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Isolation of Peripheral Blood-Derived Mesenchymal Stem Cells in Mares and Foals

Ye-Eun Oh[†]
Eun-Bee Lee[†]
Jong-Pil Seo*

College of Veterinary Medicine and Veterinary Medical Research Institute, Jeju National University, Jeju 63243, Korea

Abstract Peripheral blood-derived mesenchymal stem cells (PB-MSCs) have shown promise in cell-based therapy, as they can be harvested with ease through minimally invasive procedures. This study aimed to isolate PB-MSCs from foals and mares and to compare the proliferation and cellular characteristics of the PB-MSCs between the two groups. Six pairs of mares and their foals were used in this study. MSCs were isolated from PB by direct plating in a tissue culture medium, and cell proliferation (population doubling time [PDT], and colony-forming unit-fibroblast assay [CFU-F]), and characterization (morphology, plastic adhesiveness, colony formation, trilineage differentiation) were examined. There was no significant difference in the PB-MSC yield, CFU-F, and PDT between the mares and foals. PB-MSCs from both mares and foals showed typical MSC characteristics in terms of spindle-shaped morphology, plastic adhesive properties, formation of colonies, trilineage differentiation. These results suggest that PB-MSCs isolated from horses, both adult horses, and foals, can be used for equine cell-based therapy.

Key words peripheral blood-derived mesenchymal stem cells, MSCs isolation, horse.

[†]Ye-Eun Oh and Eun-Bee Lee contributed equally to this work.

*Correspondence: jpseo@jejunu.ac.kr

ORCID
Ye-Eun Oh:
https://orcid.org/0009-0007-1310-9303
Eun-Bee Lee:
https://orcid.org/0000-0003-3654-5654
Jong-Pil Seo:
https://orcid.org/0000-0002-6418-9813

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Introduction

Mesenchymal stem cells (MSCs) originating from the mesoderm are undifferentiated cells with low immunogenicity and the capacity for self-renewal and are also multipluripotent (4,9,10). MSCs have been successfully used in cell-based therapy and tissue engineering in humans as well as animals due to their immunomodulatory and anti-inflammatory properties (2,13). Also, in horses, MSCs have therapeutic potential in musculoskeletal diseases, including tendinopathy, and, more specifically, cartilage damage (5,10,13).

The traditional sources of MSCs have been bone marrow (BM) and adipose tissue (AT) (8). MSCs can also be harvested from umbilical cord blood (UCB), the umbilical cord matrix (UCM), and peripheral blood (PB) (2). Other possible sources are muscle, the synovial membrane, and trabecular bone (7). However, invasive methods carry the possibility of serious complications, such as cardiac puncture, pneumopericardium, arrhythmia from BM aspiration, and cosmetic defects after the surgery to remove AT (8). Also, UCB and UCM-derived MSCs cannot be collected from adults and UCM cultures are at a high risk of contamination (2,11). In contrast, PB is easy to collect at any time and the collection procedure is much less invasive. Therefore, PB is considered an appealing alternative (2,7,8,11). In an *in vivo* study by Fu et al. (4) PB-MSCs repaired cartilage defects in a rabbit model. Also, PB-MSCs are being increasingly considered for cell-based therapy in equine diseases and have been extensively studied (2,3,5-8,11).

The number of MSCs retrieved from any kind of tissue is generally inadequate for therapeutic use. Therefore, it is necessary to increase their yield *in vitro* to reach sufficient numbers prior to use (1,9). It is proven that the telomere shortens after each cell division causing cell senescence (1,12). Furthermore, the life span of cells produced by in vitro culture is affected by environmental conditions, treatment methods, culture period, and cell culture passage (1,9). The correlation between the proliferation capacity of MSCs and the age of the cell donor is still a matter of debate (1,12). In one study, a negative correlation with age was demonstrated when human MSCs between two donor groups (aged 18-29 for the young adult group and aged 66-81 for the elderly group) were compared, wherein aging was found to be associated with a decreased proliferative capacity of MSCs (12). The negative correlation between age and cell proliferation was demonstrated in other cell types as well, such as human fibroblasts and human arterial smooth muscle cells (12).

