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Effect of allogeneic platelet lysate on equine bone marrow derived mesenchymal stem cell characteristics, including immunogenic and immunomodulatory gene expression profile

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ABSTRACT

Propagation *ex vivo* of mesenchymal stem cells (MSCs) requires culture medium supplementation. Fetal bovine serum (FBS) has long been the gold standard supplement, but its use is being questioned mainly due to ethical and safety issues. The use of platelet lysate (PL) as substitute of FBS has been proposed but little is known about its effects on equine MSCs characteristics including their immune profile. The aim of this work was to investigate for the first time the effect of allogenic PL on the immunogenic and immunomodulatory gene expression profile of equine bone marrow derived MSCs (eBM-MSCs) as well as on their proliferation ability, phenotype markers, and viability post-cryopreservation. The eBM-MSCs ($n = 3$) cultures were supplemented with 20% of allogeneic pooled concentrated PL (CPL; 591×10^3 platelets/μL) or basal PL (BPL; 177×10^3 platelets/μL) from three donors, using 10% FBS supplementation as control. The proliferative ability of eBM-MSCs under the three conditions was evaluated by calculating the cell doubling times (DT) up to passage 3 (P_3) and by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at P_3. Viability of eBM-MSCs post-cryopreserved with CPL or FBS was assessed at 15, 30 and 60 days. The gene expression profile of eBM-MSCs was evaluated in P_3 by RT-qPCR for characterization, immunogenic and immunomodulatory markers. The cells cultured in CPL had significantly higher ability to proliferate than with FBS or BPL ($P < 0.001$) in the MTT assay. Post-cryopreserved viability was similar between cells cultured and preserved in FBS and CPL at all time-points. Gene expression of MSC characterization markers was similar among the three conditions. The gene expression of the immunogenic markers MHC-I, MHC-II and CD40 was slightly (non-significant) increased in CPL condition compared to FBS and BPL. The CPL condition showed higher expression of the genes coding for the immunomodulatory molecules VCAM-1 (non-significant) and IL-6 ($P < 0.05$), and similar for COX-2; whereas iNOS and IDO were not expressed under any condition. In conclusion, the replacement of FBS by allogeneic CPL as a supplement for *ex vivo* propagation of eBM-MSCs provides appropriate proliferation and cryopreservation, and mildly upregulates the gene expression of immunomodulatory markers, thus constituting a potentially suitable alternative to the use of FBS. Further studies are needed to clarify the composition and effects of CPL supplementation on equine MSCs immunological profile.

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1. Introduction

Mesenchymal stem cells (MSCs) have generated a great interest because of their therapeutic potential, primarily due to their ability to promote tissue healing mainly though their regulatory and immunomodulatory properties (Berglund et al., 2017; Cassano et al., 2018b). In the horse, cell therapy has been mainly investigated to treat orthopedic pathologies, mostly tendons and joints (de Mattos Carvalho et al., 2011; Broeckx et al., 2014). Therapy with MSCs requires *in vitro* cell expansion prior to its application to obtain a more homogeneous population and reach an appropriate cell number for the treatment. Fetal bovine serum (FBS) is usually considered as the gold standard supplement for MSC culture of different species (Bieback et al., 2009; Iudicone et al., 2014). Besides, FBS is also used as a cryoprotective agent in combination with 5–10% dimethyl sulfoxide (DMSO) for MSC banking of different species, since it provides growth factors, stabilizes the cell membrane, prevents excessive concentration of solutes, minimizes cell dehydration and reduces the formation of extracellular ice (De Rosa et al., 2009; Wang et al., 2017). Despite its advantages, the use of FBS is being questioned mainly due to ethical and safety issues. From an ethical point of view, the FBS is obtained by intracardiac puncture of the fetus after the pregnant female slaughter (Jochems et al., 2002; Seo et al., 2013), this procedure requires more than one million fetuses per year (Jochems et al., 2002; Hemeda et al., 2014). Concerning safety, there is a risk of a xeno-response as cultured cells are in contact with proteins from bovine species, plus it exists risk for prion transmission (Spees et al., 2004; Doucet et al., 2005; Chou et al., 2015).

Regarding immune xeno-response, some studies found increased anti-FBS antibody titers after receiving cell therapy in rat (Spees et al., 2004) and human, even though with low frequency (Horwitz et al., 2002). In the horse, the only study evaluating anti-FBS antibodies after MSC therapy did not find a significant titer increase, but pre-existing anti-FBS antibodies were found, likely induced by xeno-contamination of viral vaccines (Owens et al., 2016). Nevertheless Joswing et al. (2017) showed inflammatory joint reaction due to the use FBS-contaminated MSCs, indicating the relevance of developing new strategies to reduce or delete FBS xeno-antigens. The FBS proteins are internalized by the cells so repeated washing is inefficient to completely remove the xeno-contamination, but it is possible to reduce it drastically when cells are cultured with homologous serum or platelet lysate (PL) for a short period of time (Spees et al., 2004; Joswig et al., 2017).

