed because we have previously characterized MSC prepared by this standardized protocol for surface markers and trilineage differentiation (24) and a position statement by Guest et al. recommends that it is not necessary to characterize every batch that utilizes a standard protocol (25).

For the isolation of ASC, 15 g adipose tissue of the dorsal gluteal muscle were collected in same medium as BMSC. The tissue was washed, diced and incubated with 1 mg/mL collagenase I for 1 h at 37°C. After digestion, cells were recovered by centrifugation at 350 RCF for 10 min and then washed two times following resuspension in cell culture media. Cells were seeded into a 10 cm dish in cell culture media at 1,000–5,000 cells cm^2. Plastic adherent MSC were expanded, passaged and stored frozen in liquid nitrogen until used for experiments. ASCs were characterized for trilineage differentiation and expression of CD90, CD29, CD44, CD14 (neg) and CD79a (neg) using assays previously described (26).

For experiments, aliquots of cells were rapidly thawed and cultured in cell culture medium at a seeding density of 5,000 cells/cm^2. Cells were allowed to attach and recover for 24 h before stimulating with 10 ng/mL TGF-β3 (R&D Systems®, Abingdon, United Kingdom), 10 μM Y-27632 (Tocris, Bioscience, Bistrol, United Kingdom), both in combination or with 10 μM etoposide (ab120227, Abcam, Cambridge, United Kingdom) which was used as positive control to induce senescence (27). Concentrations of TGF-β3 and Y-27632 were chosen based on previous studies [(17, 28), respectively]. For the combined treatment, cells were preincubated with Y-27632 for 2 h before adding TGF-β3. Assessments were performed 48 h after stimulation.

Proliferation assay

Cell proliferation was assayed by EdU labeling with the Click-iT® Plus EdU Imaging Kit (ThermoFisher Scientific, Warrington, United Kingdom) according to manufacturer's instructions. Briefly, cells were incubated with 10 μM EdU for 2 h, fixed with 3.7% formaldehyde for 15 min and stained with the Click-iT® reaction cocktail. Cells were then stained with anti-Ki-67 antibody (ab281847, Abcam, Cambridge,

TABLE 1 Equine primers.

Gene	Primer sequence	Gene	Product length (bp)
p21	FOR: ACATACTCTGCTTGCCACCC REV: GGCCCCCTTCAAAGTGCTAT	XM_023624844.1	332
p53	FOR: ACTCCAGCCACCTGAAGTCT REV: GGGGACAGGAAGCAGAGAAT	XM_023651624.1	110
GAPDH	FOR: CATCAAATGGGGCGATGCTG REV: TGCACTGTGGTCATGAGTCC	NM_001163856.1	285

United Kingdom) for 2 h. Cell were counterstained with DAPI and then imaged with fluorescence microscopy (EVOS® FL Imaging System, ThermoFisher Scientific, Warrington, United Kingdom). Images were quantified for cell numbers with ImageJ (Version 1.53 Fiji). A minimum of 500 cells per stimulation group were counted for positive staining of each label. A ratio of EdU or Ki-67 positive cells to total cells (DAPI positive) was calculated.

Gene expression analysis

Gene expression of the senescence markers p21 and p53 (29) were analyzed by real-time PCR. GAPDH was used as a reference gene. Total RNA of equine cells was isolated using the RNeasy Mini Kit (Qiagen, United Kingdom) with additional DNase digestion (Qiagen) according to manufacturer's instructions. RNA was then converted to cDNA using the Reverse Transcriptase RevertAidH Minus kit (ThermoFisher Scientific). 50 ng cDNA was mixed with primers (Table 1) and QuantiNova™ SYBR® Green PCR kit to perform real-time PCR using the CFX96™ Real Time System (Bio-Rad, Hercules, United States). For relative quantification, gene expression ratios and fold changes were calculated with the Pfaffl method (30) and normalized to day control.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 28 software (IBM Deutschland GmbH, Ehningen, Germany). As data were not normally distributed, non-parametric Friedman tests with Bonferroni-adjusted post-hoc tests were used. Differences were considered significant at $p \leq 0.05$. Graphs were designed with GraphPad Prism 9.4.1 (GraphPad Software, San Diego, United States).