This study aimed to isolate and culture MSCs from the PB of horses, and to compare the proliferation and cellular characteristics of PB-MSCs from foals to those from mares.

Materials and Methods

Sample population

Six mares and their six foals were used for this study. Two mares were Thoroughbred, two were Haflingers, and two were Thoroughbred crosses. All six foals were mixed breeds. The age range of the mares was 3-13 years (7.83 ± 3.66 years), and that of the foals was 5-6 months (5.67 ± 0.52 months). Three foals were fillies, and the other three were colts. This experiment was approved by the Animal Care and Use Committee of Jeju National University (approval number: 2018-0025).

Isolation and culture of mesenchymal stem cells (MSCs)

The blood of each horse was collected from the jugular vein using a 20-milliliter syringe and transferred to two 10 mL heparin tubes (BD Vacutainer®, BD, USA) immediately. Subsequent steps in the laboratory were followed within 4 hours of collecting the blood. The blood was centrifuged at 1,000 × g. The blood was centrifuged at 2,089 RPM for 20 minutes (Varispin 15R, Hanil Science Medical, Republic of Korea) at room temperature to separate the mononuclear layer. Two mL of the buffy coat with mononuclear cells was collected in a tube and mixed thoroughly. And then 600 μL from 2 mL of buffy coat was distributed into three 92 × 17 mm culture dishes filled with 10 mL of tissue culture medium (TCM). The TCM contained Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (Sigma-Aldrich®, USA), 10% fetal bovine serum (FBS), and 1% antibiotics (penicillin 10,000 IU/mL, streptomycin 10,000 μg/mL) in the ratio of 9:1:0.1. The cells were cultivated in a humidified incubator at 37°C under a 5% CO_2 atmosphere. The cell medium was first changed on the 14th day from the initial plating and every 3 days thereafter. On the 23rd day, a subculture was performed. For the subculture, whole MSCs were resuspended in 1 mL with TCM and distributed equally into five 92 × 17 mm culture dishes. MSCs were dissociated from culture plates with 0.5% trypsin and counted before the subculture. On day 29, the number of MSCs was counted and they were utilized for estimating the population doubling time (PDT), the colony-forming unit-fibroblast (CFU-F) assay, trilineage cell differentiation.

Population doubling time (PDT)

For evaluating the PDT, 5×10^4 MSCs per dish were plated in 92 × 17 mm culture dishes with TCM. The medium was changed on the 2nd day after the plating and MSCs were harvested on the 4th day. The PDT calculation formula was adopted from the study by Vidal et al. (14) as follows: PDT = CT

× log2/(log Nf – log Ni), where CT is the cell culture time, Nf is the final number of cells, and Ni is the initial number of cells.

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

For the CFU-F assay, 5×10^3 MSCs per dish were cultured in two 92 × 17 mm culture dishes with TCM. The medium was changed on the 3rd and 6th days, and the MSCs were reseeded on the 7th day. Reseeding was discontinued and staining was initiated if the number of MSCs were inadequate. The medium was changed 3 days later, and growth in culture medium was halted on the 6th day from the reseeding. The MSC layer was fixed with methanol and stained with Giemsa's staining solution (Kanto Chemical Co., INC., Japan). Colonies larger than 2 mm in diameter were counted.

Osteogenic differentiation

MSCs were plated at 5×10^3 cells/cm^2 in 9.5 cm^2 6-well tissue culture plates with FBS-TCM and incubated in a humidified incubator at 37°C under 5% CO_2. The culture medium was replaced with an osteogenic medium (StemPro® Osteogenesis Differentiation Kits, Thermo Fisher Scientific Inc., Republic of Korea) on the 3rd day. The medium replacement was repeated every 4 days. On the 22nd day of the seeding, the cells were fixed with 4% formaldehyde for 30 minutes and stained with 2% alizarin red s (ARS) solution (Lifeline® Cell Technology, USA) for 2 to 3 minutes. The cells were rinsed with phosphate-buffered saline (PBS) before and after fixation. After staining, the cells were rinsed with distilled water (DW).