Due to the disadvantages of FBS as culture supplement and the need of cryobanking MSCs for therapeutic application, there are recent studies evaluating alternatives to replace the FBS, such as PL (Wang et al., 2017). Nowadays, the PL has gained interest since it allows xeno-free media and avoids the problems associated with the classic FBS supplementation. In addition, PL can be produced allogeneically, rather inexpensively and on large-scale in humans (Iudicone et al., 2014) and equines (Seo et al., 2013; Russell and Koch, 2016). In humans, several studies demonstrate that PL can replace the FBS to grow MSCs while preserving their characteristics *in vitro*, such as proliferation, multipotency and immunomodulation (Doucet et al., 2005; Iudicone et al., 2014). In equines, the effect of PL on the proliferative capacity and differentiation ability of the MSCs has been studied showing a behavior similar to the standard FBS supplementation (Del Bue et al., 2007; Seo et al., 2013; Russell and Koch, 2016). However, equine MSC immune properties have been barely investigated under this condition. One recent study evaluated the capacity of equine MSCs cultured with allogeneic PL for regulating proinflammatory cytokine synthesis by monocytes under proinflammatory stimulation in an *in vitro* coculture system, finding a suppressive capacity similar to FBS-cultured MSCs (Naskou et al., 2018). However, the immunogenic and immunomodulatory profiles of MSCs were not evaluated. Thus, we aimed to investigate for the first time the effect of allogenic PL on the gene expression profile of immunogenic and immunomodulatory markers in equine bone marrow derived MSCs (eBM-MSCs), as well as on their proliferation ability, phenotype markers, and post-cryopreservation viability.

2. Materials and methods

2.1. Study design

Equine bone marrow derived MSCs (eBM-MSCs) from three donors, cryopreserved at passage 1 (P_1), were thawed and subcultured up to P_3 with different culture media supplementation: (a) FBS: 10% FBS; (b) CPL: 20% allogeneic concentrated platelet lysate (CPL) and (c) BPL: 20% allogeneic basal platelet lysate (BPL). First, proliferative capacity was evaluated from P_1 to P_3 by calculating cell doubling times (DT) and at P_3 by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Second, we evaluated for the three culture conditions at P_3 the expression profile of genes coding for phenotype characterization surface molecules, immunogenic antigens and molecules related to the immunomodulatory ability by using real time quantitative polymerase chain reaction (RT-qPCR). Third, eBM-MSCs P_3 were cryopreserved with 90% FBS or CPL and 10% DMSO to evaluate post-cryopreservation cell viability at 15, 30 and 60 days.

2.2. Animals

Three healthy horses (400–450 kg weight, age 15–20 years, two crossbred female and one crossbred gelding) were used for the extraction of whole blood to produce the PL pool. Biological samples were obtained according to local animal welfare regulations. All procedures were carried out under Project Licence PI 31/11 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The eBM-MSCs P_1 ($n = 3$) used for this assay came from the MSC bank of LAGENBIO, so no animals were used in this study for BM harvesting. This was decided to avoid the harvesting of bone marrow with the solely purpose of this study, as well as because it has been reported that 48 h of FBS deprivation are enough to drastically reduce the internalization of FBS proteins in equine MSCs (Joswig et al., 2017) and this study aimed at culturing cells for several weeks. The eBM-MSCs were previously isolated from three healthy client-owned horses (450–500 kg weight, age 3–6 years, two Thoroughbred geldings and one Spanish purebred gelding) under previous owner consent and agreement to use the cells for scientific purposes. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.3. Allogeneic pooled PL preparation

Whole blood was aseptically collected from the jugular vein of the three donor animals using 3.8% sodium citrate (Fluka) as anticoagulant. Once the blood was extracted between a range of 1680–2280 ml/horse, syringes were placed vertically for 30 min in order to obtain plasma separation. For the extraction of PL with basal concentration of platelets (BPL), the plasma was centrifuged only once at 200*g* for 10 min to remove red and white blood cells and the supernatant was collected. To obtain PL with higher platelet concentration (CPL), a second centrifugation was carried out. The plasma supernatant collected after first centrifugation was then centrifuged at 1500*g* for 20 min to pellet the platelets. The supernatant (platelet-poor plasma) was removed and used to resuspend the pellet in a 10% of the original volume (Gilbertie et al., 2018). A sample from each animal and each preparation (basal or concentrated), separately and pooled, was taken to perform platelet and white blood cell (WBC) count using a flow cytometry hematology system (Laser-Cyte Dx, IDEXX Laboratories). Finally, both basal and concentrated platelet solutions pooled from the three donors were stored at −80 °C overnight and quickly thawed at

Table 1
Genes analyzed by real time quantitative polymerase chain reaction (RT-qPCR). GenBank accession numbers of the sequences used for primers design. Primer sequences (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, in order to facilitate the posterior analysis: Housekeeping, characterization cell surface markers, antigen presenting-related molecules and immunomodulation-related molecules.