Results

ASC were capable of differentiation into adipocytes, osteoblasts and chondrocytes. ASC were positive for CD90, CD29, and CD44 and negative for CD14 and CD79α (Figure 1). ASC and BMSC showed a spindle-shaped, fibroblast-like morphology in cell culture medium,

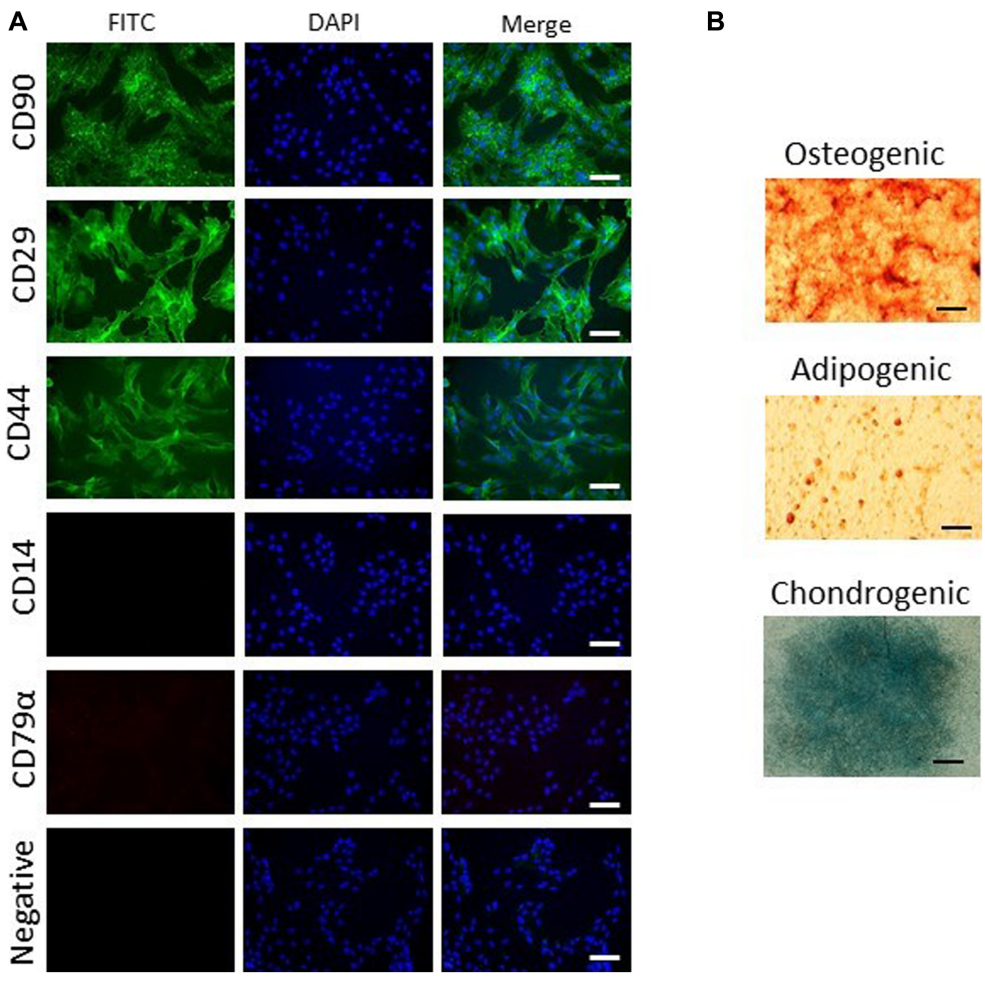

FIGURE 1
Characterization of ASCs. Representative images of three biological replicates show marker expression **(A)** and trilineage differentiation **(B)**. ASCs express CD90, CD29, and CD44 but not CD14 or CD79α (FITC, scale bar=50µm). ASCs undergo trilineage differentiation into cartilage (alcian blue staining, scale bar=1mm), bone (alizarin red staining, scale bar=50µm) and fat (oil red O staining, scale bar=50µm).

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with BMSC appearing more elongated than ASC as assessed by microscopy (Figure 2). BMSC were slower in reaching confluency than ASCs.

