Effect of extracorporeal shockwave therapy on the immunomodulatory and anti-inflammatory properties of cultured equine umbilical cord blood mesenchymal stromal cells

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Abstract

There is a knowledge gap regarding the effect of extracorporeal shockwave treatment (ESWT) on the stress response and immunomodulatory and anti-inflammatory properties of equine umbilical cord blood mesenchymal stromal cells (CB-MSCs). The objective of this study was to investigate the presence of cellular oxidative stress, inflammatory response, and production of growth factors in CB-MSCs after treatment with ESWT. We hypothesized that CB-MSCs treated with ESWT will experience higher levels of cellular stress and increased production of anti-inflammatory cytokines and growth factors compared to untreated CB-MSCs.

Résumé

Il existe un manque de connaissances concernant l'effet du traitement extracorporel par ondes de choc (ESWT) sur la réponse au stress et les propriétés immunomodulatrices et anti-inflammatoires des cellules stromales mésenchymateuses du sang de cordon ombilical équin (CB-MSCs). L'objectif de cette étude était d'étudier la présence de stress oxydatif cellulaire, de réponse inflammatoire et de production de facteurs de croissance dans les CB-MSCs après un traitement par ESWT. Nous avons émis l'hypothèse que les CB-MSCs traitées par ESWT connaîtront des niveaux plus élevés de stress cellulaire et une production accrue de cytokines anti-inflammatoires et de facteurs de croissance par rapport aux CB-MSCs non traitées.

(Traduit par Docteur Serge Messier)

Introduction

Horses engaged in athletic pursuits are consistently exposed to the potential for musculoskeletal injuries, with about 76% of the observed injuries occurring in their limbs on racetracks (1). Nearly 46% of these are directly linked to tendon-related issues (1). Tendinopathies have a high economic impact on the equine industry due to their career- or life-threatening potential. Consequently, new therapeutic approaches are needed that repair the lesion and allow the horse to return to its previous athletic function. Mesenchymal stromal cells (MSCs) have emerged as a promising therapeutic option for treating tendinopathies in horses (2), with intralesional injection improving ultrasonographic appearance, histological healing, and biomechanical properties compared to controls such as saline (3–10). Horses treated with MSCs also exhibit lower re-injury rates than with other treatment methods such as controlled exercise or hyaluronan (11,12). Extracorporeal shockwave therapy (ESWT) is another commonly used therapy (13), which enhances neovascularization and histomorphological appearance, reduces inflammation, and increases expression of transforming growth factor *beta* (TGF-β1) (14–16).

Mesenchymal stromal cells (MSCs) and ESWT are combined in equine treatment despite limited scientific evidence of their effectiveness, although studies in humans and animals suggest that tissue heals better with this combination (17–24). The reasons for these findings are unclear, but some studies have shown that stimulating MSCs with ESWT increases proliferation (25) and differentiation (26). Moreover, MSCs derived from equine umbilical cord blood (CB) that are treated with ESWT maintained their differentiation capacity and showed higher potential for differentiation towards the adipogenic and osteogenic lineages (27). Stimulation with ESWT seems to enhance the proliferation and differentiation of MSCs, potentially through activation of the cellular stress response (28,29) or by modulation of cytokines and growth factors, leading to improved tissue repair and a balanced inflammatory response (30,31).There is a knowledge gap regarding the effect of ESWT on the stress response and the immunomodulatory and anti-inflammatory properties of equine CB-MSCs. The objective of this study was to investigate the presence of cellular oxidative stress, inflammatory response, and production of growth factors in CB-MSCs after treatment with ESWT. We hypothesized that CB-MSCs treated with ESWT will experience higher levels of cellular stress and increased production

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of anti-inflammatory cytokines and growth factors compared to untreated CB-MSCs.

Materials and methods

Culture of equine CB — MSCs Cord blood was collected immediately after foaling and before the umbilical cord broke spontaneously or was broken according to farm management protocol. Venipuncture of the umbilical vein was carried out with a 16-G hypodermic needle attached to a 450-mL blood transfusion collection bag containing citrate phosphate dextrose adenine as the anticoagulant solution. The blood was then immediately transported to the laboratory at ambient temperature.