Gene	Accession number	Primer sequence (5'-3')	Amplicon size	
Housekeeping				
B2M	NM_001082502.2	F:TCGTCCTGCTCGGGCTACT R:ATTCTCTGCTGGGTGACGTGA	102	
GAPHD	NM_001163856	F:GGCAAGTTCCATGGCACAGT R:CACAACATATTCAGCACCAGCAT	128	
Characterization cell surface markers				
CD44	NM_001085435	F:CCCACGGATCTGAAACAAGTG R:TTCTGGAATTTGAGGTCTCCGTAT		95
CD73	XM_001500115	F:GGGATTGTTGGATACACTTCAAAAG R:GCTGCAACGCAGTGATTTCA		90
CD90	EU881920	F:TGCGAACTCCGCCTCTCT R:GCTTATGCCCTCGCACTTG		93
CD105	XM_001500078	F:GACGGAAAATGTGGTCAGTAATGA R:GCGAGAGGCTCTCCGTGTT		100
CD34	XM_001491596	F:CACTAAACCCTCTACATCATTTTCTCCTA R:GGCAGATACCTTGAGTCAATTTCA		150
CD45	AY_114350	F:TGATTCCCAGAAATGACCATGTA R:ACATTTTGGGCTTGTCCTGTAAC		100
Antigen presenting-related molecules				
MCH-I	AB525081	F:CGTGAGCATCATTGTTGGC R:TCCCTCTTTTTTCACCTGAGG	92	
MCH-II	NM_001142816	F:AGCGGCGAGTTGAACCTACAGT R:CGGATCAGACCTGTGGAGATGA	172	
CD40	AY514017	F:ACAAATACTGCGACCCCAACC R:TTTCACAGGCATCGCTGGA	114	
Molecules related with the immunomodulatory ability				
COX-2	AB041771	F:GTTTGCATTTTTTGCCCAGC R:ACTTAAATCCACCCCGTGACC		103
IL-6	EU438770	F:AACAGCAAGGAGGTACTGGCA R:CAGGTCTCCTGATTGAACCCA		95
VCAM-1	DQ246452	F:TCTATGCTACGCTCTGGCTACG R:TTGATGGTCTCCCCGATGA		127
iNOS	AY027883	F:CCAACAATGGCAACATCAGGT R:TGAGCATTCCAGATCCGGA		85
IDO	XM_001490681	F:TCATGACTACGTGGACCCAAAA R:CGCCTTCATAGAGCAGACCTTC		104

37 °C in a water bath to lysate the platelets (Russell and Koch, 2016). Subsequently, to remove platelet debris, pooled BPL and CPL were centrifuged at 1600*g* for 30 min and the supernatant was filtered through a 0.22 µm vacuum filter.

2.4. Culture of eBM-MSCs

The eBM-MSCs used in this study were initially isolated by BM density-gradient centrifugation as previously described (Remacha et al., 2015) and cultured in standard culture medium, consisting of Dulbecco's Modified Eagle Medium (DMEM) low glucose, 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 U/ml penicillin and 10% FBS (all from Sigma-Aldrich) until passage 1 (P_1). When these cells were cultured to use with a different purpose, part of them were cryopreserved in 90% FBS and 10% DMSO at −80 °C. For the current study, the cryopreserved cells at P_1 were thawed and plated at 5×10^3 cells/cm^2 and subsequently cultured up to P_3, under the three different conditions: (a) FBS: standard culture medium supplemented with 10% FBS (standard condition); (b) CPL: standard culture medium supplemented with 20% allogeneic pooled CPL (instead of FBS) and (c) BPL: standard culture medium supplemented with 20% allogeneic pooled BPL (instead of FBS). The PL concentration in media was chosen based on previous reports (Russell and Koch, 2016) and preliminary assays of our group (unpublished data). Heparin (2 IU/mL) was added as anticoagulant to prevent clotting to all media conditions (Seo et al., 2013). Culture media were changed twice a week and cells were subcultured until P_3 when approximately reached 80% confluence by treating with 0.25% trypsin-EDTA (Sigma-Aldrich).

2.5. Cell proliferation evaluation

At each passage when cells were detached and counted, cell doubling times (DT) were determined according to two formulae: 1) CD = ln(Nf/Ni)/ln(2) and 2) DT = CT/CD, where CD = cell doubling number; Nf = final number of cells; Ni = initial number of cells; and CT = days of culture. At P_3, the proliferative ability of eBM-MSCs cultured under the three conditions was determined by MTT assay during 7 days as previously described (Ranera et al., 2012). Briefly, 9 technical replicates of each sample were seeded in 96-well plates with each media at 1600 cells/well (5×10^3 cells/cm^2). Culture media alone (no cells) was used as a blank in each plate. The spectrophotometer (Biotek Synergy HT) was used to measure the optical density (570 nm) in each well. Viable cell numbers were determined by extrapolating from a standard curve consisting of 10 points (6 technical replicates per point) with increasing quantity of cells (0–60,000 cells/well). The total number of viable cells was determined by extrapolation from a calibration curve for each culture condition: FBS $y = 1 \times 10^{-5}x + 0.0859$, $r^2 = 0.948$; CPL $y = 5 \times 10^{-6}x + 0.0309$, $r^2 = 0.989$; BPL $y = 1 \times 10^{-5}x + 0.0124$, $r^2 = 0.990$ (y = optical density of the well; x = amount of cells).

