

Original Research Article

Effects of Wharton's jelly mesenchymal stromal/stem cells-derived conditioned medium and platelet-rich plasma on *in vitro* induced equine endometrial inflammation

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ABSTRACT

Over the years, regenerative therapies have emerged as promising alternatives for persistent breeding-induced endometritis. *In vitro* studies testing the effects of these therapies on equine endometrial cells are still scarce. This study aimed to evaluate *in vitro* the effect of Wharton's jelly (WJ) mesenchymal stromal/stem cell (MSCs)-derived conditioned medium (WJ-CM) and platelet-rich plasma (PRP) on equine endometrial cells, with or without lipopolysaccharide (LPS)-induced inflammation. The WJ-CM was obtained after 24 h of starvation in Ringer's lactate of WJ-MSCs and PRP was prepared using the double centrifugation. Endometrial epithelial cells obtained from 3 diestrus mare uteri at slaughterhouse were treated for 24 h according to six experimental groups: DMEM standard complete medium (CTRL); 10 ng/mL LPS (LPS); 10 % WJ-CM (CM); 5 % PRP (PRP); 10 ng/mL LPS and 10 % WJ-CM (LPS + CM); 10 ng/mL LPS and 5 % PRP (LPS + PRP). After 6, 12, and 24 h, endometrial cells were evaluated for viability (apoptosis and necrosis), mitochondrial activity and reactive oxygen species (ROS) generation. PGE-2 and IL-10 concentrations in spent medium were measured. The WJ-CM alone did not affect endometrial cell viability and prevented the detrimental effect of LPS on endometrial cells; it suppressed the production of PGE-2. PRP had a deleterious effect on endometrial cell viability, induced the secretion of PGE-2, as well as increased mitochondrial activity and ROS production. Endometrial benefits of the WJ-CM treatment are evident even after an LPS challenge, while unexpectedly PRP showed a deleterious effect.

1. Introduction

After insemination, mares could develop post-reproductive endometritis triggered by seminal material, debris, or bacteria. Equine endometrium responds to natural mating or artificial insemination with a physiological inflammation that typically resolves within 48 h [1,2]. However, if the inflammation persists, susceptible mares may develop a condition known as persistent breeding-induced endometritis (PBIE), which may lead to endometrial infection or progress into endometrial fibrosis, with a consequent reduction in pregnancy rates [3]. Indeed, PBIE can negatively impact fertility, as the uterus is not adequately

cleaned and prepared for the arrival of the embryo, interfering with pregnancy establishment and significantly affecting maternal recognition of pregnancy [3–5]. PBIE is a widespread and challenging disorder that is difficult to address, resulting in economic losses for the industry [4].

Over the years, various approaches have been explored as potential solutions to this challenge, utilizing both traditional and non-traditional therapies [4,6,7]. Regenerative medicine, including stem cells or their derivatives and platelet-derived products, is emerging as a promising alternative [7].

Mesenchymal stromal/stem cells (MSCs) are self-renewing, multipotent cells derived from various tissues, including bone marrow,

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Glossary			
ADU	arbitrary densitometric units	LPS	lipopolysaccharide
ANOVA	Analysis of Variance	MSC	mesenchymal stromal/stem cell
AO	Acridine orange	P	passage
CM	conditioned medium	PBIE	persistent breeding-induced endometritis
CTRL:	control	PBS	Dulbecco's Phosphate-Buffered Saline
DCF	2',7'-dichlorofluorescein	PGE	Prostaglandin E
EVs	extracellular vesicles	PI	propidium iodide
FBS	fetal bovine serum	PPP	platelet-poor plasma
H ₂ DCF-DA	H ₂ DCF diacetate	PRP	platelet-rich plasma
H ₂ DCF	2',7'-dichlorodihydrofluorescein	ROS	Reactive oxygen species
HG-DMEM	high glucose Dulbecco's Modified Eagle Medium	WJ-CM	Wharton's jelly mesenchymal stromal/stem cell-derived conditioned medium
IL:	interleukin	WJ-MSC	Wharton's jelly mesenchymal stromal/stem cell
		WJ	Wharton's jelly

adipose tissue, and extra-fetal adnexa [8,9]. The origin of cells may impact their therapeutic potential [10,11]. In general, MSCs are regarded as guardians against excessive inflammation by releasing bioactive molecules (e.g., cytokines) that can suppress and modulate the inflammatory response [12]. Moreover, the antimicrobial effect of MSCs has also been reported through indirect (stimulating the immune system) and direct mechanisms, producing antimicrobial substances [13]. The use of MSCs has been recently replaced by their conditioned medium (CM), which is the medium where the stem cells are cultured and contains the complete secretome, including microvesicles and exosomes released by MSCs. This cell-free approach avoids issues related to cell use, such as recipient rejection or regulatory concerns [14].

Another essential product for regenerative medicine is platelet-rich plasma (PRP), produced by a specific centrifugation protocol of whole blood to obtain a concentration of platelet 3–5 times higher than the individual's physiological level. It contains supraphysiologic amounts of growth factors, cytokines, and other proteins, which can help to shift the inflammatory response from an excessive state to a more controlled response [15,16]. Moreover, peptides contained in platelet granules have antimicrobial properties, including activity against bacteria isolated from mare uteri [17–19].

The application of regenerative therapies for equine PBIE with these significant regenerative products is limited. The application of MSCs or their secretome *in vivo* is supported by only two studies: Navarrete et al. [20] and Lange-Consiglio et al. [21]. In the first paper, equine endometrial and adipose MSCs significantly reduced inflammation despite a limited engraftment detectable after one month of infusion [20]. In the second paper, the combined use of extracellular vesicles (EVs) derived from amniotic MSCs and semen during artificial insemination in mares reduced polymorphonuclear neutrophil infiltration, intrauterine fluid accumulation, cytokine concentrations of interleukin (IL)-6 and increase anti-inflammatory IL-10, suggesting successful modulation of the post-insemination inflammatory response [21].

The use of PRP *in vivo* was tested for the first time in bovine species, specifically on repeat breeders, increasing pregnancy rates [22]. In equines, the *in vivo* studies exhibit high heterogeneity and present numerous limitations due to interfering factors and a lack of control over natural variables [7,23]. This can lead to inconsistent outcomes and hinder the identification of specific mechanisms of action.