The equine MSCs were isolated as described in a previous study (32). Briefly, the mononuclear cell fraction (MNCF) was cultured and non-adherent cells were removed through successive complete medium changes; the isolation medium consisted of low-glucose Dulbecco's modified Eagle medium (DMEM-LG; Lonza, Walkersville, Maryland, USA), supplemented with 30% fetal bovine serum (FBS; Invitrogen, Burlington, Ontario), low dexamethasone (10^{-7} M) (Sigma-Aldrich, Oakville, Ontario), 100 U penicillin-streptomycin (Invitrogen), and 2 mM L-glutamine (Sigma-Aldrich). When cell numbers allowed, the cells were passaged and expanded until they were cryopreserved in hypothermic biopreservation medium (CryoStor CS10; BioLife Solutions, Bothell, Washington, USA) in 1-mL aliquots at a concentration of 1×10^6 cells/mL for later use.

Mesenchymal stromal cells (MSCs) isolated from 3 different donors were used in this study [1801 (Filly, Thoroughbred), 1803 (Filly, Warmblood), and 1810 (Colt, Warmblood)]. To increase the number of technical replicates, 3 cryovials of each donor were used in the experiment for a total of 9 cryovials. The cryovials had been stored in liquid nitrogen for 2 y prior to the study and all cells were contained in passage 3. The cells were thawed, seeded at a cell density of 5000 cells/cm^2 of culture surface area, and expanded until 80 to 90% confluence to obtain sufficient cell numbers for all the experiments and assays of this study.

The culture medium consisted of low-glucose DMEM (DMEM-LG; Lonza), 10% FBS (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), and 100 U penicillin-streptomycin (Invitrogen), and passaging was done by enzymatic digestion with 0.25% trypsin-EDTA.

In-vitro extracorporeal shockwave treatment

The *in-vitro* ESWT treatment was carried out as described in previous studies (27,33) using a specially designed water bath with pre-warmed water (37°C) and an electrohydraulic shockwave generator (Versatron; Pulse Veterinary Technologies, Alpharetta, Georgia, USA). Equine MSCs were treated at 80 to 90% confluence in T-25 cell culture flasks. The flasks were placed in front of the applicator, 5 cm from the shockwave probe (R80 Trode, focal depth 50 mm to 110 mm) to the cell layer inside. The focused shock waves were applied with an energy flux density of 0.1 mJ/mm^2, a frequency of 180 pulses per min, and 300 impulses (27). The control group was maintained in the same culture conditions, consisting of the expansion medium without exposure to shock waves.

After the ESWT, the cell cultures were returned to the incubator for 3 h before oxidative stress and cytokines were determined. The 3-hour time frame was chosen to evaluate the initial effects of ESWT based on prior studies examining its early impact on oxidative stress (34,35). The experiment was conducted in technical triplicates, wherein each week, 1 cryovial of each of the 3 cell lines was cultured and subjected to the shockwave and determination of oxidative stress and cytokine levels. The experiment was repeated with new cryovials at a 1-week interval.

Production of conditioned medium of CB-MSC co-cultured with peripheral blood mononuclear cells

Before carrying out the cytokine enzyme-linked immunosorbent assays (ELISAs), a conditioned medium of equine MSCs co-cultured with peripheral blood mononuclear cells (PBMCs) was obtained as a positive control for cytokine production. For this, PBMCs from 5 unrelated equine donors were isolated and frozen as described by Lepage *et al* (36).

Frozen PBMCs from all 5 donors were thawed, pooled in equal ratios, then incubated overnight in complete Roswell Park Memorial Institute medium (RPMI 1640; Thermo Fisher Scientific, Burlington, Ontario), 2 mM L-glutamine (Sigma-Aldrich), 100 U penicillin-streptomycin (Invitrogen), and 10% horse serum.