2.6. Cell viability post-cryopreservation

The eBM-MSCs P_3 were cryopreserved with 90% of FBS or CPL and

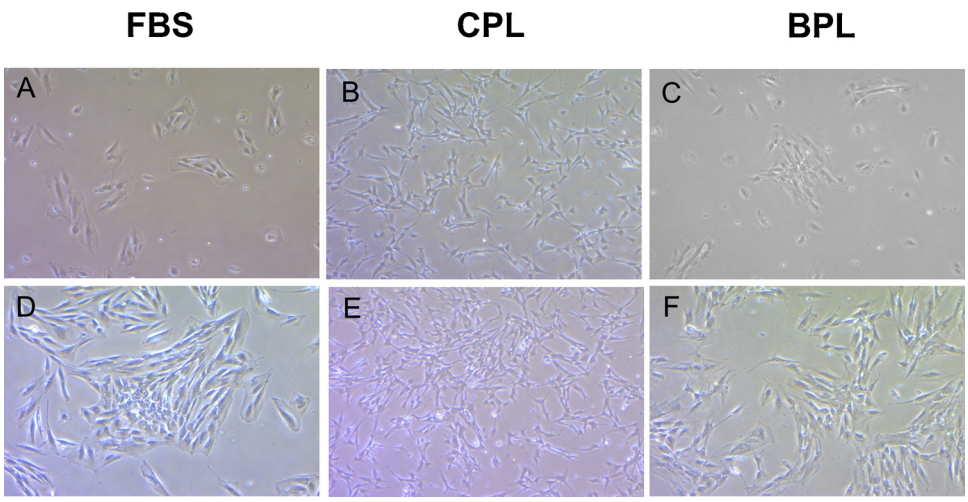


Fig. 1. Microscopic images (4×) of equine bone marrow derived mesenchymal stem cells (eBM-MSCs) in passage 3 under the three different culture conditions. (A–C) Cells with low confluence; (D–F) Cells with intermediate confluence. The media were supplemented with fetal bovine serum (FBS, A and D) or concentrated platelet lysate (CPL, B and E) or basal platelet lysate (BPL, C and F).

10% DMSO in 1 ml total volume and were stored at −80 °C. Cell viability was evaluated immediately after thawing at three different times: 15, 30 and 60 days post-cryopreservation. Cells were counted in a hemocytometer chamber using 0.4% Trypan Blue as vital stain.

2.7. RNA isolation from eBM-MSCs

Total RNA was extracted from 1×10^6 eBM-MSC P_3 cultured under the three conditions (FBS, CPL and BPL) with the commercial kit RNAspin Mini RNA Isolation Kit (GE Healthcare) following the manufacturer's instructions and DNAse Turbo (Ambion) was used to remove genomic DNA according to manufacturer's instructions. Subsequently, 1 µg mRNA was retrotranscripted into complementary DNA (cDNA) using the Superscript Reverse Trancriptase Kit (Invitrogen) following the manufacturer's instructions.

2.8. Gene expression analysis of eBM-MSCs by RT-qPCR

The gene expression of the eBM-MSCs cultured in the three conditions was assessed at P_3 for different markers: (a) genes coding for phenotype characterization surface molecules: Cluster of Differentiation (CD) 44, CD73, CD90, CD105, CD34 and CD45; (b) genes coding for the antigen presenting-related molecules: Major Histocompatibility Complex type I (MHC-I) and type II (MHC-II) and the costimulatory molecule CD40; (c) genes coding for molecules related with the immunomodulatory ability of MSCs: cyclooxygenase 2 (COX-2), interleukin 6 (IL-6), vascular cell adhesion molecule 1 (VCAM-1), inducible nitric oxide synthase (iNOS) and indoleamine 2, 3-dioxygenase (IDO). The genes beta2-microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping. Primer details, accession numbers for mRNA sequences and amplicon sizes are shown in Table 1 and have been previously validated and used in other studies, as well as the RT-qPCR methodology (Barrachina et al., 2016, 2017). Briefly, RT-qPCR was performed and monitored using the StepOne RealTime PCR System device (Applied Biosystems). All reactions were carried out in triplicate in a total volume of 10 µL with 2 µL of cDNA as a template and Fast SYBR Green Master Mix (Applied Biosystems). The cDNA was amplified following the manufacturer's protocol: 20″ at 95 °C for initial activation and denaturation, followed by 40 cycles consisting of 3″ at 95 °C and 30″ at 60 °C. A dissociation curve protocol was run after all PCR reactions. The levels of gene expression were determined by the comparative ΔΔCt method. A normalization factor (NF) was calculated as the geometric mean of the quantity of two housekeeping genes and gene expression in FBS condition (control) was used as reference to calculate fold changes.

