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Autologous point-of-care cellular therapies variably induce equine mesenchymal stem cell migration, proliferation and cytokine expression

A. KOL, N. J. WALKER, L. D. GALUPPO†, K. C. CLARK, S. BUERCHLER†, A. BERNANKE and D. L. BORJESSON*

Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, USA
†Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, USA.

*Correspondence email: dlborjesson@ucdavis.edu; Received: 21.03.12; Accepted: 25.05.12

Summary

Reasons for performing study: Autologous cellular therapy products including adipose-derived stromal vascular fraction (SVF), bone marrow mononuclear cells (BMMNs), cord blood mononuclear cells (CBMNs) and platelet rich plasma are options for treatment of acute orthopaedic lesions while mesenchymal stem cells (MSCs) are culture expanded. These products may contribute to healing by secreting matrix proteins or growth factors, but they may also act on endogenous MSCs to facilitate healing.
Objectives: To determine the effects of cell therapy products on MSCs function *in vitro*. The hypothesis was that cell therapy products promote MSCs functions including proliferation, migration and mediator release.
Methods: Fat, bone marrow (BM), cord blood and platelets were obtained from 6 Quarter Horses. The BM-MSCs and their autologous cell therapy products were co-incubated in transwells. Mesenchymal stem cells proliferation, migration, gene expression and cytokine concentrations were determined.
Results: All cell therapy products increased MSCs proliferation, but SVF induced significantly more proliferation than any other product. Also SVF elicited more MSCs chemotaxis and, along with BMMNs, significantly more MSCs chemoinvasion. Cord blood mononuclear cells stimulated MSCs to produce high concentrations of interleukin-6 (IL-6), transforming growth factor-β1 (TGF-β1), and prostaglandin E_2 (PGE_2). Stromal vascular fraction and platelet lysate did not stimulate MSCs but SVF and platelet lysate themselves contained high concentrations of PGE_2 and IL-6 (SVF) and TGF-β1 (platelet lysate).
Conclusions: Autologous cell products variably stimulate MSCs functions with 2 primary patterns apparent. Products either contained preformed mediators that may have intrinsic healing function, or products stimulated MSCs to secrete mediators.
Potential relevance: The specific clinical indications for these products may differ to include administration as a sole treatment modality prior to MSCs injection for intrinsic cell and cytokine activity (i.e. SVF) or administration concurrently with MSCs to activate MSCs for treatment of chronic lesions (i.e. CBMNs).

Keywords: horse; bone marrow mononuclear cells; chemotaxis; cord blood mononuclear cells; cytokines; mesenchymal stem cells; platelet lysate; stromal vascular fraction

Abbreviations

BM: Bone marrow
BMMNs: Bone marrow mononuclear cells
CBMNs: Cord blood mononuclear cells
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal bovine serum
IL-6: Interleukin 6
MRC: Mixed regenerative cell concentrate
MSCs: Mesenchymal stem cells
PGE_2: Prostaglandin E_2
PL: Platelet lysate
PDGF: Platelet-derived growth factor
PRP: Platelet-rich plasma
SVF: Stromal vascular fraction
TGF-β1: Transforming growth factor beta 1
TNF-α: Tumour necrosis factor alpha
UCD: University of California, Davis
VEGF: Vascular endothelial growth factor

Introduction

Equine mesenchymal stem cells (MSCs) expanded from the horse's own tissues are increasingly being used for bone, tendon, ligament and cartilage repair in veterinary medicine [1]. However, autologous MSCs cannot be administered in the acute phase of disease as MSCs expansion takes 2–3 weeks. Early treatment may facilitate healing as long as peracute cellular apoptosis and natural killer cell activity have declined [2,3].