Equine MSCs from 3 different donors (1801, 1803, and 1810) were seeded at 10 000 cells/well in a 48-well plate in MSC expansion medium and incubated overnight. The next day, PBMCs were activated with Concanavalin A mitogen (Sigma), final concentration: 5 μg/mL. MSCs were washed 1× with PBS before adding activated PBMCs in a complete RPMI medium at a ratio of 10 PBMC:1 MSC. After 5 d of PBMC:MSC co-culture, the conditioned media was collected, centrifuged at 1000 × *g* for 15 min to remove the cell debris, and was then aliquoted and stored at −80°C until carrying out the ELISAs to determine PGE2, TGF-β1, IL-1ra, and IL-10 profiles.

Measurement of mitochondrial membrane potential and reactive oxygen species

The culture medium was removed 3 h after shockwave treatment and 4 mL of a 25-nM solution of MitoTracker Red CM-H2XRos (Invitrogen) was added to the T-25 flask and incubated for 15 min to measure mitochondrial membrane potential (MMP). The cells were then rinsed twice with pre-warmed 1× PBS to remove excess stain. After that, 4 mL of a 5-μM solution of 2′,7′-dichlorodihydrofluorescein diacetate H2DCFDA (Invitrogen) for measuring reactive oxygen species (ROS) was added to the T-25 flask and incubated for 30 min. The cells were rinsed 3 times with pre-warmed 1× PBS to remove excess stain.

The stained T-25 flasks were then visualized under the fluorescent microscope at 40x and 10 field pictures of each flask were randomly obtained in areas of high cellular density. Using the Image J software (Rasband W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA), the cells in the pictures were manually highlighted; overlapping cells or those with cut margins were not included. The highlighted cells were added to the region of interest (ROI) manager and the fluorescence intensity [integrated density (intden)] was measured (Figure 1).

Table I. Values of integrated density [intden (A.U.)], indicative of fluorescence intensity, of the different cell times and treatments of ESWT-treated and control non-ESWT-treated equine CB-MSCs.

Cell line/Treatment	MitoTrack control	MitoTrack ESWT	H2DCFDA control	H2DCFDA ESWT
1801/TR1	622.92 ± 194.63	743.47 ± 193.65	336.45 ± 107.57	385.73 ± 132.44
1803/TR1	722.03 ± 211.21	788.54 ± 243.57	226.34 ± 95.75	228.330 ± 88.13
1810/TR1	635.77 ± 189.14	687.04 ± 169.34	264.33 ± 90.14	243.70 ± 79.41
1801/TR2	3108.47 ± 5928.58	812.65 ± 232.21	3373.75 ± 6224.95	895.98 ± 288.19
1803/TR2	976.83 ± 297.77	827.39 ± 274.91	987.15 ± 351.06	725.08 ± 315.52
1810/TR2	798.26 ± 234.20	723.98 ± 193.49	790.29 ± 266.24	702.48 ± 225.52
1801/TR3	644.32 ± 167.12	606.20 ± 151.43	737.90 ± 241.83	608.37 ± 201.69
1803/TR3	922.55 ± 329.22	783.48 ± 235.36	930.95 ± 407.30	658.88 ± 300
1810/TR3	593.77 ± 182.30	605.06 ± 170.78	549.93 ± 199.35	547.77 ± 217.84

Data presented as mean ± SD.

MitoTrack — Stain for mitochondrial membrane potential (MMP); H2DCFDA — Stain for reactive oxygen species (ROS); ESWT — Extracorporeal shock wave therapy; AU — Arbitrary units.

Determination of PGE2, TGF-β1, IL-1ra, and IL-10 profiles

The culture medium was collected 3 h after ESWT treatment and centrifuged at 400 × *g* for 5 min. Supernatants were then collected and stored at −80°C until further analysis. Supernatants were collected from control MSC cultures in a similar fashion. Concentrations of prostaglandin E_2 (PGE2) and transforming growth factor-β1 (TGF-β1) were determined using human PGE2 and TGF-β1 ELISA assays, previously validated for horses, according to the manufacturer's instructions (Parameter Assay Kit and Quantikine Human TGF-β1 ELISA; R&D Systems, Minneapolis, Minnesota, USA). Interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1ra) were determined using equine IL-10 and IL-1ra ELISA assays, according to the manufacturer's instructions (IL-10 Equine ELISA Kit; Invitrogen and Equine IL-1ra/IL-1F3 DuoSet ELISA; R&D Systems).