2.9. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad Prism Inc.). Data proliferation from the MTT assay was analyzed by the nonparametric ANOVA Kruskall-Wallis test with Dunn's *post-hoc* test to assess differences between conditions (FBS, CPL and BPL) at each time-point and study differences along time (day 1 to 7) within each condition. The paired Friedman test followed by the *post-hoc* Dunn's test was used to compare the DTs from each condition along passages and among conditions at each passage, as well as for studying differences in post-cryopreservation viability along the different time-points. Post- cryopreservation viability data from FBS and CPL conditions was compared with Wilcoxon paired test at each time-point. The expression of each gene was compared between different conditions (FBS, CPL and BPL) by using the paired Friedman test followed by the *post-hoc* Dunn's test. Results are expressed as mean ± SD. Significance was set at $P < 0.05$ for all analysis.

3. Results

3.1. Characteristics of the allogeneic PL

The CPL presented a concentration of 591×10^3 platelets/µL and 590 WBC/µL, whereas the BPL consisted of 177×10^3 platelets/µL and 130 WBC/µL. The ratio platelet:WBC was 1002:1 for CPL and 1361:1 for BPL. Furthermore, the efficiency of obtaining CPL was 5.3% and BPL 40% of the blood volume extracted.

3.2. Morphological characteristics and proliferation ability of eBM-MSCs cultured with PL

The eBM-MCs showed fibroblast-like spindle shape under the three culture conditions (Fig. 1). Regarding proliferation ability, the DTs obtained during successive passages P_1–P_3 were overall lower (faster proliferation) for CPL cultured cells compared to standard condition (FBS) and BPL (Table 2). Significantly shorter DT was observed in P_3 when comparing CPL *vs* BPL (1.7 ± 0.1 *vs* 3.9 ± 0.6 days, respectively). Proliferation of eBM-MSCs on P_3 was quantified along one week by MTT assay, showing that CPL condition produced a significantly higher amount of cells from day 5 to 7 when compared to FBS ($P < 0.001$) and from day 4 to 7 compared to BPL ($P < 0.001$) (Fig. 2).

3.3. Viability of eBM-MSCs post-cryopreservation with CPL

The percentage of viable eBM-MSCs observed post-cryopreservation

Table 2
Summary of cell doubling times (DT), in days, of equine bone marrow derived mesenchymal stem cells (eBM-MSCs) cultured under the three different conditions from passage 1 (P_1) to passage 3 (P_3). The results are expressed as mean ± SD (n = 3) for each condition and each passage. Different letters mean significant differences ($P < 0.05$).

Supplement	DT (days)		
	P_1	P_2	P_3
FBS	3.0 ± 0.5[a]	2.8 ± 0.3[a]	2.1 ± 0.1[a,b]
CPL	2.5 ± 0.4[a]	2.0 ± 0.1[a]	1.7 ± 0.1[a]
BPL	2.6 ± 0.0[a]	4.5 ± 1.6[a]	3.9 ± 0.6[b]

was similar when CPL was used compared to FBS at all the time-points analyzed. Furthermore, significant differences were not observed among different time-points within any condition (Table 3). The supplement BPL was not assessed for cryopreservation because of the low amount of cells obtained under this culture condition, which was insufficient to perform all the assays. Therefore, we decided to prioritize the assessment of proliferation and gene expression.

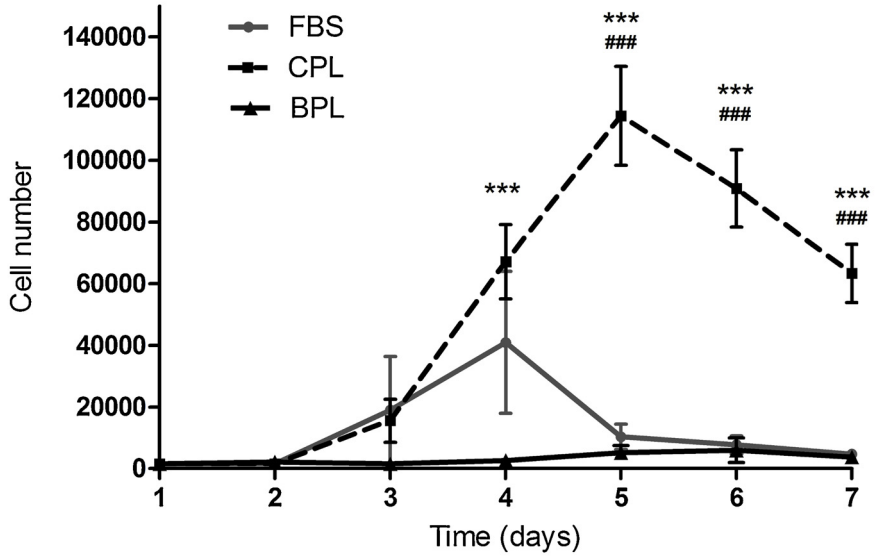
Table 3
Summary of post-cryopreservation cell viability (percentage of viable cells) of equine bone marrow derived mesenchymal stem cells (eBM-MSCs) in passage 3 frozen with 90% of fetal bovine serum (FBS) or concentrated platelet lysate (CPL) and 10% dimethyl sulfoxide (DMSO). The results are expressed as mean ± SD (n = 3) for each condition and each passage. No significant differences were found.