Early treatment options include allogeneic MSCs or autologous mixed regenerative cell concentrate (MRC) products that can be produced within hours of sample collection. Mixed regenerative cell concentrate products currently include adipose-derived stromal vascular fraction (SVF), bone marrow mononuclear cells (BMMNs), cord blood mononuclear cells (CBMNs) and platelet-rich plasma (PRP). Mixed regenerative cell concentrate products may contribute scaffolding proteins, growth factors, cells and cytokines to the healing process. Stromal vascular fraction, CBMNs and BMMNs contain a variable number of MSCs, endothelial progenitor cells, monocytes/macrophages, haematopoietic stem and progenitor cells, and other haemic and stromal cells. Mixed regenerative cell concentrate products are minimally manipulated and can be produced near patient-side (BMMNs, PRP and SVF) or thawed from storage for near immediate administration as either sole treatment modalities or in combination with other therapies (for example PRP can be mixed with MSCs or PRP can be mixed with BMMNs) [4]. As they are autologous and minimally manipulated, MRC products pose no risk of an immune response and receive less stringent regulation by the Federal Drug Administration. Stromal vascular fraction has been used in dogs and horses to treat lameness due to osteoarthritis [5–7]; allogeneic PRP has proven useful in a critical-size bone defect in rabbits [8]; and both autologous PRP and BMMNs have been proposed as a patient-side treatment for equine tendonitis [9–11].

Early treatment with autologous MRC products may facilitate healing by acting on and recruiting endogeneous MSCs. Local, resident MSCs have been described in nearly every tissue examined [12–14]. Recent data suggest that MSCs arise from pericytes and may proliferate in response to local injury [14,15]. Mesenchymal stem cells have a profile of bioactive trophic factors, inflammatory mediators and adhesion molecules that function to decrease inflammation, inhibit scar formation, inhibit apoptosis and increase angiogenesis [15]. Mesenchymal stem cells need to be activated to perform these functions [15,16]. Factors known to activate MSCs include tumour necrosis factor α (TNF-α) and interferon gamma (IFN-γ) among others [17,18]. Factors known to be secreted by MSCs in

response to activation include interleukin-6 (IL-6), prostaglandin E_2 (PGE_2) and transforming growth factor β1 (TGF-β1) [1,15,19]. The addition of MRC products (with their heterogeneous cellular and bioactive factors) may be one method to stimulate local MSCs function.

For this study, we developed an *in vitro* model to mimic how MRC products may alter MSCs function. The objective was to compare 4 MRC products for their efficacy in stimulating autologous MSCs migration, proliferation and immunomodulatory factor gene expression and secretion *in vitro*. We hypothesised that MRC products would promote MSCs functions including proliferation, migration and mediator release.

Materials and methods

Horses and sample collection

Seven healthy 1–2-year-old Quarter Horses were used in this study. All tissues (fat, BM, cord blood and platelets) were available from 5 horses (matched samples). Only platelets and cord blood were available from one horse and only fat and BM were available from another horse, therefore, there was an n = 6 for each MRC product. All horses were housed at the Center for Equine Health at the University of California, Davis (UCD). Fat for SVF preparation, BM for mononuclear cell isolation, and whole blood for platelet lysate (PL) preparation were collected from March to July 2010 according to an approved institutional animal care and use protocol.

Cell isolation and processing

Subcutaneous adipose tissue (6–15 g) and heparinised BM (100 ml) were collected from the tailhead and sternum or *tuber coxae*, of each horse, respectively and were processed exactly as previously described and used immediately [19]. Cord blood had been previously collected, processed and stored from these horses at foaling [20]. Bone marrow for MSCs isolation had been collected, expanded and frozen the year prior to the study exactly as previously described [19]. Characterisation of equine MSCs was carried out by our laboratory prior to the commencement of this study [21–23].

Platelet lysate: A 60 ml sample of whole blood was collected via jugular venipuncture and placed into tubes containing acid citrate dextrose. After sedimentation of red blood cells, the plasma was transferred into polypropylene round-bottomed tubes and pelleted (420 ***g***, 15 min). The supernatant (platelet-poor plasma) was removed. The platelet pellets were resuspended to 6 ml using platelet-poor plasma. Platelets were depleted of white blood cells and red blood cells by centrifugation (100 ***g***, 15 min, slow deceleration). Platelets were adjusted to a final concentration of 500–600 × 10^9/l using plasma. To generate PL, 3 u/ml bovine thrombin[a] was added and permitted to incubate at 37°C for 1 h after which the tube was centrifuged (1800 ***g***, 8 min) and the supernatant (PL) plated in experimental wells.