To the author's knowledge, *in vitro* studies are underutilized for testing the effects of regenerative products for equine endometritis. Indeed, to date, there are no papers about the *in vitro* effect of PRP on equine endometrial cells. PRP was tested on bovine endometrial cells [24] and human endometrial cells [25]. The *in vitro* effects of CM, or EVs derived from MSCs, have primarily been studied by Corradetti et al. [26] and Perrini et al. [27] on equine endometrial cells. All these *in vitro* studies showed a pronounced effect of these regenerative products (PRP, CM, and EVs) on endometrial cells, resulting in increased cell

proliferation rates, enhanced expression of regenerative enzymes, cell migration, and upregulation of genes critical to reproduction.

An *in vitro* evaluation of regenerative treatments could provide crucial insights into their mechanisms of action and help optimize the design and application of these therapies *in vivo*. Therefore, the aim of this study was to evaluate *in vitro* the effect of Wharton's jelly (WJ) MSC-derived CM and PRP on equine endometrial cells, with or without lipopolysaccharide (LPS)- induced inflammation.

2. Material and methods

2.1. Experimental design

To test the *in vitro* effect of PRP and WJ-CM, endometrial epithelial cell cultures were established by enzymatic digestion of uteri collected at a local slaughterhouse. PRP was prepared following the double centrifugation method, and WJ-CM was obtained after 24h of starvation in Ringer's lactate solution of MSCs from WJ. Then, endometrial epithelial cells were treated according to six experimental groups: DMEM standard complete medium (CTRL); 10 ng/mL LPS (LPS); 10 % WJ-CM (CM); 5 % PRP (PRP); 10 ng/mL LPS and 10 % WJ-CM (LPS + CM); 10 ng/mL LPS and 5 % PRP (LPS + PRP). Three independent experiments were carried out.

A dose-response curve was previously performed to elect the optimal concentrations of LPS, CM and PRP to be used. After 6, 12, and 24h of incubation, cell viability, apoptosis and necrosis were evaluated, together with cell mitochondrial activity and reactive oxygen species (ROS) concentrations. Moreover, PGE-2 and IL-10 concentrations in incubation media were assessed.

2.2. Endometrial cell isolation and culture

For the preparation of a pool of epithelial endometrial cells, three fresh uteri were recovered at the horse slaughterhouse, intended for human consumption and unrelated to our studies, and then sent to the laboratory on ice. Uteri selected for endometrial culture were collected from post-pubertal mares in the early/mild diestrus phase of estrus with an obvious corpus luteum on the ovary and no evidence of genital disease.

In the laboratory, endometrial cell isolation and primary culture were performed according to the protocol described by Perrini et al. [27], a modification of the technique described by Donofrio et al. [28] for bovine cells. Briefly, uterine horns ipsilateral to the corpus luteum were opened with scissors lengthwise from the oviduct to the bifurcation of the uterine horns. Then, the endometrium was washed with sterile saline three times and isolated from the underlying tissues with scissors. Isolated endometrium was cut into strips and placed in sterile-filtered Hank's buffered salt solution supplemented with 1 mg/mL collagenase

II, 4 mg/mL bovine serum albumin, and 0.4 mg/mL DNase I for 3 h at 38.5 °C in a shaking bath for digestion. Endometrial cells were subsequently filtered with an 80 µm pore size membrane, followed by centrifugation at 200×g for 10 min, and then subjected to two washes in PBS.

A primary culture was initiated using HG-DMEM (high glucose Dulbecco's Modified Eagle Medium) supplemented with 10 % FBS (fetal bovine serum), penicillin (100 UI/mL), streptomycin (100µg/mL), 0.25 µg/mL amphotericin B, and two mM L-glutamine. Before seeding, the total number of viable cells was counted using a Bürker chamber after Trypan blue staining.

Cells obtained after digestion were plated in T-75 flasks at a density of 1×10^5 cells/cm² and incubated at 38.5 °C in a humidified atmosphere with 5 % CO₂. After 18 h of incubation, endometrial connective cells adhere to the bottom of the flask, while endometrial epithelial cells remain in suspension. So, the culture medium rich in the epithelial population was removed and seeded again. This way, it was possible to obtain connective and epithelial cells separately. Both populations of cells were detached using 0.05 % trypsin-EDTA just before reaching 80 % confluence and then were cryopreserved at passage (P) 1. A detailed characterization of cells was conducted according to Corradetti et al. [26].

2.3. Preparation of WJ- CM

WJ samples were recovered immediately after the parturition of three healthy mares, housed at the Department of Veterinary Medical Sciences, University of Bologna, for attended delivery. After recovery, MSCs from WJ were isolated as previously described by Iacono et al. [29]. Cells were cultured *in vitro* until P 2, then frozen as previously reported by Merlo et al. [30]. For conditioned medium (WJ-CM) preparation, frozen WJMSCs were thawed, plated in a T-75 flask at a density of 1×10^6 cells/flask, and cultured in DMEM+10 % FBS until 80–90 % confluence. At this time, cells were washed three times with Dulbecco's Phosphate-Buffered Saline (PBS).

Then, cells were cultured with Ringer's lactate solution following a protocol for human [31] and canine [32] MSCs, with the duration extended to 24 h. After starvation, the solution was recovered and centrifuged at 4000×g for 30 min at 25 °C to eliminate cellular debris. The supernatant was then recovered and frozen at –80 °C until use.

2.4. Preparation of PRP

PRP was prepared from blood collected from three healthy, not pregnant, donor mares. A total of 300 mL of blood was drawn from each mare via the jugular vein using a 16-gauge needle connected to a blood bag containing citrate-phosphate-dextrose-adenine (CPDA-1). The samples were transported to the laboratory at 4 °C within 2 h of collection and immediately processed.

PRP was prepared using a double centrifugation method. Blood was placed into 50 mL conical tubes (Falcon®, EuroClone SpA, Milan, Italy) and centrifuged at 100×g for 30 min. The plasma was then pipetted into new tubes for further centrifugation at 1500×g for 10 min.

Afterward, the supernatant (platelet-poor plasma, PPP) was aspirated, and the remaining fraction was collected and diluted with PRP to achieve a final concentration of 1×10^9 platelets/mL. Platelet concentration of PRP was performed by an automatic hematology analyzer HeCo Vet SEAC (Florence, Italy). To release platelet-derived factors, three cycles of freezing at –80 °C and thawing at 37 °C were performed [33]. To prevent coagulation and clot formation, the fibrinogen was removed as reported by Mojica-Henshaw et al. [34]. Briefly, after thawing, PRP was centrifuged at 4000×g for 20 min, the supernatant was collected, and calcium chloride (20 % w/v) at a ratio of 1:100 was added. The product was left overnight at 4 °C to allow clot formation. The coagulated product was centrifuged at 4000×g for 20 min, and the supernatant was collected to obtain PRP fibrinogen-free.