Condition	Time cryopreservation		
	15 days	30 days	60 days
FBS	64.8 % ± 9.0	63.9 % ± 12.3	70 % ± 9.3
CPL	64.5 % ± 9.6	65.3 % ± 12.5	62.3 % ± 9.3

3.4. Gene expression profile of phenotype characterization markers

The gene expression of surface markers generally used for eBM-MSCs characterization was assessed at P_3. In the three culture conditions, the cells presented similar expression of positive markers CD44, CD73, CD90 and CD105, and did not show gene expression of negative markers CD34 and CD45. Thus, the culture under both CPL and BPL supplements did not seem to change the gene expression of phenotype characteristic markers (Fig. 3 A).

A



B

Supplement	Days						
	1	2	3	4	5	6	7
FBS	1600 ±0	1600 ±0	19004 ±29988	40958 ±32512	10366 ±5821	7739 ±5035	4717 ±2172
CPL	1600 ±0 [a]	1600 ±0 [a]	15537 ±12070 [a]	67095 ±20874 [b]	114442 ±27853[c]	90897 ±12791[d]	63326 ±20845[e]
BPL	1600 ±0	2128 ±608	1600 ±0	3467 ±2117	5222 ±3890	5978 ±6942	3736 ±1866

Fig. 2. Proliferation of equine bone marrow derived mesenchymal stem cells (passage 3) cultured with fetal bovine serum (FBS) or concentrated platelet lysate (CPL) or basal platelet lysate (BPL) along 7 days in the MTT assay. The results are expressed as mean ± SD (n = 3) of the cell number reached at each time-point for each condition. A) Growth curve (FBS, gray line; CPL, black dotted line; BPL, black continuous line). Significant differences between CPL and BPL are indicated with* (***=$P < 0.001$); significant differences between CPL and FBS are indicated with # (###=$P < 0.001$). B) Table showing the cell numbers (numeric data) along culture period within each condition. Significant differences were found only for CPL condition; different letters indicate statistically significant differences between time-points ($P < 0.001$).

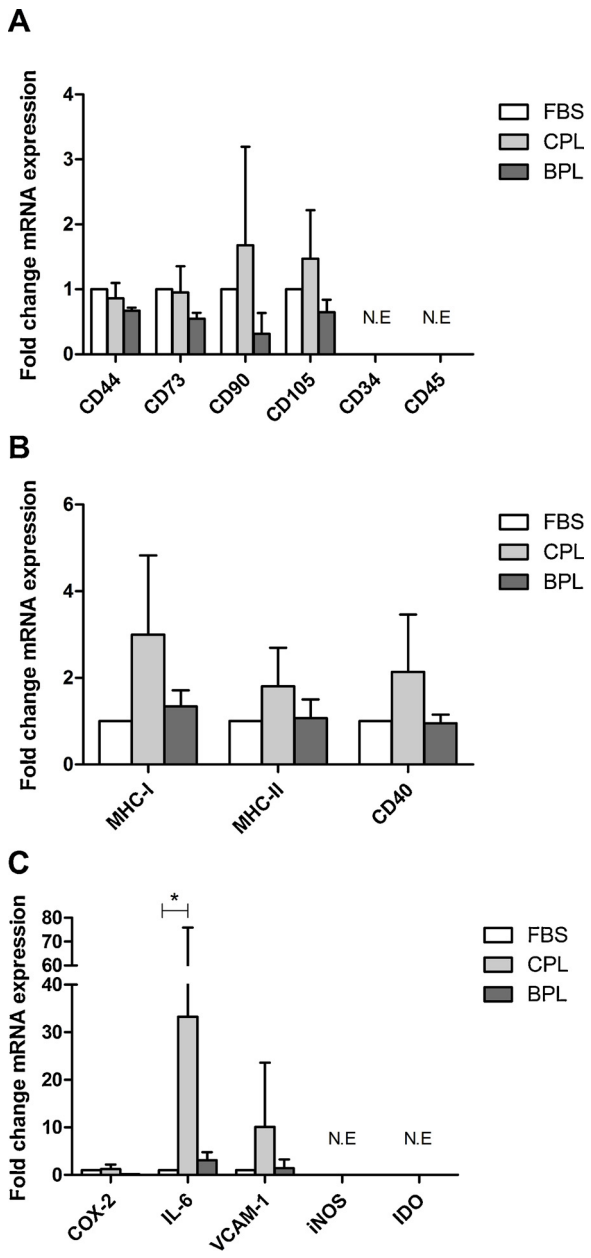


Fig. 3. Equine bone marrow derived mesenchymal stem cells (eBM-MSCs) gene expression studied by real time quantitative polymerase chain reaction (RT-qPCR) under the three culture conditions: fetal bovine serum (FBS), concentrated platelet lysate (CPL) and basal platelet lysate (BPL). Gene expression of all genes is shown as mean ± SD ($n = 3$) fold change regarding standard condition (FBS). (A) Expression of genes coding for cell surface markers related with MSC phenotype characterization. (B) Expression of genes coding for immunogenic-related molecules. (C) Expression of genes coding for immunomodulatory-related molecules. *= $P < 0.05$; N.E = no expression.