Overview of study design

Bone marrow MSCs and their autologous MRC products were co-incubated in 24-well plates with transwell inserts. Complete culture media (Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution) was used as a control. For each experimental well, MRC products were added as follows: BMMNs: 1 × 10^7 cells/well; SVF: 5 × 10^5 cells/well; CBMNs: 3 × 10^6 cells/well; PL derived from 150 × 10^6 platelets. The number of cells used for each MRC product assay was based on average cell recovery from a typical BM collection, cord blood collection, SVF administration or platelet recovery (from the standard 60 ml of blood used to produce autologous PRP) to mimic current preparation and therapeutic use of these products at the Regenerative Medicine Laboratory, UCD.

MSCs proliferation

To determine the effect of mediators secreted by MRC products on equine MSCs proliferation, MSCs were plated at 1 × 10^4 cells/well and a 0.4 μm-pore transwell insert (Costar)[b] was added to each well (except for PL). Each MRC product (resuspended in 250 μl of complete culture media) was added on top of each insert. Platelet lysate was added to the well directly. Control wells were prepared identically except an additional 250 μl aliquot of complete culture media was added to each well. Plates were incubated at 37°C for 72 h, after which cell proliferation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation assay[c] per manufacturer's instructions [24]. Colour changes were measured on a microplate reader (Synergy HT Multi-Mode microplate reader with Gen5 software)[d]. A calibration curve was run in triplicate for each MSCs line and each experiment. Data were normalised and presented as the fold increase in MSCs proliferation compared with MSCs in complete culture media alone with no added MRC products.

MSCs chemotaxis and chemoinvasion

Chemotaxis assay: Preliminary data demonstrated that maximal protein secretion by MRC products or MSCs *in vitro* generally occurs 48–72 h after plating (after which mediators become adequately concentrated for measurement creating 'conditioned media'). As such, MRC products were plated for 48 h at 37°C to permit media conditioning. At 48 h, a 8.0 μm transwell insert[b] was added and 2 × 10^5 MSCs in 100 μl of media were placed on each insert and incubated at 37°C. MSCs migration towards the MRC products was measured at 24 h. A well with MSCs migrating towards fresh complete culture media (with 10% FBS) served as a positive control. To measure MSCs migration through the filter, inserts were rinsed twice and placed in wells containing 350 μl of 2 μg/ml calcein AM (labels MSCs)[e] in 1x cell dissociation solution[f] and incubated at 37°C for 1 h. At 1 h, all MSCs previously adhered to the bottom of the inserts had disassociated. Inserts were removed and the plates that contained the labelled MSCs were read immediately (Synergy HT plate reader)[d]. A calibration curve was run in triplicate for each MSCs line and each experiment.

Chemoinvasion assay: These assays were run in parallel and exactly as described for the chemotaxis assays except that a 8.0 μm pore matrigel coated transwell insert (BD Matrigel invasion chambers)[g] replaced the Costar transwell inserts used for chemotaxis. Data were normalised and presented as fold increase in chemotaxis and chemoinvasion compared with MSCs migration towards media alone.

Enzyme-linked immunosorbent assays

At 72 h, the transwell insert was removed and the fluid supernatant from each well was aspirated and centrifuged at 300 *g* × 10 min to pellet any contaminating cells. The supernatant was frozen at -80°C until analysis. Complete culture media controls were prepared and run in parallel to experimental samples. All enzyme-linked immunosorbent assay (ELISA) wells were run in duplicate. Factors measured included transforming growth factor-β1 (TGF-β1)[h] [25], interleukin-6 (IL-6)[h] [26], prostaglandin E_2 (PGE_2)[h] [27], and TNF-α (Equine TNFα Screening Set)[i] [28]. The ELISAs were completed per manufacturer instructions and were read spectrophotometrically on a microplate reader (Synergy HT Multi-Mode)[d] with Gen5 software[d].