2.5. *In vitro* effect of LPS and treatment with CM and PRP

To study the *in vitro* effect of different regenerative treatments on endometrial cells, thawed epithelial endometrial cells at P1 were plated in 24-well plates at a density of 20,000 cells/well and incubated at 38.5 °C in a humidified atmosphere with 5 % CO₂. At 80 % confluency, six different experimental conditions were tested: CTRL, LPS, CM, PRP, LPS + CM, LPS + PRP.

Endometrial cells and spent medium were evaluated after 6, 12, and 24 h of incubation. Endometrial epithelial cells were evaluated for cell viability (apoptosis and necrosis), mitochondrial activity, and ROS generation at each time point. Moreover, cell-free spent medium was collected and stored at –20 °C for subsequent measurement of PGE-2 and IL-10 concentrations.

As a preliminary experiment, dose-response curves of LPS, CM, and PRP on endometrial cells were conducted. The results indicated that 10 ng/mL of LPS was the most effective concentration in inducing cellular stress, as evaluated by apoptosis. Similarly, the optimal concentration of CM was found to be 10 % and for PRP 5 %.

2.6. Endometrial cell viability, apoptosis, and necrosis

Acridine orange (AO) and propidium iodide (PI) double staining was used to identify viable, apoptotic, and necrotic endometrial cells. Briefly, 3 mg of PI and 5 mg of AO were diluted separately in 1 mL of absolute ethanol and each solution was stored at 4 °C. One µL of PI solution, one µL of AO, and 998 µL of PBS were mixed to obtain the working solution. The cell monolayer was trypsinized, and the cell suspension was centrifuged at 250×g for 10 min. Aliquots of 50 µL of pellets were diluted with 50 µL of working staining solution. The freshly stained cell suspension was immediately examined under a fluorescence light microscope (Olympus BX51, Japan) at a magnification of × 40. The excitation maximum shifts to 460 nm, and the emission maximum shifts to 650 nm for AO, while PI was excited at 535 nm while the emission wavelength was set at 617 nm. The percentages of viable, apoptotic, and necrotic endometrial cells were determined in more than 200 cells, according to the literature [35]. Indeed, AO and PI are dyes binding to DNA and emitting green and orange fluorescence, respectively. Meanwhile, AO crosses the plasma membrane of viable and early apoptotic cells, and PI can only enter dead cells with poor membrane integrity. The criteria for the identification are as follows: viable cells appear to have an intact nucleus stained with green chromatin; apoptotic cells exhibit a green or orange nucleus showing condensed or fragmented chromatin and budding of the cell membrane; necrotic cells have similar intact orange or red nucleus.

2.7. Cell ROS concentrations and mitochondrial activity

Endometrial cells cultured were stained with MitoTracker Orange CMTM Ros (Molecular Probes, OR, USA) for mitochondrial distribution and apparent energy status and with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) for ROS generation, as reported by Lange-Consiglio et al. [36]. MitoTracker Orange CMTM Ros is a cell-permeant, mitochondrion-selective dye containing a thiol-reactive chloromethyl moiety. The probe passively enters the cell membrane and is selectively sequestered by active mitochondria, where it can react with accessible thiol groups on peptides and proteins to form an aldehyde-fixable conjugate. The H₂DCF-DA can penetrate the cell membrane where it is hydrolyzed by intracellular esterase and converted to 2',7'-dichlorodihydrofluorescein (H₂DCF), which is a polar molecule and thus retained inside the cell. This H₂DCF reacts with intracellular hydrogen peroxide (H₂O₂) or other oxidizing ROS to give the fluorescent 2',7'-dichlorofluorescein (DCF). The fluorescence of DCF is proportional to the concentration of peroxide levels [37].

Briefly, endometrial cells were first incubated with a 280 nM MitoTracker Orange CMTM Ros (Molecular Probes, OR, USA) in the cultured

medium for 30 min at 38.5 °C under 5 % CO₂ [38]. After washing in PBS with 0.3 % BSA three times, cells were incubated for 15 min in the same media containing 10 µM H2DCF-DA [39]. Then, cells were fixed with 2 % paraformaldehyde 4 in PBS for 2 h and stained with 2.5 mg/mL Hoechst 33258 in 3:1 (v/v) glycerol/PBS. Cells were mounted onto glass slides and kept at 4 °C in the dark until observation. sSlides were examined at 600× magnification in oil immersion with Nikon C1/TE2000-U laser scanning confocal microscope to assess the fluorescence intensities. For each sample, five to eight randomly selected fields, totaling approximately 100 cells, were captured to quantify the fluorescence intensities using the EZ-C1 Gold Version 3.70 image analysis software platform for the Nikon C1 (Nikon Instruments) confocal microscope. Results of fluorescence intensity are expressed as arbitrary densitometric units (ADU).

2.8. PGE-2 and IL-10 concentrations in spent media

Spent medium of each condition at each time point was centrifuged at 250×g for 5 min and stored at -20 °C until the PGE-2 and IL-10 concentrations assessment. The concentrations of PGE-2 and IL-10 were quantified via commercially available ELISA Kit [40,41]. The equine PGE-2 and equine IL-10 ELISA Kits (Assay Genie, Dublin, Ireland) were specifically designed for equine species and validated by the manufacturer for use with equine cell culture media. Preliminary experiments were conducted to determine the optimal dilution factor for the whole experiment. All analyses were carried out following the manufacturer's instructions. Results are expressed in pg/mL.

2.9. Statistical analysis

The results are expressed as mean ± standard deviation values from three replicates. A General Lineal model with time and conditions as categorical variables followed by Fisher's least-square difference test was used to evaluate significant differences among conditions at each time point and among time points within each condition.

Statistical Package for the Social Sciences (IBM SPSS v. 29.0, Chicago, IL, USA) was used and P-value <0.05 was considered statistically significant.