3.5. Gene expression profile of immunogenic and immunomodulatory markers

The eBM-MSCs P_3 cultured with CPL showed slightly increased (non-significant) gene expression of the immunogenic markers MHC-I, MHC-II and CD40 compared to control (FBS), whereas the cells cultured with BPL showed expression of these markers similar to control (FBS) cells (Fig. 3 B). The cells cultured with CPL showed overexpression of genes coding for the immunomodulatory molecules IL-6 ($P < 0.05$ compared to FBS) and VCAM-1 (non-significant), whereas COX-2 expression was similar among the three conditions. Gene expression of iNOS and IDO was not detected under any of the three culture conditions (Fig. 3 C).

4. Discussion

In this study, three main findings were observed. When analyzing the growth kinetics of eBM-MSCs with different supplements, a higher proliferative capacity was shown when using CPL compared to BPL and FBS by MTT assay, similarly to that previously reported (Horn et al., 2010; Hemeda et al., 2014; Russell and Koch, 2016). Secondly, the use of CPL for cryopreservation provided cell viability similar to FBS at the three time-points assessed, suggesting that it is a good candidate for reducing the xeno-contamination also during MSC banking and being this study the first evaluating MSC viability post-cryopreservation with PL in the horse. Third, when the gene expression profile was explored, a similar expression of surface markers was observed under the three culture conditions, but when evaluating genes linked to immunogenicity and immunomodulation, some differences could be observed between CPL and traditional supplement (FBS), including significant upregulation of the gene coding for IL-6. The profile of these markers in eBM-MSCs cultured in standard conditions (FBS) was similar to previous reports (Remacha et al., 2015; Barrachina et al., 2016). However, there are no previous reports evaluating the gene expression of phenotype characterization, immunogenic and immunomodulatory profile markers in equine MSCs cultured with allogeneic PL.

In this study, platelets were concentrated 3.3 fold more than the basal concentration, reaching 591 × 10^3 platelets/μL for the CPL. Other authors assessing the use of CPL reported even higher concentrations of up to 1000 × 10^3 platelets/μL in horse (Seo et al., 2013; Russell and Koch, 2016), dog (Russell et al., 2015) and human (Iudicone et al., 2014). The difference between the amount of platelets that we obtained and previous reports could be explained by different methodologies used to obtain PL. Despite of this fact, this lower platelet concentration was enough to promote MSC proliferation following an easy and economic preparation method. Moreover, there is evidence that not only the amount of platelets is important, but also the concentration of certain growth factors, the percentage of PL that is added to the culture media or the age of the PL donor may affect the cell culture as described in human species (Lohmann et al., 2012).

Cells cultured with CPL provided rather faster expansion capacity than BPL based on the shorter DTs observed along passages and the growing kinetics reflected by the MTT assay, thus indicating that CPL is a superior supplement than BPL for *ex vivo* propagation of eBM-MSCs. This different potential may be due to the higher concentration of growth factors achieved when concentrating platelets, such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) or transforming growth factor-beta1 (TGF-β1) (Iudicone et al., 2014; Russell and Koch, 2016). However, this hypothesis was not tested in this work by measuring growth factors. Regarding the percentage of PL added to the culture media, we used a concentration of 20% (V/V) of CPL or BPL according to the concentration ranges previously described for equine MSCs culture (Horn et al., 2010; Russell and Koch, 2016). In addition, and based on previous unpublished data of our group, this concentration allowed the comparison between both supplements since lower percentages of BPL do not promote consistent MSC growth. It has been described that increasing concentrations (up to 20%) of both PL and FBS can increase MSC proliferation (Horn et al., 2010). However, we chose not to increase FBS concentration but to keep it at 10% to allow straight comparison with standard culture condition. This could have influenced the higher proliferation observer under CPL (20%) compared to FBS (10%) in the MTT assay. Nevertheless, based on our results, we could hypothesize that 10% of CPL might be sufficient to promote eBM-MSC proliferation. In addition, 2 IU/mL heparin were added to avoid clotting of the PL as other reports (Seo et al., 2013; Naskou et al., 2018). The heparin frequently used range is 0.6–2 IU/mL, since lower concentrations do not avoid PL gelification and higher than

2 IU/mL may negatively affect *in vitro* proliferation (Hemeda et al., 2013; Burnouf et al., 2016). Thus, providing that heparin can influence cell proliferation, the same concentration was also added to the FBS media.

Cell viability was evaluated post-cryopreservation with equine PL during different times and no differences were found between the use of CPL and FBS, indicating that its replacement would be viable, not only for culture but also for cryopreservation. There is only one recent report of Naskou et al. (2018) which mentions the use of equine PL and DMSO (90% and 10%, respectively) as freezing medium for the cryopreservation of eBM-MSCs. However, it is not mentioned the effect on the viability of cryopreserved cells after cryopreservation. On the other hand, the use of human allogeneic PL has been shown to have similar cryoprotective effect than FBS in terms of cell viability of human MSCs (Wang et al., 2017). Finally, in both human and domestic animals, limited reports exist about the effect of PL as cryopreservant on the properties of MSCs, therefore it seems important to continue studying this subject to develop xeno-free cryopreservation for MSC banking.

