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Comparison of allogeneic platelet lysate and fetal bovine serum for *in vitro* expansion of equine bone marrow-derived mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are promising candidates for cell-based therapy and tissue engineering approaches. Fetal bovine serum (FBS) is commonly used for *in vitro* MSC expansion; however, the use of FBS may be associated with ethical, scientific, and safety issues. This study aimed to compare the ability of allogeneic platelet lysate (PL) and FBS to cause equine bone marrow-derived MSC expansion. MSCs were isolated from bone marrow aspirate in media supplemented with either PL or FBS, and cell proliferation properties and characteristics were examined. There were no significant differences in MSC yield, colony-forming unit-fibroblast (CFU-F) assay, and population doubling time between PL and FBS cultures. In addition, both PL-MSCs and FBS-MSCs showed similar results in term of ALP staining, osteogenic differentiation, and RT-PCR, although there were subtle differences in morphology, growth pattern, and adhesive properties. These results suggest that PL is a suitable alternative to FBS for use in equine MSC expansion, without the problems related to FBS use.

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1. Introduction

Mesenchymal stem cells (MSCs) are undifferentiated cells with the ability of self-renewal and differentiation into several cell lineages, such as bone, cartilage, adipose tissue, muscle, and tendon (Pittenger et al., 1999; Spencer et al., 2011). Therefore, MSC-based cell therapies have been widely investigated and successfully applied for several years in equine and human medicine (Koch et al., 2009; Chanda et al., 2010). For most therapeutic purposes, MSCs are expanded *in vitro* before putting them to therapeutic use, because they are rare in their tissue of origin (Pittenger et al., 1999; Koch et al., 2009).

Fetal bovine serum (FBS) is the most commonly used supplement for *in vitro* MSC culture. It provides the MSCs with hormones, vital nutrients, attachment factors, and growth factors (van der Valk et al., 2004; Bieback et al., 2009). However, the use of FBS may involve ethical, scientific, and safety problems. FBS is harvested from bovine fetuses taken from slaughtered pregnant cows. The current methods of FBS harvesting may cause suffering to bovine fetuses (Jochems et al., 2002; van der Valk et al., 2004). The composition of FBS is undefined and varies between batches; therefore, its application in culture media may interfere with the reproducibility of experiments (Jochems et al., 2002). In addition, FBS is a potential source of xenogenic immune reactions and transmission of bovine pathogens such as viruses, bacteria, and prions (Jochems et al., 2002; Sundin et al., 2007). Recently, because of these problems, various supplements to replace FBS in cell culture have been tested. These include growth factors, plant constituents, autologous or allogeneic serum, plasma, and platelet derivatives (van der Valk et al., 2004; Tonti and Mannello, 2008; Bieback et al., 2009).

Platelets are known as a rich source of growth factors (GF), such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), which play many important roles in the tissue healing process (Blair and Flaumenhaft, 2009). Platelet concentrates (i.e., platelet rich plasma: PRP) have been widely used for wound healing and tissue regeneration in equine and human medicine (Sutter et al., 2004; Argüelles et al., 2008; Bosch et al., 2010; Lacci and Dardik, 2010). In recent years, platelet lysate (PL) has gained interest as a substitute for FBS in cell culture. It is derived from platelet concentrates by lysing the platelet bodies (Doucet et al., 2005; Bieback et al., 2009). In human medicine, many studies on the use of PL as a supplement for use in MSC culture have been conducted to solve the problems related to FBS use, and these studies showed that PL can replace FBS in MSC expansion. (Doucet et al., 2005; Bernardo et al., 2007; Bieback et al., 2009; Cholewa et al., 2011).

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Table 1
Primers used for gene specific RT-PCR.

Primer name	Primer sequence
Act	F-CTCTTCCAGCCCTCCTTCCT
	R-GTCCCACCCGACAGCACT
CD34	F-CAAACCAAAATCAAGGGAGAA
	R-AAGGAGCAAGGAGCACACC
Oct4	F-GTCCTCACTTCACTACGCTGT
	R-ACTTCACCTTCCCTCCAACC
Nanog	F-TACCTCAGCCTCCAGCAGAT
	R-CGTTCCCAGCAGTGTTCA
Sox2	F-TGGTTACCTCTTCCTCCCACT
	R-GGGCAGTGTGCCGTTAAT
Klf4	F-AAGAGGGGGAGAAGGTCAT
	R-CGGTGTGTTTGCGGTAGT

PL is also expected to be used as an FBS substitute in equine MSC expansion, considering the increasing use of MSCs in equine patients and the problems related to FBS. However, there are no studies comparing PL and FBS in terms of the ability to cause equine MSC expansion. Therefore, this study aimed to compare PL and FBS in terms of their abilities to cause equine bone marrow-derived MSC expansion.

2. Materials and methods

2.1. Sample population

Six healthy thoroughbred horses (three males and three females) aged 1.5–10 years (6.08 ± 4.31 years) and weighting 340–516 kg (470 ± 66 kg) were used in this study. Prior to the start of the experiment, the health of the animals was evaluated on the basis of physical examination, complete blood cell count, and blood chemical analysis findings. This experiment was approved by the Experimental Animal Committee of Obihiro University of Agriculture and Veterinary Medicine.