Real-time polymerase chain reaction

At 72 h, assay supernatant was removed (for ELISAs) and the MSCs were washed twice with DPBS (with Ca^{2+} and Mg^{2+}) and then lysed with total RNA lysis solution[j] and the lysate was stored at -20°C. RNA was extracted using the ABI 6100 PrepStation (ABI Prism 6100 Nucleic Acid PrepStation, Applied Biosystems) according to manufacturer's instructions. The cDNA was synthesised using the Qiagen Quantitect kit[k] and TaqMan analysis was done using the low density array format exactly as described previously [29] at the Real-Time PCR Research and Diagnostics Core Facility, UCD. The equine Real Time Taqman PCR assays used for IL-6 (AF005227), TGF-β (X99438), TNF-α (EU438779) and GAPDH (AF097179; housekeeping gene) were designed using AB Primer Express 3 with the sequences from the listed Genebank accession numbers. The samples were amplified in an automated fluorometer (7900 HT FAST Real Time PCR System)[l]. Changes were depicted as fold change in gene expression compared with a control well (MSCs in complete culture media with no MRC product added).

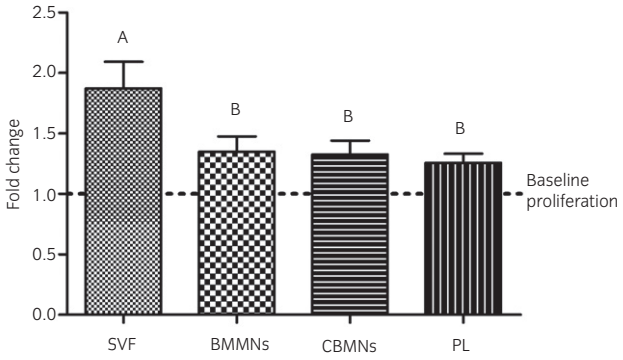

Fig 1: Fold change in MSCs proliferation in response to SVF, CBMNs, BMMNs and PL. Baseline MSCs proliferation in complete culture media is depicted with a dashed line. Bars indicate the mean ± s.e. All MRC products significantly enhanced MSCs proliferation compared with baseline (P<0.05). Significant differences between MRC products are indicated by A/B (n = 6).

Data analyses

Statistical analyses were performed using a mixed-model ANOVA (SAS Statistical Software)[m] with a Tukey-Kramer *post hoc* test. ELISA data were log-transformed prior to analysis. Data were tested for normality using a Shapiro-Wilk test. For pair-wise comparisons, a paired *t* test was used. A value of P<0.05 was considered significant for all analyses.

Results

MSCs proliferation in response to MRC products

All MRC products significantly increased MSCs proliferation (~1.3-fold increase) compared with baseline MSCs proliferation in complete culture media (P<0.05 for all comparisons, Fig 1). However, SVF induced significantly more MSCs proliferation compared with CBMNs, BMMNs and PL (P = 0.008, Fig 1).

MSCs chemotaxis and chemoinvasion in response to MRC products

While MRC products had no significant effect on MSCs chemotaxis, SVF consistently induced the strongest MSCs chemotactic response (5/6 horses, Fig 2a), although this response was not statistically significant. Due to high variation between horse samples, there was no statistical difference between the 4 MRC products in their ability to elicit MSCs chemotaxis. Both SVF and BMMNs significantly increased MSCs chemoinvasion compared with PL and CBMNs (P<0.005, Fig 2b).

MSCs gene transcription and mediator secretion

TNF-α: TNF-α was not detected when MSCs or any MRC product were incubated alone or after co-incubation of MSCs and any MRC product (data not shown). Mesenchymal stem cells did not upregulate TNF-α gene transcription in any condition (data not shown).

IL-6: IL-6 was not detected when MSCs were incubated alone (Fig 3a), although it was detected when SVF was incubated alone (Fig 3a). The concentration of IL-6 detected in cultures of SVF alone was significantly greater than all other MRC products where IL-6 was not detected (P = 0.002, Fig 3a). The co-incubation of MSCs with SVF resulted in detectable IL-6 levels however the amount produced was not statistically greater than that produced by SVF alone (Fig 3a). Although neither MSCs nor CBMNs produced IL-6 on their own, co-incubation of CBMNs with MSCs markedly induced IL-6 secretion (P<0.001, Fig 3a).

Upregulation of MSCs IL-6 gene transcription was highly variable. After MSCs-SVF co-incubation, IL-6 transcripts were markedly increased in 2/6 horses (Fig 3b), although this upregulation was inconsistent. The MSCs–CBMNs co-incubation caused a marked upregulation (P = 0.006) of MSCs IL-6 gene transcription (in 6/6 horses, Fig 3b) suggesting that CBMNs stimulated MSCs. The absence of IL-6 secretion after MSCs co-incubation with PL or BMMNs was confirmed by the absence of MSCs gene transcription for IL-6 under these conditions (Fig 3b).