Statistical analysis

Data normality was assessed using normal probability Q-Q plots and the Kolmogorov-Smirnov test. Descriptive statistics, such as mean and standard deviation (SD), were employed. The impact of ESWT on mitochondrial membrane potential (MMP), reactive oxygen species (ROS) (MitoTracker and H2DCFDA), and PGE2 concentration was analyzed using a mixed model analysis of variance (ANOVA) test to account for random effects.

The model for stain intensity (MitoTracker and H2DCFDA) included the fixed effect of treatment (ESWT) and random effects of the cell line (1801, 1803, and 1810), image, and subsample within the image. Random effects for the PGE2 model included the date. The normality of residuals was assessed using the Kolmogorov-Smirnov test. Statistical significance was defined at $P < 0.05$.

Results

Effect of ESWT on mitochondrial membrane potential and reactive oxygen species

The different values of Integrated Density [intden (Arbitrary Units)], reflecting the fluorescence intensity, for the various treatments and cell lines of ESWT-treated and non-treated CB-MSCs are presented in Table I. No significant difference was observed in the levels of oxidative stress, as indicated by changes in mitochondrial membrane potential (MMP) or reactive oxygen species (ROS), between ESWT-treated CB-MSC cells (MMP = intden 729 AU ± 64 and ROS = intden 560 AU ± 69) and non-treated cells (MMP = intden 995 AU ± 397 and ROS = intden 903 AU ± 483) (MMP: $P = 0.36$; ROS: $P = 0.29$).

Effect of ESWT on PGE2, TGF-β1, IL-1ra, and IL-10 concentrations

The multivariable mixed model analysis revealed that there were no statistically significant differences in the concentration of PGE2 between the non-treated (1203.95 pg/mL ± 199.43) and ESWT-treated (1095.29 pg/mL ± 199.43) CB-MSCs ($P = 0.5416$) (Figure 2). In addition, no TGF-β1, IL-1ra, or IL-10 production was observed in non-treated or ESWT-treated CB-MSCs. The CB-MSCs preconditioned with peripheral blood mononuclear cells (PBMCs), which served as a positive control for the ELISA tests, exhibited the production of IL-1ra or IL-10 and showed an increase in the amount of secreted PGE2 (Figure 3).

Discussion

This study examined the effects of extracorporeal shockwave therapy (ESWT) on equine cord blood mesenchymal stromal cells (CB-MSCs) *in-vitro.* Results indicate that ESWT did not induce the anticipated increase in cellular oxidative stress or the production of PGE2, TGF-β1, IL-1ra, and IL-10, nor did it elevate mitochondrial membrane potential or ROS production, thus rejecting our initial hypothesis.

In previous studies, treating adipose-derived MSCs with ESWT has increased cellular proliferation and differentiation (25,26). These observed effects in equine MSCs may be associated with mechanotransduction driven by ESWT, as applying shockwave to human MSCs triggers the release of ATP-activating Erk 1/2 and MAPK signalling leading to proliferation and differentiation (28,29).

It has also been reported that treating human mesenchymal progenitor cells with ESWT results in increased cellular oxidative stress,