3. Results

3.1. Effects on endometrial cell viability, apoptosis, and necrosis

The effects of the different conditions on endometrial cell viability, apoptosis, and necrosis at each time point are shown in Fig. 1. After 6 h of incubation, LPS and LPS + PRP showed lower ($P < 0.05$) endometrial cell viability compared to CTRL and CM, while the viability in PRP and LPS + CM did not differ from all other conditions. At 12 h, cell viability was higher ($P < 0.05$) in CTRL and CM compared to all other conditions, and LPS + PRP showed lower ($P < 0.05$) percentages compared to PRP and LPS + CM. At 24 h, the number of live cells remained higher ($P < 0.05$) in CTRL and CM compared to LPS, PRP, and LPS + PRP but similar to LPS + CM. Differences in endometrial cell apoptosis among groups were observed only at 12 h, with lower ($P < 0.05$) values in the CTRL and CM than in the LPS, PRP, and LPS + PRP groups. The proportion of apoptotic cells in LPS + CM was similar to the other groups but lower than in LPS + PRP. After 6 h of incubation, necrosis was lower ($P < 0.05$) in CTRL, CM, and LPS + CM compared to LPS, PRP, and LPS + PRP. At 12 h, lower values ($P < 0.05$) of necrotic cells were observed in CTRL and CM than in all other conditions, with LPS + PRP showing the highest levels ($P < 0.05$). At 24 h, the percentages of necrotic cells were lower ($P < 0.05$) in CTRL, CM, and LPS + CM than in LPS, PRP, and LPS + PRP, with PRP being higher than in all other conditions.

The conditions that affect ($P < 0.05$) viability, apoptosis, and necrosis over time were LPS, PRP, LPS + CM, and LPS + PRP, while CTRL and CM remained unaffected by incubation time. LPS caused a decrease

($P < 0.05$) in live cells and an increase ($P < 0.05$) in apoptotic cells at six and 12 h, while necrotic cells increased ($P < 0.05$) only at 6 h. In the PRP condition, viability decreased ($P < 0.05$) at six and 12 h, apoptosis increased ($P < 0.05$) only at 12 h, whereas necrosis increased ($P < 0.05$) at both six and 24 h. In LPS + CM, viability decreased ($P < 0.05$) and apoptosis increased ($P < 0.05$) only at 6 h, while necrosis increased ($P < 0.05$) only at 12 h. In LPS + PRP, viability decreased ($P < 0.05$) at six and 12 h but increased ($P < 0.05$) again at 24 h, while necrotic and apoptotic cells increased ($P < 0.05$) at six and 12 h and then decreased ($P < 0.05$) at 24 h.

3.2. Effect on cell mitochondrial activity and ROS concentrations

Fig. 2 shows photomicrographs of staining for intracellular ROS concentrations and mitochondrial activity and obtained under the different experimental conditions at each time point.

Fig. 3 shows the intracellular ROS concentrations and cell mitochondrial activity under the different tested conditions within each time point.

3.2.1. Effect on intracellular ROS

At 6 h, intracellular ROS concentrations were lower ($P < 0.05$) in CTRL than in all the other groups, and the highest concentrations were recorded in CM. However, at 12 h, intracellular ROS concentrations were higher ($P < 0.05$) in PRP compared to CTRL and LPS. After 24 h of incubation, the highest ($P < 0.05$) ROS concentrations were observed in LPS and CM, followed by LPS + CM and LPS + PRP ($P < 0.05$), then CTRL ($P < 0.05$), with the lowest values in PRP ($P < 0.05$).

Intracellular ROS concentrations followed the same pattern over incubation time in all conditions: ROS concentrations increased ($P < 0.05$) at 6 h, decreased ($P < 0.05$) at 12 h, and increased ($P < 0.05$) again at 24 h.

3.2.2. Effect on mitochondrial activity

After 6 h of incubation, the cell mitochondrial activity was lower ($P < 0.05$) in CTRL than in all the other groups. Within the treated groups, PRP and LPS + CM had lower ($P < 0.05$) mitochondrial activity than LPS, CM, and LPS + PRP. At 12 h, CTRL, CM, and LPS + CM showed a higher ($P < 0.05$) mitochondrial activity than in all other conditions, with LPS + PRP higher ($P < 0.05$) than PRP.

At 24 h, cell mitochondrial activity was lower in CTRL and LPS + PRP compared to all other conditions, with CM lower ($P < 0.05$) than LPS, PRP, and LPS + CM.

Changes in mitochondrial activity were observed over incubation time, varying across different conditions. The CTRL and LPS conditions exhibited opposite patterns of mitochondrial activity: in CTRL, mitochondrial activity decreased ($P < 0.05$) at 6 h, increased ($P < 0.05$) at 12 h, and decreased ($P < 0.05$) again at 24 h, whereas in LPS, it increased ($P < 0.05$) at 6 h, decreased ($P < 0.05$) at 12 h, and increased ($P < 0.05$) again at 24 h. In the CM condition, mitochondrial activity increased ($P < 0.05$) at 6 h and decreased ($P < 0.05$) at 24 h; while in PRP, it decreased ($P < 0.05$) at 12 h and increased ($P < 0.05$) at 24 h. In LPS + CM, mitochondrial activity increased ($P < 0.05$) over time, whereas in LPS + PRP, it increased ($P < 0.05$) only at 6 h.

3.3. Effect on PGE-2 and IL-10 concentrations

Fig. 4 shows the *in vitro* effects of the different conditions on PGE-2 and IL-10 concentrations at each time point.

3.3.1. Effect on PGE-2

After 6 h of incubation, PGE-2 concentrations were higher ($P < 0.05$) in LPS and LPS + PRP compared to all other conditions, with LPS + CM showing higher ($P < 0.05$) concentrations than CTRL. At 12 h, PGE-2 concentrations were higher ($P < 0.05$) in LPS, PRP, and LPS + PRP compared to LPS + CM, which gave higher ($P < 0.05$) values than CTRL