TGF-β: MSCs alone produced TGF-β (average = 181 ng/l, range = 0–1000 ng/l, Fig 4a). Platelet lysate was the only MRC product that contained abundant TGF-β (up to 11,000 ng/l). The concentration of TGF-β in PL was significantly higher than that of BMMNs, CBMNs and SVF (P<0.002, Fig 4a). The concentration of TGF-β was highest in the MSCs–PL co-incubation, but the concentration was not higher than with PL alone (Fig 4a). Given that MSCs did not significantly upregulate TGF-β gene transcription above baseline after incubation with PL (Fig 4b), TGF-β secretion was considered to be primarily of PL origin. Co-incubation of MSCs with CBMNs resulted in a TGF-β concentration that was significantly higher than that of BMMNs and SVF (P<0.001, Fig 4a). However, similar to PL, co-incubation did not result in significant increase in TGF-β secretion by ELISA (Fig 4a) or upregulation of MSCs gene expression (Fig 4b) compared with production by CBMNs cells alone. As such, we concluded that PL and CBMNs both produce TGF-β but do not enhance the production of TGF-β by MSCs.

The co-incubation of MSCs with MRC products did not result in a significant increase in MSCs TGF-β gene transcription for any condition (Fig 4b). Mesenchymal stem cells constitutively produce TGF-β, although this production is not augmented after incubation with MRC products.

PGE_2: MSCs alone produced some PGE_2 (average = 247 ng/l, range = 75–400 ng/l, Fig 5). The SVF was the only MRC product that secreted a

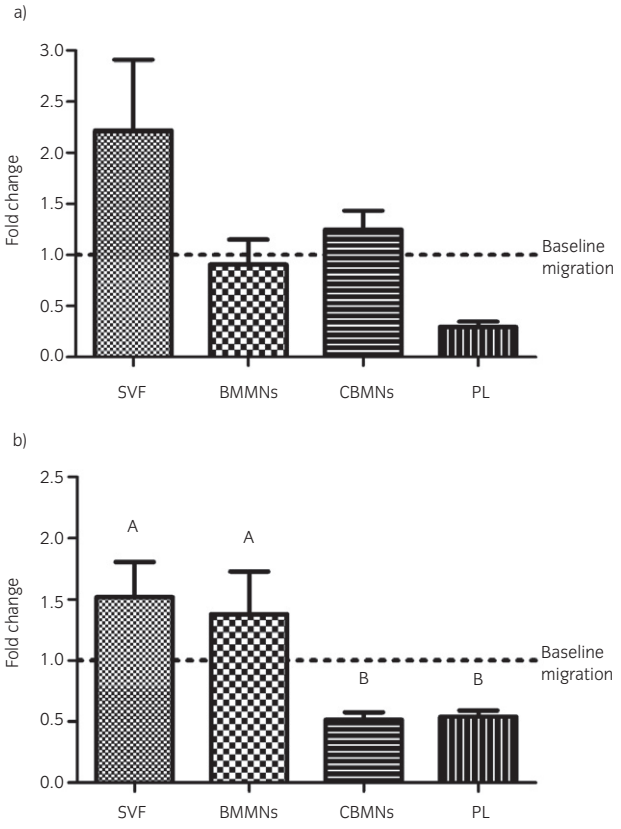

Fig 2: Fold change in MSCs migration: (a) chemotaxis and (b) chemoinvasion, in response to SVF, BMMNs, CBMNs and PL. Baseline MSCs migration towards depleted media is depicted with a dashed line. Bars indicate the mean ± s.e. Significant differences between MRC products are indicated by A/B (n = 6).