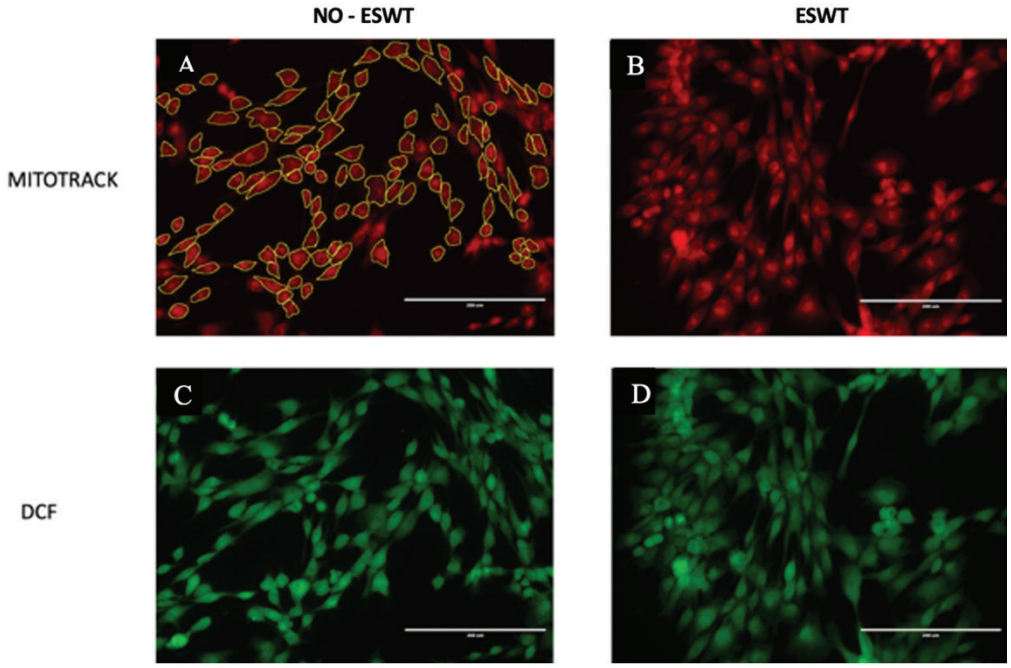

Figure 1. Equine CB-MSCs stained with Mitotracker (red) and H2DCFDA (green). A — Control non-ESWT-treated CB-MSCs stained with Mitotracker and highlighted with Image J/Fiji for measurement of the integrated density. B — ESWT-treated CB-MSCs stained with Mitotracker. C — Control non-ESWT-treated CB-MSCs stained with H2DCFDA. D — ESWT-treated CB-MSCs stained with H2DCFDA.

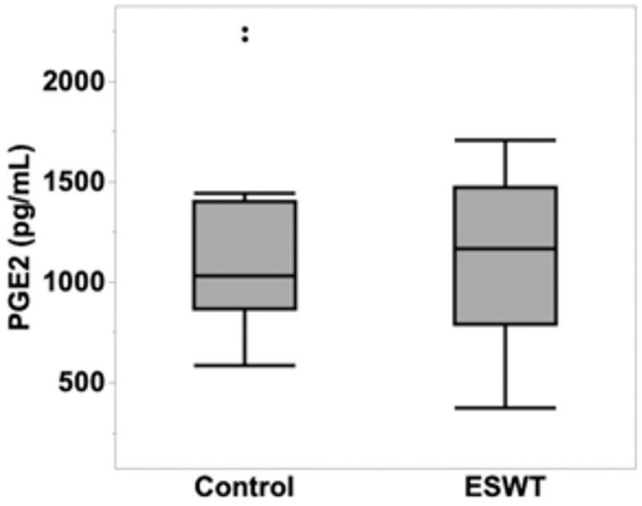

Figure 2. Concentrations of PGE2 in control non-ESWT treated CB-MSCs and ESWT-treated CB-MSCs. There were no differences in the concentration of PGE2 of both groups of MSCs ($P = 0.5416$).

which has been linked with dose-dependent increased osteogenic potential of these cells (31). This is consistent with other literature describing the association between oxidative stress and proliferation and differentiation of human MSCs (37–39), which provided a rationale to examine the role of ROS in equine MSCs treated with ESWT.

Our previous study showed increased proliferation and differentiation of equine CB-MSCs after ESWT treatment (27). However, the results from our current study suggest that applying shockwave with an energy flux density of 0.1 mJ/mm^2, frequency of 180 p/m, and 300 impulses did not appear to affect oxidative stress in equine MSCs 3 h after the exposure. This contradicts the results from a similar study in human cord blood-derived mesenchymal progenitors described above that demonstrated an increase in ROS within 60 min of exposure to ESWT (31).