Chondrogenic differentiation

MSCs were plated at 8×10^6 cells/well in each well of the 4-well culture plates. The cells were incubated in a humidified 5% CO_2 incubator at 37°C for 2 hours. The medium was first replaced after 2 hours with chondrogenic medium (StemPro® Adipogenesis Differentiation Kits, Thermo Fisher Scientific Inc., Republic of Korea) and the change of medium was repeated every 3 days. On the 15th day of the seeding, the cells were fixed with 4% formaldehyde for 30 minutes and stained with 1% alcian blue (10 mL HCl (0.1N) + 0.1 g alcian blue (Alcian Blue 8GX powder, Sigma-Aldrich, USA)) for 30 minutes. The cells were rinsed with PBS before and after fixation. Staining was followed by rinsing with 0.1 N HCl and neutralizing with DW.

Adipogenic differentiation

MSCs were plated in 9.5 cm^2 6-well tissue culture plates at 1×10^4 cells/cm^2 with FBS-TCM and incubated under 5% CO_2 at 37°C. The medium was replaced on the 3rd day with adipogenic medium (StemPro® Adipogenesis Differentiation Kits, Thermo Fisher Scientific Inc., Republic of Korea). The

Table 1. Characteristics of MSCs isolated from peripheral blood

Horse	Breed	Sex	Age	MSC yield (cells)	CFU-F assay (colonies)	PDT (hours)
M1	TB	f	6 yr	-	-	-
M2	TB X	f	11 yr	1.92×10^6	427.5	13.404
M3	TB X	f	13 yr	-	-	-
M4	TB	f	6 yr	-	-	-
M5	H	f	8 yr	2.70×10^7	770	26.515
M6	H	f	3 yr	-	-	-
Mean ± SD				$1.45 \pm 1.77 \times 10^7$	598.5 ± 242.2	19.960 ± 9.271
F1	TB X	f	6 mo	1.90×10^7	307	27.149
F2	Mixed	m	6 mo	6.00×10^4	2	25.567
F3	Mixed	m	6 mo	-	-	-
F4	Mixed	f	5 mo	9.89×10^6	986.5	54.374
F5	Mixed	m	6 mo	1.10×10^7	276	22.402
F6	Mixed	f	5 mo	-	-	-
Mean ± SD				$9.99 \pm 7.76 \times 10^6$	392.9 ± 418.8	32.373 ± 14.780
Mann-Whitney U test				0.800	0.533	0.533

MSC, Mesenchymal stem cell; CFU-F, Colony-forming unit-fibroblast; PDT, Population doubling time; M, Mare; F, Foal; F1, The foal of M1; TB, Thoroughbred; TB X, Thoroughbred crossed; H, Haflinger; f, Female; m, Male.

Cells were counted to collect MSC yield data on the 29th day from the initial seeding PDT = CT × log2/(log N_f – log N_i).

CT, The cell culture time; Nf, The final number of cells; Ni, The initial number of cells.

$p < 0.05$.

medium was changed every 4 days. On the 15th day from the seeding, the cells were fixed with 10% formaldehyde for 10 minutes and with fresh 10% formaldehyde for 1 hour. The cells were rinsed with DW followed by rinsing with 60% isopropanol for 5 minutes. The cells were dried with a hair dryer before staining and they were stained with Oil Red O Solution (Oil Red O solution 0.5% in isopropanol, Sigma, USA) for 10 minutes. Then the cells were rinsed with DW.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics Subscription (11-2018, IBM, USA). The data of the mares and foals were compared through descriptive statistics and the nonparametric test, Mann-Whitney, with $p < 0.05$. All data were expressed as mean ± standard deviation (mean ± SD).