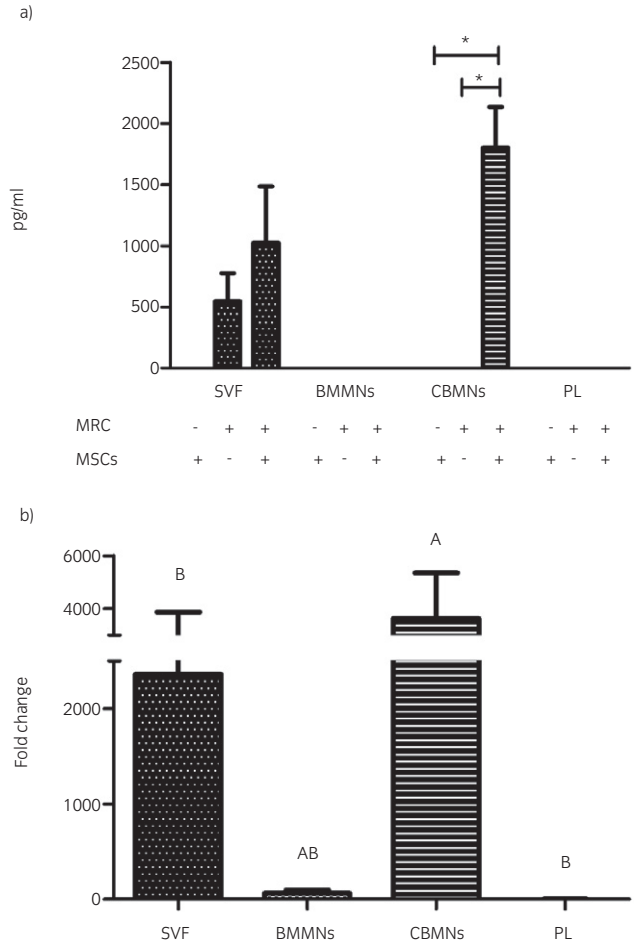

Fig 3: Interleukin 6 secretion and gene expression. a) IL-6 protein secretion *in vitro* by unstimulated MSCs alone (first column for each MRC product), MRC products alone (second column for each MRC product) and by MSCs and MRCs after co-incubation (third column for each MRC product). Significant differences between MRC products are indicated by a letter (A/B). Significant differences within groups are indicated by * (n = 6). b) MSCs IL-6 gene expression after co-incubation with SVF, BMMNs, CBMNs, and PL. Data are represented as fold change above baseline gene expression (MSCs grown in complete culture media). Bars indicate the mean ± s.e.

significant amount of PGE_2 (average = 6500 ng/l) and this concentration was significantly higher than all other MRC products (P<0.001, all comparisons, Fig 5). Mesenchymal stem cells co-incubated with CBMNs resulted in the greatest production of PGE_2. The concentration produced was significantly higher than that produced by MSCs after incubation with BMMNs, SVF and PL (P<0.005, all comparisons, Fig 5). The concentration of PGE_2 was also increased in the SVF–MSCs co-incubation, but was not increased over the concentration produced by SVF alone. Co-incubation of MSCs with BMMNs and PL mildly increased MSCs secretion of PGE_2 (~1500 ng/l, Fig 5).

Discussion

Mixed regenerative cell products clearly enhanced MSCs proliferation and variably induced MSCs migration, mediator secretion and gene expression. Our novel finding is that soluble factors released from adipose-derived SVF induced significantly more MSCs proliferation and migration than any other MRC product, including PL. It is not known if SVF would alter the differentiation capacity of equine MSCs. Given its potent stimulatory effect on MSCs, this avenue may be worth pursuing for tissue regeneration applications, as SVF did not have any negative effects on MSCs function or viability. As such, further *in vivo* studies looking at the administration of SVF as a sole, early therapy, while awaiting MSCs expansion, or mixing SVF with MSCs prior to injection as a combination therapy may be useful to augment MSCs function.

Administration of MRC products may have local, positive effects on endogenous MSCs function and tissue healing. Mixed regenerative cell concentrate products clearly enhanced MSCs proliferation, although the role of MRC products on MSCs migration was less clear. To date, there are no data on the mechanisms of equine MSCs migration and recruitment to sites of injury. Mesenchymal stem cells migration is dependent on local environmental signals that upregulate chemokine receptors. Chemotaxis and/or chemoinvasion of human MSCs can be induced by TNF-α [18], vascular endothelial growth factor-A (VEGF-A) [30,31], the CXCR4/CXCL12 axis [30], TGF-β1, platelet-derived growth factor (PDGF), and basic fibroblast growth factor [31,32]. Human and equine BM–MSCs express CXCR4 (D.L. Borjesson, unpublished data) and the CXCR4/CXCL12 axis is important for MSCs migration and recruitment from BM [33]. Stromal vascular fraction and BMMNs significantly induced MSCs chemoinvasion and SVF was the most potent inducer of chemotaxis, although not statistically significant, probably due to a large interhorse variation. We did