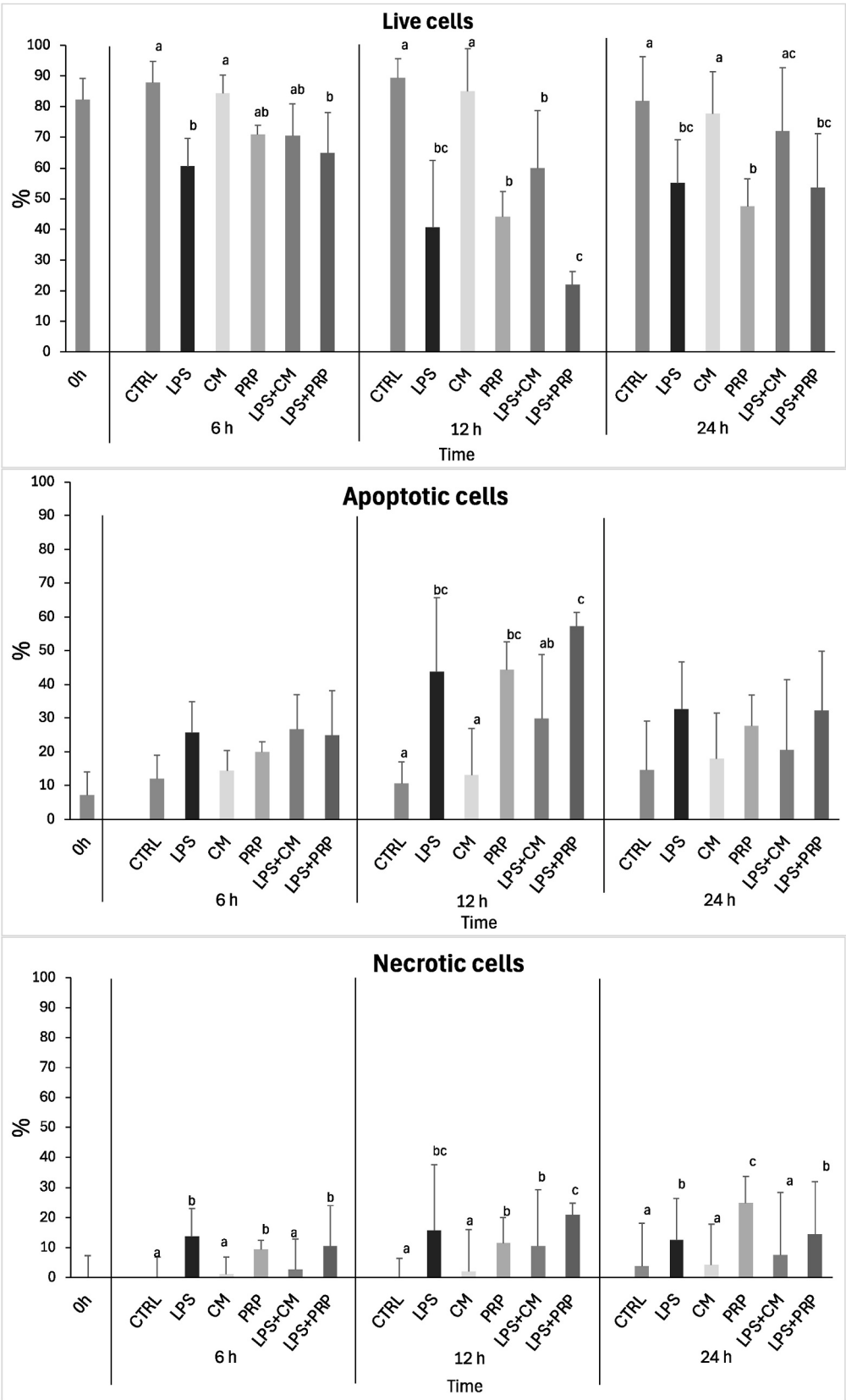


Fig. 1. Percentages of live, apoptotic, and necrotic cells of three replicates in different conditions (CTRL, LPS, CM, PRP, LPS + CM, LPS + PRP) after six, 12, and 24 h of incubation; different superscript letters indicate significant differences ($P < 0.05$) among groups within each time point (a–d).
Legend: CTRL: control; LPS: DMEM with the addition of 10 ng/mL LPS lipopolysaccharide; CM: 10 % of Wharton’s jelly mesenchymal stromal/stem cells-derived conditioned (WJ-CM); LPS + CM: 10 ng/mL of LPS and 10 % WJ-CM; PRP: 5 % PRP; LPS + PRP: 10 ng/mL of LPS and 5 % PRP.