Interestingly, whole cord blood was initially exposed to shockwave in the study by Wang *et al* (31), whereas in the present study, only isolated CB-MSCs were exposed to ESWT. Although isolated CB-MSCs are more relevant clinically, as they represent the cell product being transplanted, the oxidative stress observed in their study could have resulted from stimulation of cellular populations other than MSCs present in the cord blood. Further studies are required to determine the origin of oxidative stress.

Secondly, ROS concentrations were determined using different methods. In our current study, H2DCFDA was used as an indicator of intracellular ROS, whereas in the study by Wang *et al* (31), Lucigenin chemiluminescence was used, which only detects extracellular ROS. More specifically, the H2DCFDA assay is more sensitive for measuring concentrations of hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), whereas Lucigenin chemiluminescence is more sensitive to extracellular superoxide (40). As we would expect intracellular ROS to be associated with a response to external mechanical stimuli such as ESWT, this is what we probed for. However, it is unknown if extracellular ROS levels increased with ESWT-treated CB-MSCs.

In our current study, cultured CB-MSCs produced detectable concentrations of PGE2 in both ESWT-treated and non-ESWT-treated cells. This finding aligns with previous research documenting the

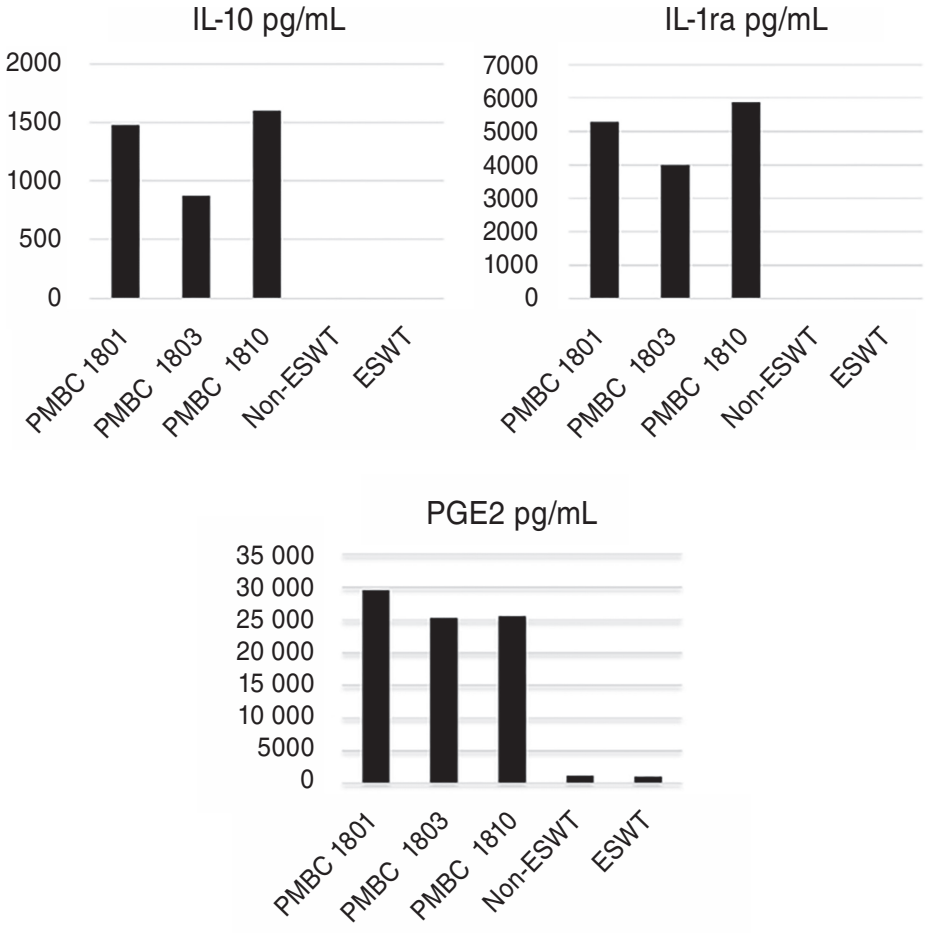

Figure 3. Concentrations of IL-10, IL-1ra, and PGE2 in control non-ESWT treated CB-MSCs, ESWT-treated CB-MSCs and the conditioned medium of CB-MSCs co-cultured with PMBCs.

production of PGE2 by bone marrow-derived MSCs without any external stimulation (41). It is unknown how this could explain the effect of MSCs on tissue repair. However, PGE2 has been associated with the activation of endogenous mesenchymal cells, immunomodulation, and stimulation of neovascularization by activating prostanoid receptors (42).