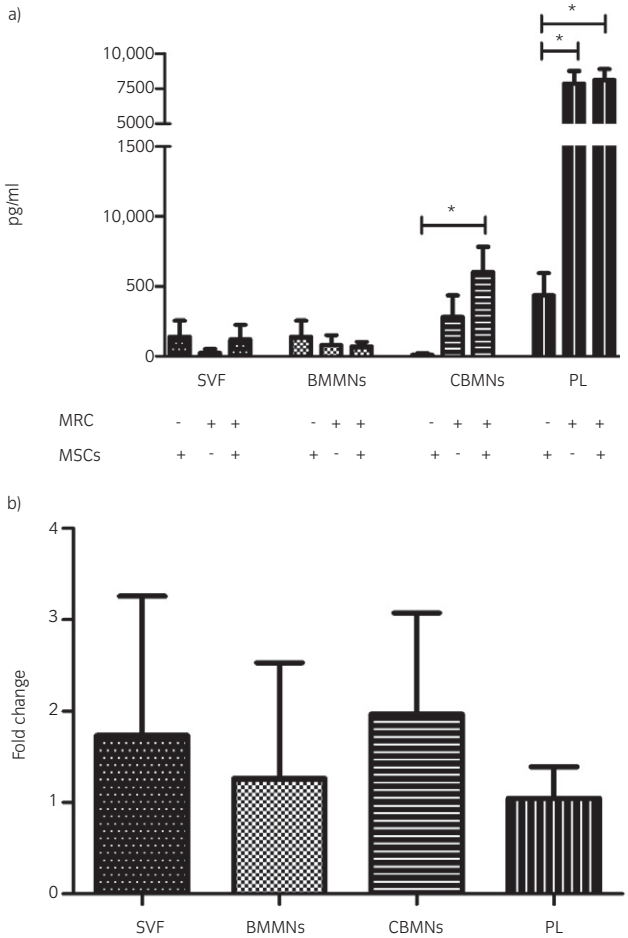

Fig 4: Transforming growth factor beta 1 secretion and gene expression. a) TGF-β1 protein secretion *in vitro* by unstimulated MSCs alone (first column for each MRC product), MRC products alone (second column for each MRC product) and by MSCs and MRCs after co-incubation (third column for each MRC product). Significant differences within groups are indicated by * (n = 6). b) MSCs TGF-β1 gene expression after co-incubation with SVF, BMMNs, CBMNs, and PL. Data are represented as fold change above baseline gene expression (MSCs grown in complete culture media). Bars indicate the mean ± s.e.

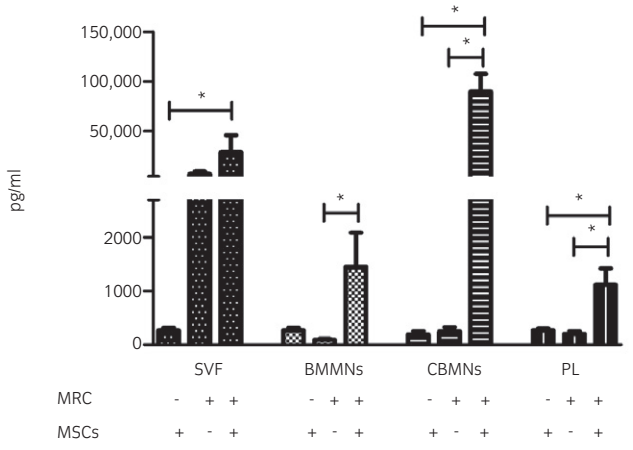

Fig 5: Prostaglandin E_2 secretion *in vitro* by unstimulated MSCs alone (first column for each MRC product), MRC products alone (second column for each MRC product) and by MSCs and MRCs after co-incubation (third column for each MRC product). Significant differences between MRC products are indicated by a letter (A/B). Significant differences within groups are indicated by * (n = 6). Bars indicate the mean ± s.e.

not specifically investigate the mediators responsible for induction of MSCs migration; however, there was no TNF-α present in our study and, in our PL samples, TGF-β1 and PDGF-BB were very high, although PL did not induce any MSCs migration. Similarly, the concentration of IL-6 was not connected to the success of MSCs migration. As such we speculate that the most likely candidate chemokines and growth factors that may be linked to equine MSCs migration include CXCR4/CXCL12, VEGF-A and/or basic fibroblast growth factor.