In the presence of TGF-β3 alone or with Y-27632, BMSC exhibited a change in morphology which was more marked than in ASC. ROCK inhibition with Y-27632 resulted in the loss of the elongated spindle shape

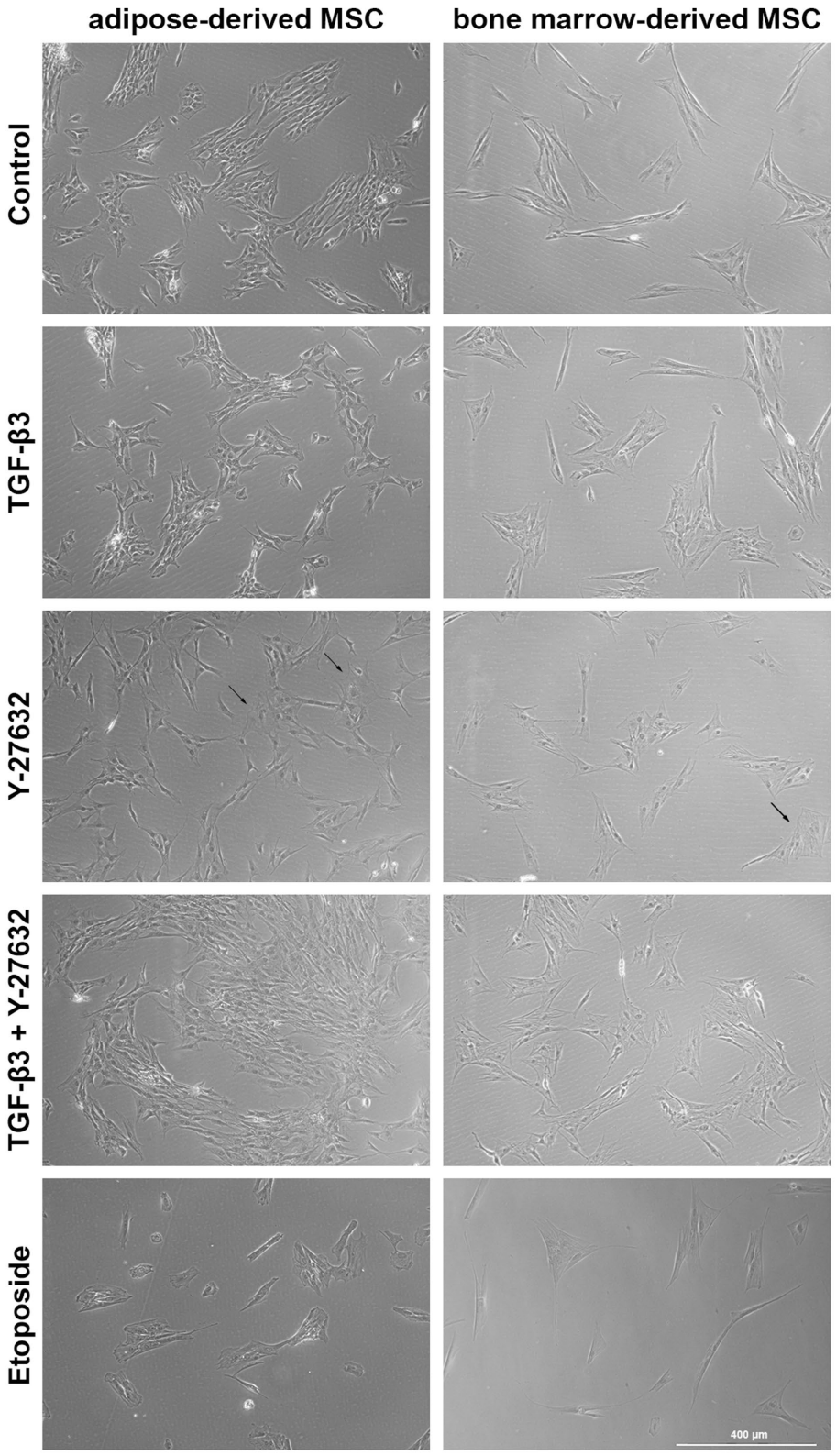

FIGURE 2
Brightfield microscopy of ASC and BMSC. Cells were treated with 10ng/mL TGF-β3 or 10µM Y-27632, and in combination or with 10µM etoposide and analyzed after 48h incubation. Representative images show cell morphology and confluency after stimulation. Arrows highlight single flattened cells with poor contrast.

