Evaluation of equine peripheral blood apheresis product, bone marrow, and adipose tissue as sources of mesenchymal stem cells and their differentiation potential

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Objective—To evaluate effects of apheresis on mesenchymal stem cells (MSCs) and compare those MSCs with MSCs obtained from adipose tissue or bone marrow (BM).

Sample Population—Samples obtained from 6 adult horses.

Procedures—Samples of blood from a peripheral vein, adipose tissue, and BM aspirate were obtained from each horse. Samples were processed via apheresis of blood and techniques reported elsewhere for adipose tissue and BM. Cultures were maintained until adherence and subsequently were subjected to differentiation protocols to evaluate adipogenic, osteoblastic, and chondrogenic potential.

Results—Apheresis product had a significantly higher mononuclear percentage, higher platelet count, and lower RBC count, compared with values for peripheral blood. No cell adherence to the tissue culture plates was detected for the apheresis product. Adherence was detected for 6 of 6 adipose-derived and 4 of 6 BM-derived samples. Variations in efficiency were detected for differentiation of adipose- and BM-derived cells into adipocytes, chondrocytes, and osteoblasts.

Conclusions and Clinical Relevance—Apheresis was able to concentrate mononuclear cells and reduce RBC contamination. However, the apheresis product was unable to adhere to the tissue culture plates. In matched horses, adipose- and BM-derived MSCs were capable of producing lipids, glycosaminoglycan, and mineral. The BM was vastly superior to adipose tissue as a source of MSCs with osteoblastic potential in matched horses. Additional studies will be necessary to optimize apheresis techniques for horses before peripheral blood can be considered a suitable source for multipotential cells for use in cell-based treatments. (Am J Vet Res 2011;72:127–133)

Musculoskeletal injury is a common cause of lameness and decreased performance in horses. Use of stem cells is currently being developed and evaluated. However, it has the potential to be a novel efficacious treatment option for a large range of conditions in humans and horses. Stem cells can be characterized as embryonic or adult stem cells. Research on adult stem cells has generated excitement about the potential clinical use of these cells because under appropriate conditions, they have the ability to differentiate into a number of different cell types. Adult stem cells typically generate the cell types of the tissue in which they reside. For example, hematopoietic stem cells would typically give rise to erythrocytes, leukocytes, and platelets. Other studies have revealed that these cells can undergo osteoblastogenesis, adipogenesis, and chondrogenesis.

In human medicine, stem cell treatment has been primarily experimental, but stem cells have been used to achieve regeneration of tissues ranging from damaged cartilage to bone, muscle, tendon, ligament, and fat. In horses, some major injuries and conditions (eg, superficial digital flexor tendi-
Isolation of stem cells from peripheral blood is an enticing concept. Harvesting blood is a safer, less invasive procedure associated with lower morbidity, compared with the risks associated with the current criterion-referenced standard of BMA. Investigators in a study of horses found that it was possible to isolate and propagate fibroblastoid, progenitor-type cells from equine peripheral blood and subsequently determined that these cells are able to differentiate into different mesenchymal lineages. The authors of that study concluded that there are low numbers of PBPs in the mononuclear cell fraction and that future experiments should focus on obtaining higher numbers of these cells.

The PBPs are commonly isolated by use of gradient techniques, such as density gradient centrifugation techniques. A technique that has been used in humans and only recently became available to veterinary scientists is stem cell apheresis. This system allows for the processing of large volumes of blood to collect high numbers of mononuclear cells. Automatic centrifugation maintains a constant separation factor, which allows for a more homogenous harvest with minimal cross-cellular contamination. For equine patients, which have a relatively large blood volume (approx 36 L for a 500-kg horse), it may be possible to obtain sufficient PBPs from a single collection (maximum volume, 7 to 8 L/horse) to enable direct treatment without the need for time-consuming cell culture techniques. An alternative is to connect an equine patient to an apheresis system and selectively harvest the PBPs, with the blood components that are not harvested being returned to the patient. As a result, a potential benefit of apheresis would be to isolate a large enough number of PBPs during a single collection to allow them to be used immediately as a raw product. This would avoid the lag period currently required for culture of cells to obtain a sufficient number for use in clinical situations.