Results

Cell proliferation

MSCs were successfully harvested from two out of the six mares ($1.45 \pm 1.77 \times 10^7$ cells) and four out of the six foals ($9.99 \pm 7.76 \times 10^6$ cells). The yields are listed in Table 1. The MSCs from mares formed marginally more colonies (598.75 ± 242.18 colonies) and required less time (19.96 ± 9.27 hours) to proliferate than the MSCs from the foals (392.88 ± 418.81 colonies and 32.37 ± 14.80 hours). The data are listed in Table 1; however, the difference was not significant. One of each mare and foal's MSCs grew into "fingerprint" CFUs, and the other showed dispersed CFUs (Fig. 1).

CFU

The difference between the mares and the foals was not significant. Both the most number and the least number of colonies occurred in the foal group; the greatest number of colonies was as many as 986.5 ± 418.8 and the least was as few as 2 ± 418.81. The 2nd and the 3rd largest number of colonies grew from the mares; the numbers were 427.5 ± 242.2 and 770 ± 242.2, respectively.

Characterization of MSCs

The collected cells from both groups were spindle-shaped, adhered to the plastic cell culture plates, and formed colonies. Calcium deposits were detected by ARS staining, acidic polysaccharides such as glycosaminoglycans were observed by alcian blue staining, and lipid presence was confirmed by Oil Red O (ORO) staining. These results are summarized in Fig. 2.

Discussion

Cell-based therapy using MSCs has shown promise in musculoskeletal diseases in horses. Musculoskeletal diseases are the most prevalent health problem in horses and are associ-

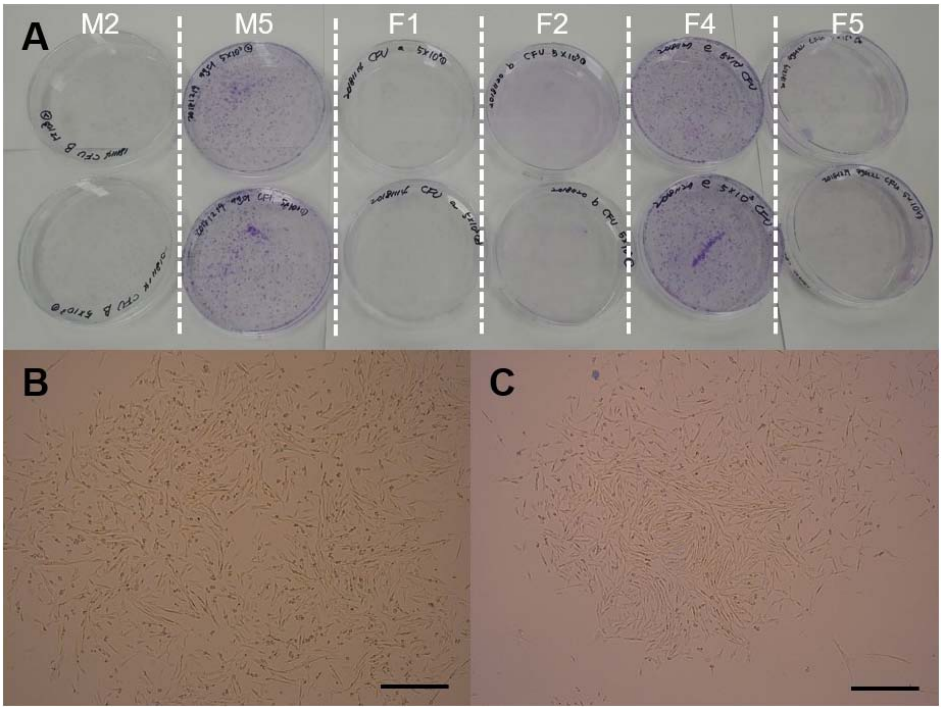

Fig. 1. Macroscopic and microscopic view of colony-forming units (CFUs). Macroscopic (A) and microscopic images of CFUs. (B): dispersed CFUs from foal number 1. (C): 'fingerprint' CFUs (crystal violet stain) from mare number 2. Scale bar: 250 μm.