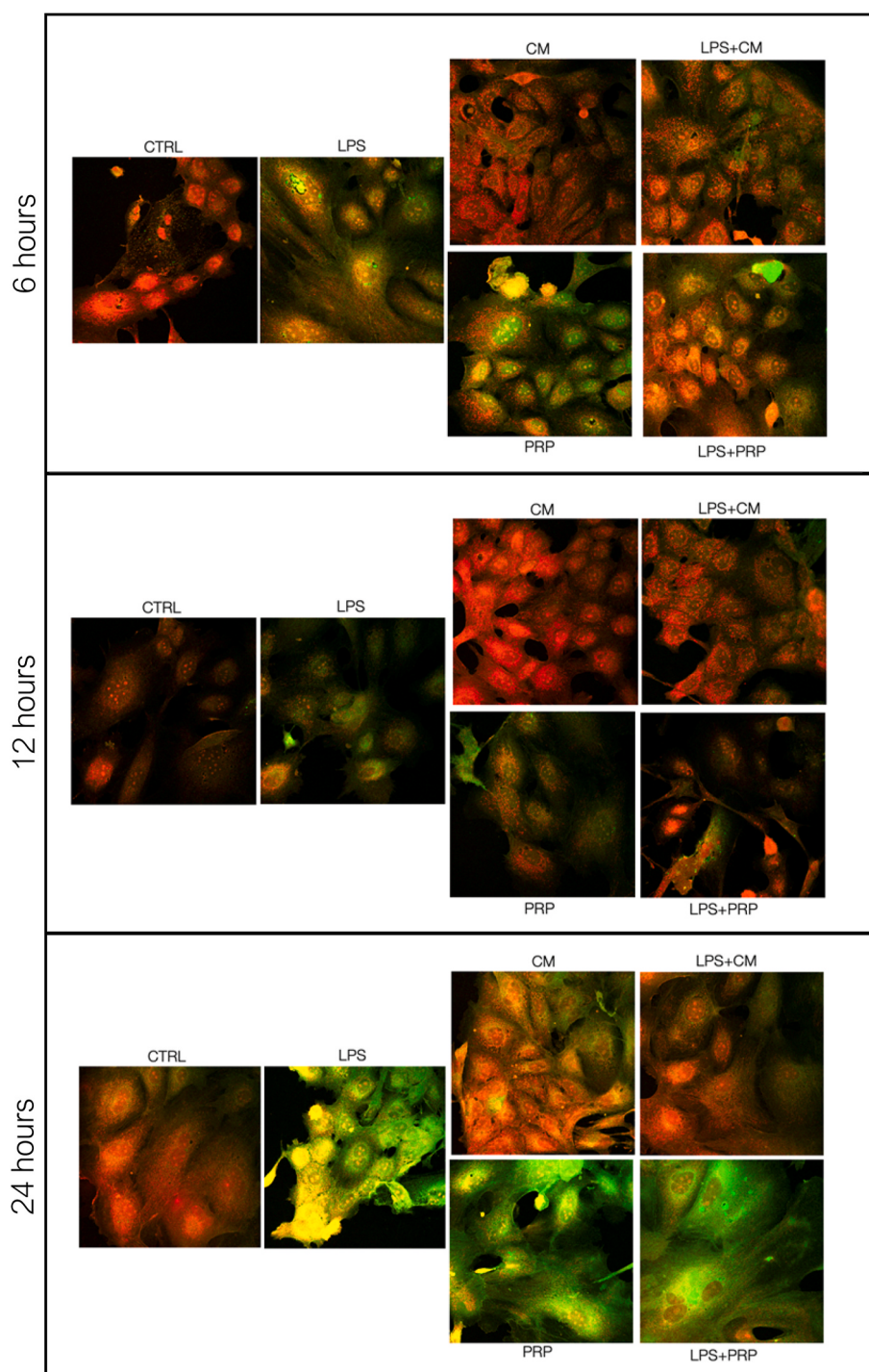


Fig. 2. Representative photomicrographs of endometrial cells stained with MitoTracker Orange CMTM Ros for mitochondrial activity and with 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) for intracellular ROS concentrations across different experimental conditions (CTRL, LPS, CM, LPS + CM, PRP, LPS + PRP) at each timepoint (6, 12, and 24 h). The scale bar represents 10 μ m.

Legend: CTRL: control; LPS: DMEM with the addition of 10 ng/mL LPS lipopolysaccharide; CM: 10 % of Wharton's jelly mesenchymal stromal/stem cells-derived conditioned (WJ-CM); LPS + CM: 10 ng/mL of LPS and 10 % WJ-CM; PRP: 5 % PRP; LPS + PRP: 10 ng/mL of LPS and 5 % PRP. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and CM. At 24 h, the highest ($P < 0.05$) concentrations of PGE-2 were recorded in LPS, while the lowest ($P < 0.05$) concentrations were found in CTRL and LPS + CM. The PRP and LPS + PRP gave higher ($P < 0.05$) concentrations than CM. Regarding the differences among time points, PGE-2 concentrations increased ($P < 0.05$) at 6 h in all conditions except PRP, which showed an increase ($P < 0.05$) at 12 h. At 24 h, PGE-2 concentrations continued to increase ($P < 0.05$) in LPS and LPS +

PRP, whereas they decreased ($P < 0.05$) in CTRL and LPS + CM.

3.3.2. Effect on IL-10

At 6 h of incubation, IL-10 concentrations were higher ($P < 0.05$) in CTRL, CM, and PRP compared to LPS and LPS + CM. At 12 h, IL-10 concentrations were higher ($P < 0.05$) in LPS compared to CTRL, PRP, and LPS + PRP. Lower ($P < 0.05$) IL-10 concentrations were found in

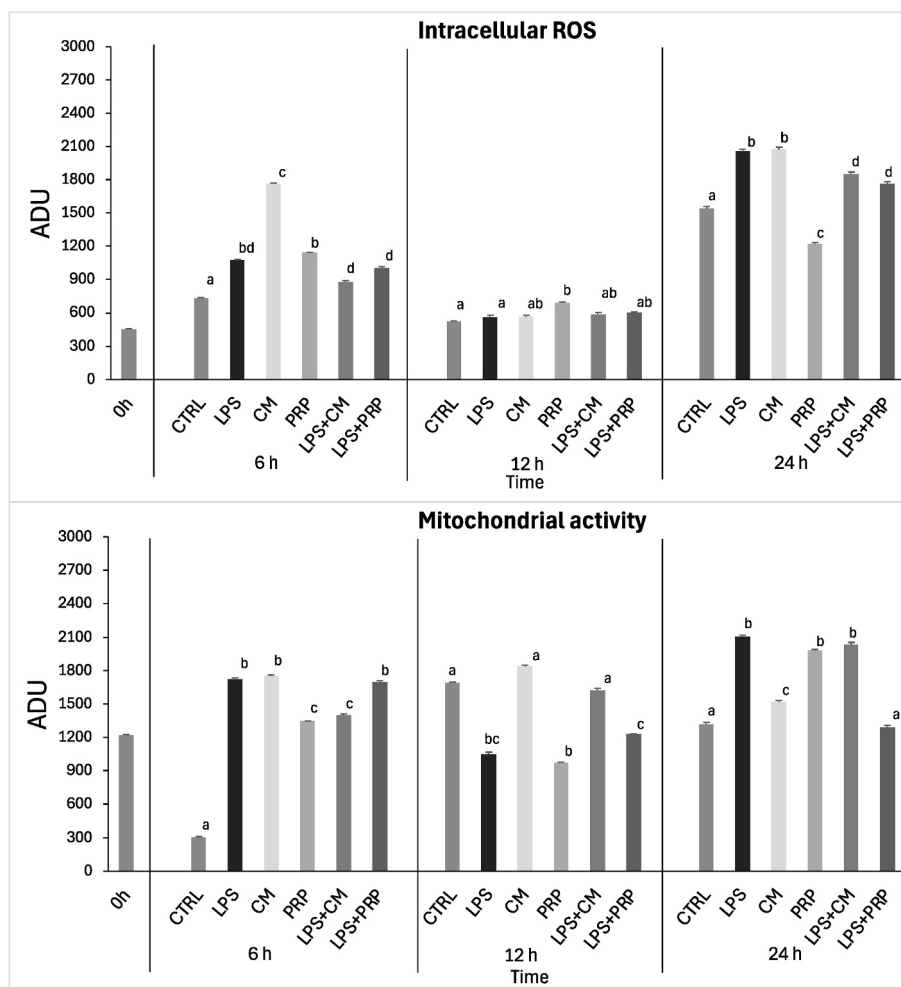


Fig. 3. Intracellular ROS (A) and mitochondrial activity (B) of three different replicates in different conditions (CTRL, LPS, CM, PRP, LPS + CM, LPS + PRP) after six, 12, and 24 h of incubation; different superscript letters indicate significant differences ($P < 0.05$) among groups at each time point (a–d).

Legend: ADU: arbitrary densitometric unit; CTRL: control; LPS: DMEM with the addition of 10 ng/mL LPS lipopolysaccharide; CM: 10 % of Wharton's jelly mesenchymal stromal/stem cells-derived conditioned (WJ-CM); LPS + CM: 10 ng/mL of LPS and 10 % WJMSC-CM; PRP: 5 % PRP; LPS + PRP: 10 ng/mL of LPS and 5 % PRP.