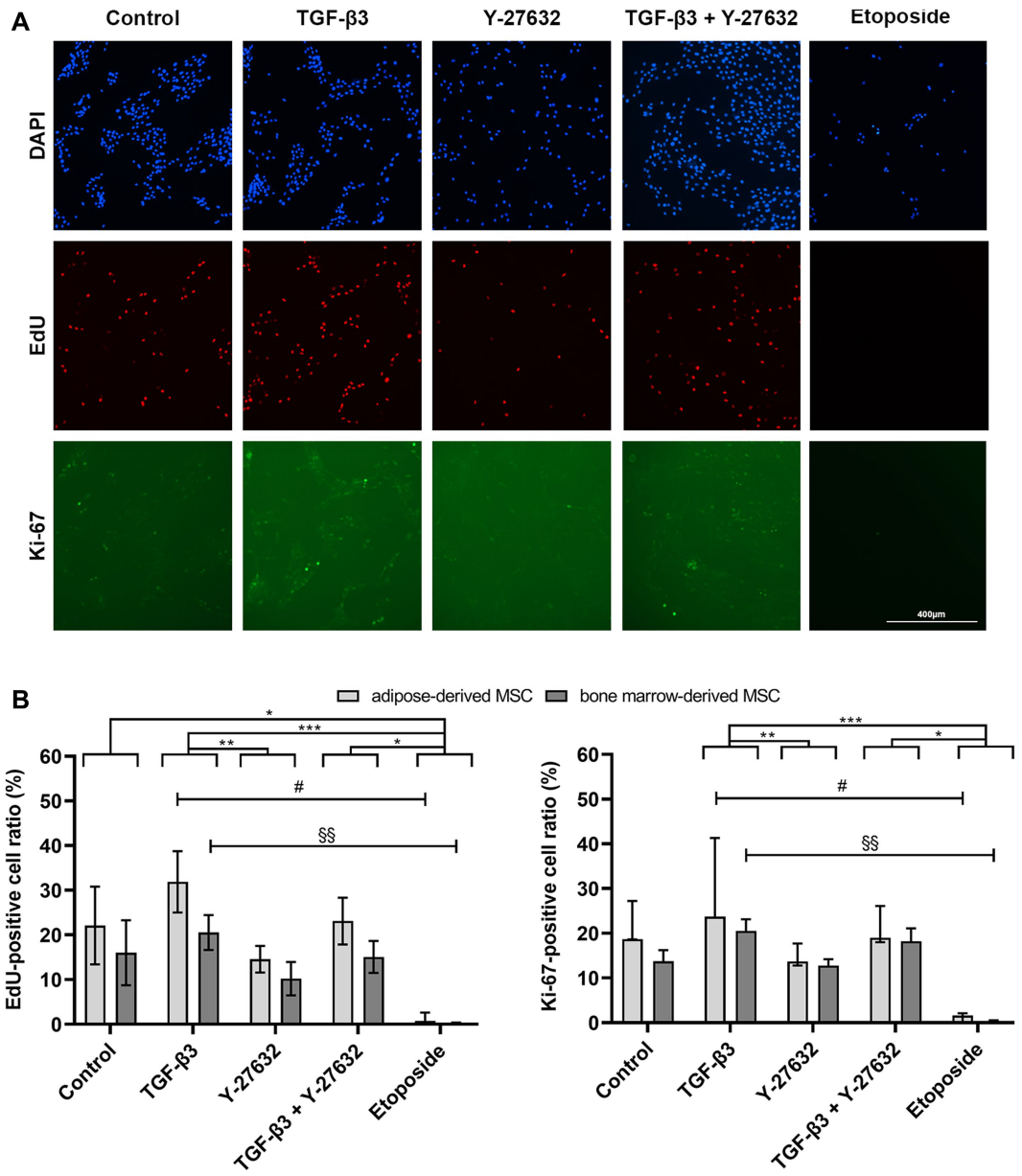

FIGURE 3
Fluorescence images of EdU- and Ki-67-labeling. ASC and BMSC were treated with 10ng/mL TGF-β3 or 10µM Y-27632, and in combination, or with 10µM etoposide and analyzed after 48h incubation. Representative images of ASC show nuclei staining (DAPI) and EdU- and Ki-67-labeling **(A)**. The diagrams represent quantitative image analysis results of proliferation activity of ASC and BMSC treatment groups **(B)**. Bars represent the median values, error bars the 95% confidence intervals. The asterisks indicate significant differences between the corresponding groups when the data of ASC and BMSC are combined (* corresponds to $p<0.05$; ** corresponds to $p<0.01$; *** corresponds to $p<0.001$; $n=7$). The hashtag and paragraph symbols mark differences between the indicated groups for ASC and BMSC alone (#: ASC; $n=3$; §: BMSC; $n=4$).

and into a broader rectangular cell shape in both cell types. In addition, some cells had a flattened circular appearance with poor contrast in phase contrast microscopy. These morphological changes were less marked with TGF-β3. The addition of etoposide induced a rounded cell shape and arrested cell proliferation, as indicated by reduced cell confluency.