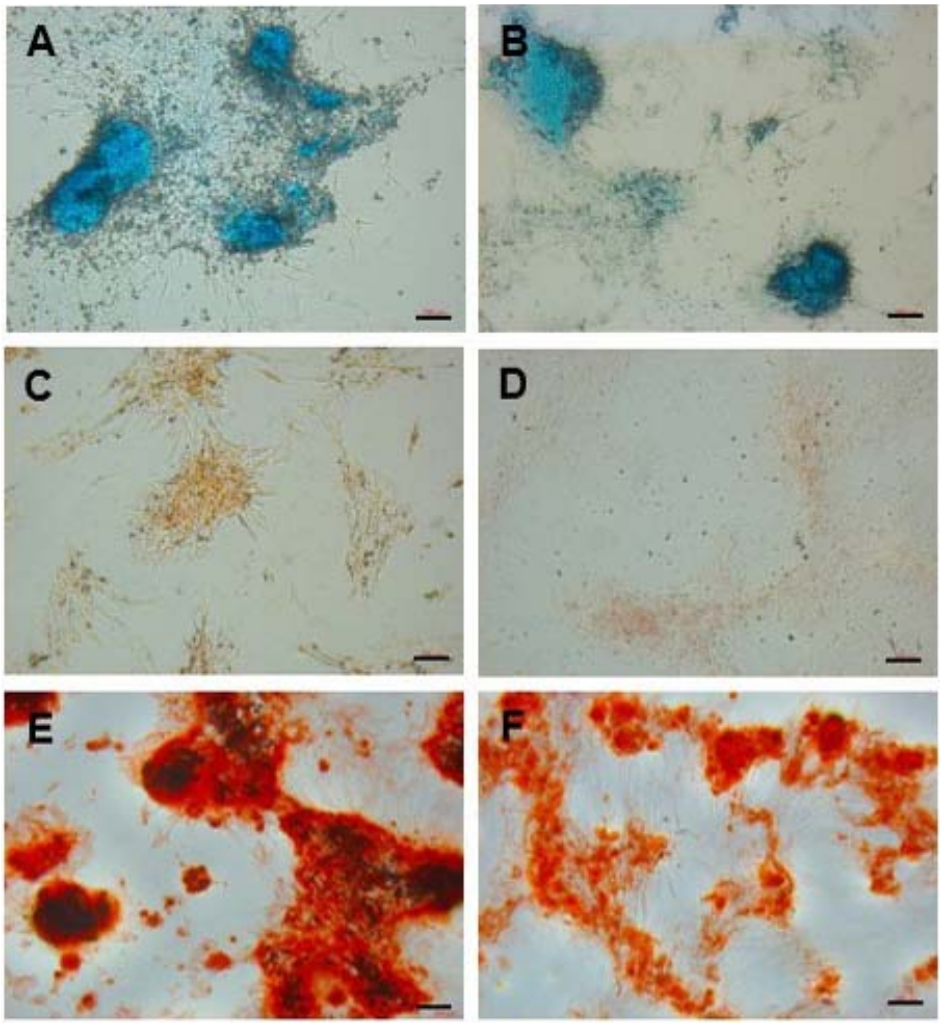

Fig. 2. Cellular characterizations of trilineage differentiation. Trilineage differentiation of equine peripheral blood-derived mesenchymal stem cells (PB-MSCs). Alcian blue staining of developing chondrogenic pellets of foal number 1 (A) and mare number 2 (B). Oil Red O staining of developing adipogenic lineage of foal number 1 (C) and mare number 2 (D). Osteogenesis Differentiation Medium demonstrated differentiation into Alizarin Red S staining of foal number 1 (E) and mare number 2 (F). Scale bar: 100 μm.

ated with significant mortality (13). However, recent studies have attempted to find alternative sources to isolate MSCs because of the drawbacks of conventional collection methods (8). PB-MSCs are receiving renewed attention because they can be harvested with ease through minimally invasive techniques thereby offering simplicity in sampling (2,7,8,11). In this study, we successfully isolated PB-MSCs from mares and foals without any of the complications associated with conventional sources and methods.