LPS + CM compared to CTRL, LPS, and CM. At 24 h, IL-10 concentrations were higher ($P < 0.05$) in LPS and LPS + PRP compared to CTRL, PRP, and LPS + CM, and the lowest ($P < 0.05$) values were recorded in LPS + CM.

IL-10 concentrations changed over time only in groups containing LPS: they decreased ($P < 0.05$) at 6 h in both LPS and LPS + CM, increased ($P < 0.05$) at 12 h in LPS, and increased ($P < 0.05$) at 24 h in LPS + PRP.

4. Discussion

The post-breeding inflammatory response is an extremely complex and intertwined process involving the interaction of pro- and anti-inflammatory cytokines [42]. Although pro-inflammatory cytokines are essential for fighting endometrial infections and challenges, an excessive or prolonged inflammatory response can lead to tissue damage and play a role in the development of fibrosis [43–45]. Anti-inflammatory cytokines, regulating the inflammatory response, contribute to managing the inflammatory process in endometritis by inhibiting the transcription of pro-inflammatory cytokines through various mechanisms [46–48]. A balance between pro- and anti-inflammatory interleukins is essential for modulating the exacerbated inflammatory response occurring in mares susceptible to PBIE [42,49], who display an endometrial upregulation of pro-inflammatory

cytokines lasting for more than 72 h post-insemination or mating [1,2,50].

Conditioned medium and PRP are rich in cytokines, chemokines, and growth factors that could drive immunomodulation via paracrine signaling. In addition to soluble factors, both CM and PRP contain EVs [51,52] that vehiculate active molecules, such as lipids, proteins, DNAs, mRNAs, and microRNAs (miRNAs) to target cells. The released molecules act as signaling complexes by promoting proliferation and angiogenesis, preventing apoptosis, modulating the immune system, and suppressing fibrosis [53].

In mares susceptible to PBIE, the regenerative therapies based on the use of MSCs and their secretome are very limited [20,21], while the use of PRP is becoming very popular, however, the results are controversial [7]. Only a few *in vitro* studies have investigated the effects of these treatments on equine endometrial cells: Perrini et al. [27] used amniotic-derived EVs that were able to reduce apoptosis rate, increase cell proliferation values, downregulate pro-inflammatory gene expression, and decrease secretion of pro-inflammatory cytokines after the detrimental action of LPS; Cespedes et al. [54] showed the migration of MSCs and epithelial cells after scratch. However, to date, there are no *in vitro* studies on the effect of PRP on equine endometrial cells. Therefore, for the first time in this study, the impact of PRP and WJ-CM was evaluated with or without LPS-induced inflammation.

To provide insight into the mechanisms of action of these treatments,

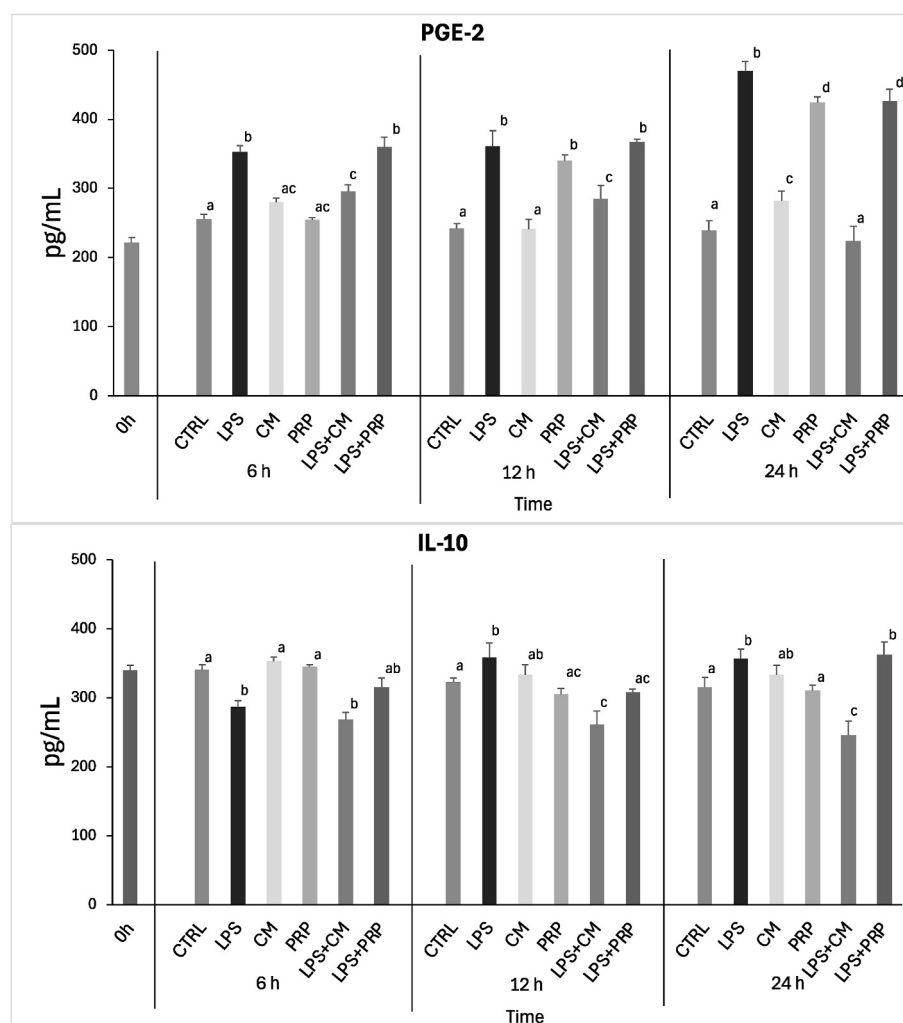


Fig. 4. Concentrations of PGE-2 (A) and IL-10 (B) of three different replicates in different conditions (CTRL, LPS, CM, PRP, LPS + CM, LPS + PRP) after six, 12, and 24 h of incubation; different superscript letters indicate significant differences ($P < 0.05$) among groups at each timepoint (a–d).