Consistent with this, both EdU and Ki-67 labeling was almost completely inhibited by etoposide stimulation in both BMSC and ASC (EdU and Ki-67 label: $p<0.05$ compared to control, TGF-β3 + Y-27632, and TGF-β3; data of BMSC and ASC combined; Figure 3). Although there was a trend for an increase in proliferation with TGF-β3 compared to controls, this was not significant for BMSC or for ASC. There was a small decrease in proliferation with Y-27632, but was not significant compared to the control. There was a significant difference in proliferation between TGF-β3 and Y-27632 (EdU label: $p<0.01$ for TGF-β3 compared to Y-27632; data of BMSC and ASC combined). When both compounds were used in combination, proliferation recovered to control levels (no significant difference). Proliferation of ASC and BMSC in the etoposide groups remained significantly lower as compared to TGF-β3 stimulation (EdU/Ki-67 label for BMSC: $p<0.01$, for ASC: $p<0.05$). Although there were no significant differences between ASC and BMSC in controls or treatment groups, ASC showed a tendency for higher proliferation activity than BMSC.

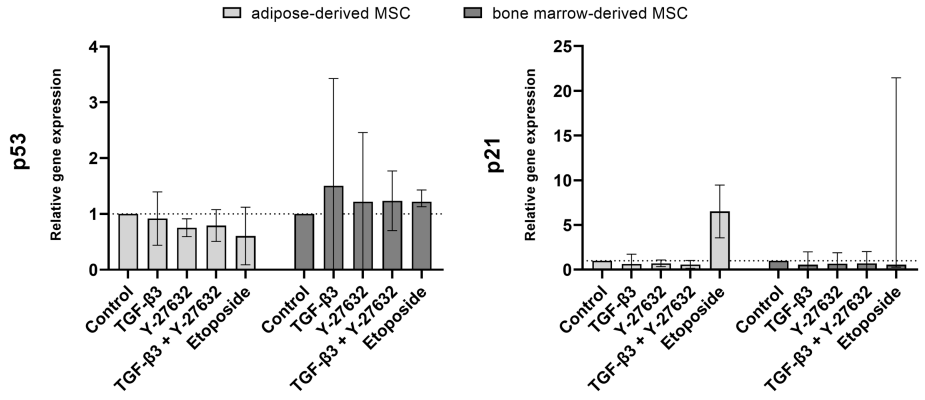

FIGURE 4
Gene expression of p53 and p21. ASC and BMSC were treated with 10ng/mL TGF-β3, 10μM Y-27632, in combination or 10μM etoposide and analyzed after 48h incubation. Bars represent the median values, error bars the 95% confidence intervals.

Gene expression of p21 and p53 were examined to assess senescence induction (Figure 4). Stimulation with TGF-β3, Y-27632, or in combination did not significantly alter the expression of p21 in ASC or BMSC. The addition of etoposide resulted in an upregulation of p21 expression in ASCs but was variable between BMSC donor horses with only one donor responding with an upregulation. Similarly, no significant regulation by TGF-β3, Y-27632, or in combination was detected for p53 gene expression.

Discussion

Adipose tissue and bone marrow represent important sources for obtaining MSC for therapeutic purposes. Several studies show that ASC and BMSC are comparable in many biological responses. Nevertheless, differences between the two cell types have been reported in several studies where comparisons have been made. In this study, while cell morphology was found to be similar between the two cell types, BMSC tended to show lower proliferation activity than ASC. These results are consistent with other studies, where the MSC showed the same cell morphology as ASC but the doubling time of ASC was significantly higher than that of BMSC (9, 31, 32). Y-27632 induced an enlarged, less spindle-shaped cell shape which may be a result of cytoskeletal changes from inhibition of the Rho/ROCK pathway (33, 34). The effect on morphology is reversible (35) although this was not investigated in this study.