Donor age is a major factor determining the lifespan and quality of MSCs as cells from aged donors have reduced proliferative capacity and differentiation potential (12). We, therefore, conducted this study based on the hypothesis that young horses would have a higher possibility of higher yield of MSCs with greater proliferative capacity. Six pairs of a mare and a foal each were divided into two groups with the following age conditions: mares should be no less than 3 years of age and foals should be no more than 6 months of age. The isolation was successful from 2 of 6 mares (approximately 33%) and 4 of 6 foals (approximately 66%) in this study; in total, it was 6 of 12 horses (50%). However, the small sample size was a limitation of this study.

Success percentages from earlier studies have varied from 30% (3 of 10 horses) to 100% (4 of 4 horses or 6 of 6 horses) (2,3,5,7,11). The percentage variation seen in these studies could be attributed to the differences in the methods used to harvest MSCs. Specifically, differences were seen based on the lymphocyte separation medium (Ficoll or Percoll) used. Studies that used Ficoll showed 30% (3 of 10 horses) to 67% (8 of 12 horses) success in MSC isolation (3,5,7), and the studies that used Percoll had a 100% success rate (2,11). We used direct plating and achieved a total result of 50% (6 of 12 horses). In addition, there was the risk of losing MSCs during seeding since we only used 600 μL from 2 L of buffy

coat. Limiting the number of dishes would have lowered the budget by reducing labor and instruments. However, to increase the yield, the use of more buffy coat should be considered. In addition, in this study, the success rate of stem cell isolation through direct plating in foals was higher than that in mares. Further studies are needed to increase the sample size and compare the success rates of stem cell isolation from mares to that from foals using lymphocyte separation media.

Characterization of the collected cells is required to prove that they are MSCs. Common methods include cell morphology, cell adhesion, colony formation, trilineage differentiation, alkaline phosphatase staining, RT-PCR, and immunophenotyping (2,3,5-7,9-11). The cells from our study were spindle-shaped which is the typical shape of stem cells, adhered to the plastic dish, and were composed of colonies. The cells were also capable of differentiating into osteocytes, chondrocytes, and adipocytes. These results suggest that the cells from both mares and foals were MSCs and that there was no difference in characteristics of cell morphology, cell adhesion, and colony formation between the two groups of cells.

The number of cells had to be expanded to reach the optimal cell number for therapeutic use (1,6). Therefore, the proliferation ability of MSCs is important. In earlier studies by Seo et al. (9) and Ishikawa et al. (6) the approximate PDT was 21.26 hours to 25.2 hours respectively. In our study, the mean PDT of foals was 19.960 hours and that of mares was 32.373 hours. The cell colonies from foals expanded faster and those from mares expanded at a slower rate than what has been observed in earlier studies. However, a study with a larger sample size is necessary to clarify the age-dependent differences in MSC yield.

This is the first report on the isolation and evaluation of MSCs from PB in foals under 6 months of age. We also compared the properties of stem cells from mares and their foals for the first time. In conclusion, there was no significant difference in the MSC yield, expansion (CFU-F, PDT), and cellular characteristics (trilineage differentiations) between the two groups. However, further study with a larger sample size is needed. These results suggest that PB-MSCs isolated from horses, irrespective of whether the horses are adults or foals, can be used as a source of MSCs for equine cell-based therapy.

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Conflicts of Interest

The authors have no conflicting interests.

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