When evaluating the expression of genes coding for eBM-MSC phenotype associated-markers (Ranera et al., 2011), it was observed that cells cultured under both PL conditions presented similar expression profile between them and compared to the standard condition. Studies on human MSCs have also shown similar immunophenotype profile between cells cultured with FBS or PL (Horn et al., 2010; Hemeda et al., 2014; Iudicone et al., 2014), but there are only few descriptions of the phenotype of equine MSCs cultured with PL. Recently, Naskou et al. (2018) observed a significantly higher percentage of CD90 and CD45 positive equine MSCs cultured with FBS compared to PL culture. In contrast, we observed similar CD90 expression in cells cultured under the three culture media supplementation and did not find CD45 expression under any condition. These differences could be explained by the use of different techniques for phenotype assessment (surface protein expression *vs* gene expression). The gene expression of these markers has been previously used by other authors for eBM-MSCs characterization (Remacha et al., 2015; Barrachina et al., 2016).

When evaluating gene expression of molecules related to allorecognition such as MHC-I, MHC-II and CD40, we found a slightly increased expression (non-significant) of the three of them under CPL culture compared to the other conditions. Other authors have seen a similar profile in MSCs after receiving a pro-inflammatory stimulus (Barrachina et al., 2016; Berglund et al., 2017).

Furthermore, we evaluated for the first time the expression profile of genes coding for molecules closely related to eBM-MSCs immune properties cultured with allogeneic PL. Provided that RT-qPCR is an acknowledged approach and a well validated technique that has been widely used to study immune properties of equine MSCs because of its feasibility (Ranera et al., 2011; Remacha et al., 2015), we considered relevant to assess how culture with allogeneic PL may influence eBM-MSC gene expression profile of related markers. Cells in CPL condition showed increased gene expression of VCAM-1 (non-significant) and IL-6 (significant) and, as aforementioned, also a slight non-significant increase of immunogenic markers. Non-significant results must be cautiously interpreted, but these findings suggests a similar profile shown by cells cultured under pro-inflammatory conditions, such as cytokines stimulation (Ren et al., 2010; Barrachina et al., 2016; Berglund et al., 2017; Cassano et al., 2018a), activation of TLR-4 pathway or pro-inflammatory macrophages exposure (Cassano et al., 2018b). Pro-inflammatory *in vitro* stimulation of eBM-MSCs with tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) resulted in an upregulation of MHC-II, IL-6 and VCAM-1 (Barrachina et al., 2016, 2017), similar to our CPL condition profile. On the other hand, these studies also described an upregulation of COX-2, IDO and iNOS, whereas we did not find changes for COX-2 expression among the three conditions and IDO and iNOS expression was not induced.

Therefore, the upregulation of these immunogenic and immunomodulatory genes might be related with the pro-inflammatory priming of equine MSCs. We hypothesize that these findings in the immune profile of eBM-MSCs might be due to the presence of certain cytokines from residual leukocytes in the PL. Furthermore, the alpha-granules of platelets contain growth factors needed to promote the proliferation of MSCs, but can also carry proinflammatory cytokines such as IL-8 (Burnouf et al., 2016). Therefore, the presence of bioactive molecules in the CPL could explain why the eBM-MSCs cultured with CPL showed an inflammatory stimulation-like profile. On the other hand, iNOS and IDO are not expressed by MSCs in basal conditions but induced by inflammatory stimuli (Ren et al., 2010; Barrachina et al., 2016, 2017; Cassano et al., 2018a). The lack of expression induction of these genes might indicate low inflammatory level of the CPL, not enough to significantly upregulate several immune-related markers. This hypothesis was not tested in the present study as we did not measure in the PL potentially implied mediators, but warrants further investigation to thoroughly assess its composition and how it may influence the properties of equine MSCs. Since MSCs can respond to their environment, the possibility of priming these cells to increase their immunomodulatory potential (Szabó et al., 2015; Barrachina et al., 2017, 2018; Cassano et al., 2018b) could be an additional advantage of the CPL as culture supplement.

5. Conclusion

The use of allogenic pooled concentrated PL as culture media supplement instead of FBS was suitable for the *ex vivo* propagation of eBM-MSCs during successive passages with similar growth and phenotype characteristics, as well as for cryopreservation. The cells cultured with CPL slightly increased the expression of immunogenic genes and significantly upregulated IL-6 as immunomodulatory marker. This study provides, for the first time, information about the effect of replacement of FBS by PL as cell culture supplement on the immune profile of eBM-MSCs. Further studies are warranted to gain deeper knowledge into the effect of PL on equine MSC immune properties and to develop xeno-free media, provided their key role in the effectiveness and safety of cellular therapies.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of paper.

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