2.2. PL preparation

PL was prepared from the blood of four different horses by a two-step centrifugation. Whole blood was collected from the jugular vein into 60-ml syringes loaded with 6 ml of the anticoagulant, acid citrate dextrose A (ACD-A). The blood was centrifuged at 230 *g* for 10 min at 10 °C to separate platelet-containing plasma from red cells. The platelet-containing plasma was then centrifuged at 900 *g* for 15 min at 10 °C. The supernatant (platelet-poor plasma: PPP) was separated out, and the platelet pellets were re-suspended in PPP to a final concentration of 1×10^6 platelets/μl. The platelet suspension was frozen at −80 °C, subsequently thawed at 37 °C to release platelet-derived growth factors, and centrifuged at 1600 *g* for 30 min at 10 °C to remove platelet fragments. Next, the supernatant was filtered through 0.22 μl syringe driven filters (Millex-GV Filter Unit, Millipore Ireland Ltd., IRL) and stored at −30 °C.

2.3. MSC isolation and culture

Horses were placed in stocks and sedated by intravenous administration of 5 μg/kg medetomidine hydrochloride (Domitor, Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan). The sternum was aseptically exposed, and 10 ml of 2% lidocaine (Xylocaine, Astra Zeneca Japan, Osaka, Japan) was infiltrated into the subcutaneous tissue and muscle over the 4th and 5th sternebrae. Bone marrow was aspirated from the 4th or 5th sternebra through 8G, 10 cm bone marrow biopsy/aspiration needles (Jamshidi, Cardinal Health, Illinois, USA) into heparin-coated syringes (100 IU/ml of the bone marrow aspirate). The bone marrow aspirate was cultured (bone marrow aspirate:medium = 100 μl:10 ml) on 92 × 17 mm culture dishes (Nalge Nunc International, Tokyo, Japan) in tissue culture medium (TCM) containing Dulbecco's modified Eagle's medium (Sigma–Aldrich Japan, Tokyo, Japan), 50 U/ml penicillin–streptomycin (Sigma–Aldrich Japan, Tokyo, Japan), and 10% FBS (Biowest, Nuaille, France) (FBS-TCM) or 10% allogeneic PL (PL-TCM). To prevent *in vitro* gel formation, 2 IU/ml heparin was added to the PL-TCM. Cells were cultured in a humidified incubator at 37 °C under 5% CO_2 for 10 days. The medium was changed at 3, 6, and 8 days after initial plating. At 10 days, MSCs were dissociated from culture plates using 0.25% trypsin/2.21 mM EDTA-4Na solution (Mediatech, Herndon, USA) and counted using a hemocytometer.

Table 2
Summary data of mesenchymal stem cell (MSC) yield, colony-forming unit-fibroblast (CFU-F) assay, and population doubling time (PDT).

	MSC yield after 10 days (cell number)	CFU-F assay		PDT (h)
		Colony number	Colony size (mm)	
PL	$5.99 \pm 4.82 \times 10^5$	108.16 ± 60.08	2.25 ± 0.91	22.94 ± 2.33
FBS	$5.90 \pm 4.81 \times 10^5$	99.33 ± 60.49	2.78 ± 0.53	22.70 ± 1.44

Cell number indicates the number of MSCs isolated from 0.1 ml of bone marrow aspirate. Data are expressed as mean ± standard deviation ($n = 6$). There were no significant differences between the two groups.

2.4. Colony-forming unit-fibroblast (CFU-F) assay

For CFU-F assays, cells were cultured in 92 × 17 mm culture dishes with either FBS-TCM or PL-TCM. The medium was changed every 2–3 days and culture was stopped on day 10. The cell layer was fixed in methanol and stained with a Giemsa stain solution (Merck KGaA, Darmstadt, Germany). Colonies composed of at least 50 cells were counted and colony size measured using a graduated ruler. The mean colony size was calculated for 20 colonies chosen randomly.

2.5. Population doubling time (PDT)

To calculate PDT, 50,000 MSCs were plated in 92 × 17 mm culture dishes with either FBS-TCM or PL-TCM. The medium was changed every 2 days. The MSCs were harvested after 4 days of culture, and PDT was calculated using the following previously described formula (Vidal et al., 2006): $PDT = CT * \log 2 / (\log N_f - \log N_i)$, where CT is the cell culture time, N_f is the final number of cells, and N_i is the initial number of cells.

2.6. Alkaline phosphatase (ALP) staining

MSCs were plated at 3×10^4 cells/well on 12-well culture plates (Iwaki Glass Co. Ltd., Chiba, Japan) and cultured with either FBS-TCM or PL-TCM in a humidified incubator at 37 °C under 5% CO_2 for two days. The MSCs were then stained with ALP staining solution (NBT/BCIP stock solution, Roche Diagnostics Japan, Japan).

2.7. Osteogenic differentiation

MSCs were plated at 3×10^4 cells/well on 12-well culture plates and cultured with either FBS-TCM or PL-TCM in a humidified incubator at 37 °C under 5% CO_2 for two days. The medium was then replaced with osteogenic medium containing dexamethasone 10 nM, ascorbic acid 0.1 mM, and β-glycerophosphoric acid 10 mM. The