Legend: CTRL: control; LPS: DMEM with the addition of 10 ng/mL LPS lipopolysaccharide; CM: 10 % of Wharton's jelly mesenchymal stromal/stem cells-derived conditioned (WJ-CM); LPS + CM: 10 ng/mL of LPS and 10 % WJMSC-CM; PRP: 5 % PRP; LPS + PRP: 10 ng/mL of LPS and 5 % PRP.

viability, pro- and anti-inflammatory cytokine concentrations, oxidative stress, and mitochondrial activity of cells were evaluated. In view of future clinical applications, this study used CM obtained by incubating equine WJ-MSCs with Ringer's Lactate, with slight modifications to reported protocols for human and canine MSCs [31,32]. Ringer's lactate is commonly used for uterine infusion. More importantly, the *in vivo* use of CM obtained with commercial cell culture media (e.g., PBS, M199, DMEM) is inappropriate, as these are not approved vehicles for safe infusion into patients [31].

The addition of only CM to the culture media did not affect endometrial cell viability or induce apoptosis or necrosis over time; furthermore, the CM alleviated LPS-induced necrosis at 6 and 24 h of treatment. Placental-derived MSCs, especially umbilical cord WJ-MSCs, are renowned for their distinctive immunomodulatory and anti-inflammatory properties [55–57]. These functions have been attributed to MSCs ability to influence inflammatory cytokine secretion [55, 58]. The literature suggests that umbilical cord MSCs possess the ability to skew the cytokine secretion profiles from pro-inflammatory to anti-inflammatory by the inhibition of critical factors for endometritis [59]. Our results confirmed the anti-inflammatory effect of WJ-CM, as it limited endometrial production of PGE-2 both alone and during LPS-induced inflammation at all time points. The reduction in PGE-2 synthesis contributes to a more regulated and anti-inflammatory

environment. The complex and multifaceted composition of WJ-CM, which includes cytokines, growth factors, EVs, and microRNAs, can modulate inflammatory signaling pathways, downregulate COX-2 expression, and reprogram immune cells [60–62]. Thus, the intrauterine application of WJ-CM could help control the excessive and prolonged inflammatory response observed in susceptible mares.

The *in vitro* effect of PRP on equine endometrial cells was tested for the first time. However, our results show that PRP alone was detrimental for endometrial cells, inducing apoptosis and necrosis, compared to the effect of LPS. These results are inconsistent with the data obtained on bovine endometrial cells [24]. In that study, the same concentrations of PRP stimulated endometrial cell proliferation through an anti-inflammatory effect, reducing pro-inflammatory cytokines. In contrast, our results did not demonstrate an anti-inflammatory effect but revealed pro-inflammatory properties of PRP. The sustained increase in PGE-2 under PRP conditions lasted up to 24 h; it is known that endometrial secretion of PGE-2 has been associated with endometritis in mares [63]. The similar production of IL-10 in PRP and the CTRL groups suggests the lack of an anti-inflammatory effect of PRP on equine endometrial cells *in vitro*.

The negative effect of PRP could be explained by chemokines, some of which act in synergy with white blood cells. In contrast, others are chemotactic and can recall monocytes and macrophages at the site of

inflammation [64]. In addition to the chemotactic action of PRP for the mobilization of macrophages to the site of damage or infection, it also promotes their activation [65]. *In vivo*, the macrophages, attracted by the chemotactic action of growth factors, would be able to resolve the inflammation phase, promoting tissue remodeling by the secretion of factors that stimulate the production of fibroblasts and regulate the synthesis of connective tissue and by angiogenic factors.

All these interactions are missing in the *in vitro* study and could explain the deleterious effect of PRP in our research. Indeed, it is also evident from our results the increased production of pro-inflammatory cytokines that could be responsible for the heightened mitochondrial activity and excessive ROS production found in PRP-treated endometrial cells, in most cases similar to or even worse than LPS treatment. These conditions lead to oxidative stress, resulting in cellular damage and apoptosis. Oxidative stress is one of the main pathophysiological mechanisms for some inflammatory conditions [66]. The results of this study confirm that LPS induces oxidative stress and mitochondrial dysfunction, characterized by a decrease in expression, mitochondrial mass, and reduction in the mitochondrial membrane potential [66]. It seems that cytokines, especially pro-inflammatory types, could induce ROS production via mitochondrial-induced activity [67]. While intracellular ROS have a role in host defense against infectious agents, in redox-sensitive signal transduction, and other cellular processes, excess ROS can lead to significant cellular and tissue damage and contribute to chronic inflammation [68,69]. An increase in ROS in endometrial cells treated with LPS was expected. Still, the most controversial result of this study is the increase of ROS and mitochondrial activity in CM-treated endometrial cells. Previous studies have reported an antioxidant effect of CM by MSCs isolated from various sources and its ability to preserve cell viability by reducing ROS generation [36,70,71]. In our results, there is a disagreement between cell viability and data obtained from the evaluation of oxidative stress in WJ-CM-treated cells. Since the antioxidant properties of WJ-CM were not tested in this study, it will be interesting to investigate and deepen our results to explain why an increase in intracellular ROS did not lead to increased endometrial cell necrosis and apoptosis. It is worth noting that all conditions followed the same pattern of intracellular ROS concentrations over incubation time, likely reflecting the oxidative stress induced by the cell culture process [72]. Indeed, it has been reported that, during incubation, components of the culture media tend to generate ROS and other oxidative byproducts [72].

In conclusion, this study demonstrated that WJ-CM treatment benefits endometrial cell viability following an LPS challenge, presumably due to its anti-inflammatory action, as suggested by the reduced concentrations of the pro-inflammatory cytokine PGE-2. Unexpectedly, PRP exhibited a deleterious effect on endometrial cells, promoting the secretion of pro-inflammatory cytokines. These findings support the potential therapeutic use of WJ-CM *in vivo* for treating PBIE in mares. Further studies are needed to investigate the composition and the mechanism of action of WJ-CM.

CRedit authorship contribution statement

Chiara Del Prete: Writing – original draft, Methodology, Data curation, Conceptualization. **Giulia Gaspari:** Writing – review & editing, Methodology, Investigation. **Michał Andrzej Kosior:** Writing – review & editing, Methodology, Investigation. **Barbara Merlo:** Methodology, Conceptualization. **Eleonora Iacono:** Methodology, Conceptualization. **Consiglia Longobardi:** Investigation. **Nicola Antonio Martino:** Investigation. **Maria Elena Dell'Aquila:** Investigation. **Sara Damiano:** Investigation. **Nataschia Cocchia:** Supervision. **Bianca Gasparri:** Writing – review & editing, Conceptualization. **Anna Lange-Consiglio:** Writing – original draft, Methodology, Investigation, Conceptualization.

Data availability

All data generated or analyzed during this study will be available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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