TGF-β3 is a well-established tool for initiating and supporting MSC differentiation. The Rho/ROCK inhibitor Y-27632 has been reported to have beneficial effects, particularly on cell proliferation and senescence, in the culture of embryonic stem cells and multipotent cells. Based on this, combined stimulation with Y-27632 and TGF-β3 was used to investigate potential synergistic effects as previously reported with respect to differentiation (17, 36). In our study, no significant effects on proliferation or senescence markers were found. This is in contrast to studies describing an increase of proliferation in human urine-derived mesenchymal stromal cells, periodontal ligament stem cells and other mesenchymal progenitor cells (19, 20, 37, 38), or a decrease in human adipose-derived MSC and CD34+ hematopoietic progenitor cells (28, 39) with Y-27632 stimulation. These varied responses suggest that Y-27632 likely targets multiple cellular pathways depending on the tissue origin of the cells.

Differences in senescence have also been reported for Y-27632-stimulated cells. Studies on primary keratinocytes showed a protective effect of Y-27632 that prevented cell senescence (16, 19), whereas it drove senescence in primary fibroblasts (40). In the current study, no significant regulation of senescence by Y-27632 was noted in either cell type with respect to p21 and p53 expression. It is possible that the age of the cells, in terms of passage number and population doubling level, and the different analyses used to investigate senescence between studies could play a role in varied cellular responses. The cells used in this study were at low passage number where they are at a young cellular age and likely to be more resilient to be driven towards a senescence phenotype and thus the potential protective effect of Y-27632 may not be readily apparent. It is possible that equine ASC and BMSC with higher cellular age (passage number > 15) may be more responsive to the effects of inhibiting the Rho/ROCK pathway. Further analysis would be interesting at late passage to rule out the senescence, as senescence is not be mediated solely by the p53/p21 signaling pathway.

A limitation of the study is the timepoint of investigations. This study focused on the initial effect on proliferation activity and senescence by Y-27632 and TGF-β3 stimulation at 48 h. Further experiments are necessary to investigate effects at longer time points.

Conclusion

The Rho/ROCK inhibitor Y-27632 has no significant effect on proliferation and does not induce senescence in equine ASC and BMSC. Consequently, the inhibitor does not appear to be suitable as a proliferation-promoting supplement for cell cultivation. Nevertheless, no adverse effects are expected for short-term use for differentiation of equine MSC.

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.

Author contributions

MM created the study design, conducted the study, performed the statistical analysis, and wrote the first draft of the manuscript. JB and JD contributed to the conception and design of the study. JD wrote a section (parts of the methods and discussion) of the manuscript. DG provided the characterization data and prepared the related part of the manuscript. All authors contributed to the article and approved the submitted version.

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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.

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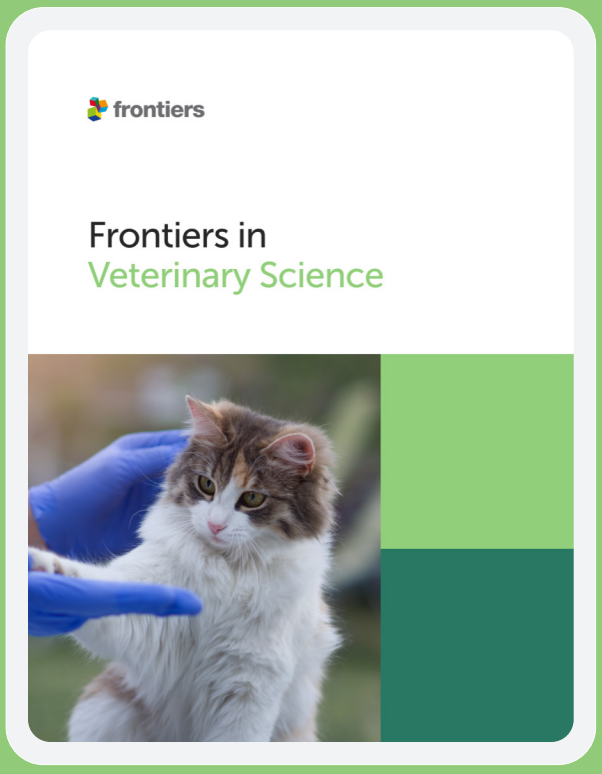

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