Interleukin-6 and PGE_2 are pleiotropic cytokines implicated as major players in immune modulation by MSCs [15]. In this study, IL-6 and PGE_2 were markedly induced in MSCs by CBMNs, whereas SVF was the only MRC product that contained measurable amounts of PGE_2 and IL-6. These findings may, in part, explain some of the immediate, anti-inflammatory benefits reported after SVF injection even in chronic osteoarthritis [5,6]. Our data suggest that SVF contains immunomodulatory mediators (IL-6 and PGE_2) but SVF does not specifically stimulate MSCs to produce these mediators.

Cord blood mononuclear cells induced gene transcription and mediator secretion by MSCs to a greater extent than any other MRC product. Cord blood mononuclear cells are an interesting and heterogeneous population of cells. Compared with peripheral blood mononuclear cells, CBMNs have increased gene expression for a number of transcription factors, signal transduction molecules and cytokines (including IL-6) [34]. When normalised for MSCs number, the addition of CBMNs to MSCs in this study resulted in comparable MSCs IL-6 secretion to that induced by activated T cells [19]. These data suggest that the addition of CBMNs to MSCs may be an alternative way to activate MSCs prior to injection into chronic lesions that may lack activating stimuli for MSCs. *In vivo* experiments are warranted to assess the differential effect of MRC products on the local environment in injured tissues.

Platelet lysate is currently being used as a substitute for FBS in MSCs manufacturing for clinical trials in human patients [35–39] and PRP has been evaluated as a therapeutic cellular product to treat wounds and orthopaedic disorders [38,40]. Similar to our findings, PL increased proliferation of human BM, fat and cord-tissue derived MSCs compared with baseline MSCs proliferation in complete culture media [35–39,41,42]. The platelet factors TGF-β1 and PDGF-AB have been implicated for this increased human BM–MSCs proliferative response [42]. Others have shown that incubation of PL with MSCs does not alter MSCs differentiation capacity [37–39] or immunosuppressive activity [37]. Our study confirmed that equine PL does contain abundant TGF-β1, and it has previously been demonstrated that equine PL contains PDGF-BB (D.L. Borjesson, unpublished data) [25,43]. In equine medicine, PRP has been shown to contain anabolic factors that support matrix production by equine tendon and ligament explants *ex vivo* [11,44,45]. However, PL does not appear to activate MSCs *in vitro* with resultant increases in protein secretion or gene transcription. These findings concur with an *in vivo* model of a critical size bone defect in rabbits where the combination of PRP and MSCs did not further enhance bone healing [8]. Thus, although PRP/PL may serve as a source of growth factors and MSCs scaffolding, unlike SVF and CBMNs, PL does not appear to activate MSCs or promote MSCs migration, protein secretion or gene transcription *in vitro*.

Authors' declaration of interests

No conflicts of interest have been declared.

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Manufacturers' addresses

[a]Sigma, St. Louis, Missouri, USA.
[b]Corning Inc., Corning, New York, USA.
[c]Promega Corporation, Madison, Wisconsin, USA.
[d]Biotek, Winooski, Vermont, USA.
[e]Invitrogen, Carlsbad, California, USA.
[f]Trevigen, Gaithersburg, Maryland, USA.
[g]Becton Dickinson, Franklin Lakes, New Jersey, USA
[h]R&D systems Inc., Minneapolis, Minnesota, USA
[i]Thermo Scientific, Rockford, Illinois, USA.
[j]Applied Biosystems, Foster City, California, USA.
[k]Qiagen, Valencia, California, USA.
[l]ABI, Carlsbad, California, USA.
[m]SAS Institute Inc., Cary, North Carolina, USA

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