Our results also indicate that stimulation of CB-MSCs with ESWT did not alter the production of PGE2, TGF-β1, IL-1ra, or IL-10, which suggests that ESWT might not influence the production of these cytokines. This finding agrees with a previous study showing that ESWT does not increase VEGF, TGF-β1, and PGE2 production (41). In contrast to the present study, stimulation of human tendon cells and mesenchymal progenitors with ESWT has increased the production of IL-10 and TGF-β1, respectively. These changes in the concentration of IL-10 and TGF-β1 are observed from 15 to 24 h after exposure to ESWT (30,31).

In the present study, PGE2, TGF-β1, IL-1ra, and IL-10 concentrations were measured 3 h after treatment with ESWT to assess the early response of CB-MSCs to ESWT exposure. In addition, this timepoint reduced the risk of other sources of cellular stress, such as trypsinization and over-confluency, which have been found to increase cytokine expression and apoptosis, respectively (43,44).

It is possible that any ESWT-associated production of PGE2, TGF-β1, IL-1ra, and IL-10 required more than 3 h to be observed. Further studies with different time points are necessary to estimate when these mechanisms to produce PGE2, TGF-β1, IL-1ra, and IL-10 start unveiling.

As a part of our study, we used CB-MSCs preconditioned with PBMCs as a positive control when carrying out the ELISA to determine the concentrations of PGE2, TGF-β1, IL-1ra, and IL-10. The production of different cytokines, such as VEGF, HGF, IGF-1, TGF-β1, PGE-2, IDO, iNOS, and interleukins including IL-1, IL-1ra, IL-3, IL-6 IL-10, upon preconditioning of MSCs has been demonstrated (45–48).

Similarly, we discovered that equine CB-MSCs preconditioned with PBMCs produced IL-10 and IL-1ra and increased production of PGE2 compared to non-preconditioned CB-MSCs. To the authors' knowledge, this is the first study reporting the secretion of PGE2 and interleukins by equine CB-MSCs preconditioned with PBMCs. Further studies are required to categorize the cytokine and interleukin profiles of equine CB-MSC.

We acknowledge that our study was limited by a small sample size, which may have hindered our ability to detect smaller differences between the groups. A *post-hoc* power analysis revealed that, due to the small differences in fluorescence intensity between the groups, our study had a power of only 40%. Although our study's sample size may have influenced our results, it is important to consider the cumulative evidence from similar studies that have achieved notable findings despite similar limitations (27,33).

Additional limitations warrant mentioning. Firstly, our study only assessed a limited number of cytokines. Moreover, these cytokines were measured at a single point after the MSCs were exposed to ESWT. This approach might have hindered our ability to detect cytokines with early expression patterns or those essential to the mechanism of action of ESWT.

Secondly, we acknowledge the existence of numerous ESWT protocols varying in energy flux densities, frequencies, and shock numbers for both *in-vitro* and *in-vivo* applications. The findings from our study are confined to the experimental settings of ESWT chosen here and, importantly, should not be extrapolated to clinical scenarios.

In conclusion, we did not observe an increase in MMP, production of ROS, or the production of PGE2, TGF-β1, IL-1ra, and IL-10 by CB-MSCs 3 h after treatment with ESWT. Further *in-vitro* and *in-vivo* studies are warranted to elucidate the previously reported mechanism that has influenced the changes in metabolic activity and differentiation of MSCs treated with ESWT. These studies should include calculating a proper sample size based on our findings, incorporating different sampling times after treatment with ESWT, encompassing a larger spectrum of cytokines, and including multiple ESWT protocols.

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