The number of mononuclear cells that could be harvested from equine blood by use of an apheresis system is unknown. Furthermore, the proportion of mononuclear cells that would be stem cells is also unknown.

We hypothesized that mononuclear cells could be harvested in substantial numbers from equine peripheral blood and that these apheresis-derived cells would be capable of adherence to tissue culture plates and able to differentiate into chondrogenic, adipogenic, and osteoblastogenic lineages in cell culture. Additionally, we hypothesized that cells obtained from apheresis product, adipose tissue, and BM would have similar abilities to differentiate into chondrogenic, adipogenic, and osteoblastogenic lineages in cell culture.

### Materials and Methods

**Sample population**—Samples of blood obtained from a peripheral vein, BM, and fat were obtained from 6 skeletally mature mix-bred horses (mean ± SD age, 6.8 ± 1.9 years). All horses were sedated by administration of detomidine hydrochloride (0.05 mg/kg, IV) and butorphanol tartrate (0.05 mg/kg, IV) immediately prior to any procedures. The study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

**Apheresis-derived isolation of cells**—A blood sample was collected from a peripheral vein into a tube containing EDTA; this sample was used for a CBC. Blood then was collected, and MSCs were isolated as described in other reports. Four liters of blood was collected via a 12-gauge catheter inserted in a jugular vein; the blood was collected in a single sterile blood bag that contained acid citrate dextrose solution as the anticoagulant (1 part anticoagulant:9 parts blood). After the blood collection bags were filled, they were placed on ice for transport (2 to 3 hours) until further processing by use of an apheresis machine. The machine had been programmed originally to collect mononuclear cells, and it was manually adjusted as the processing progressed to optimize the collection of mononuclear cells and reduce erythrocyte contamination. At completion of apheresis, a sample of the apheresis product was collected for a CBC. The product was counted via a hemacytometer with trypan blue dye exclusion (used to indicate live cells). Live cells (5 × 10^6 cells, 10 × 10^6 cells, 25 × 10^6 cells, 50 × 10^6 cells, and 100 × 10^6 cells) and 10 and 13 mL of undiluted apheresis product were seeded onto 10-cm² plates that contained DMEM-F12, 20% FBS, and 1% penicillin-streptomycin. Cells were incubated at 37°C in a humidified 5% CO₂ environment. The first change of medium was 10 days after seeding. Subsequently, medium was changed twice weekly.

**Adipocyte-derived isolation of cells**—Adipose tissue (10 to 20 g of fat) was obtained from the tail head adipose pad, placed in DMEM solution, and stored on ice for transportation and subsequent processing. The adipose pad was washed with PBS solution, minced, and then digested by incubation for 1.5 hours with DMEM containing 0.1% collagenase. Nucleated adipose cells were pelleted, washed, and then plated at a cell density of 3.26 × 10^5/cm² in medium that contained DMEM, 10% FBS, and 1% penicillin-streptomycin.

**Isolation of BM MSCs**—An 11-gauge Jamshidi needle was used to aspirate BM (10 to 20 mL) from the iliac crest. The aspirate was placed in tubes that contained sodium citrate; tubes were placed on ice for transportation and subsequent processing. The aspirate pad was washed with PBS solution, minced, and then digested by incubation for 1.5 hours with DMEM containing 0.1% collagenase. Nucleated adipose cells were pelleted, washed, and then plated at a cell density of 3.26 × 10^5/cm² in medium that contained DMEM, 10% FBS, and 1% penicillin-streptomycin.

**Adipogenic differentiation**—Cells were plated and grown to confluence (day of confluence was designated as day –2) in DMEM and 10% FBS. On day 0 (ie, 2 days after confluence was achieved), cells were exposed to...
induction medium that contained DMEM, 1% 100mM minimum essential medium, sodium pyruvate, 1% 100X penicillin-streptomycin-glutamine, 1% isobutylmethylxanthine, 0.1% insulin, 0.01% dexamethasone, and 0.01% troglitazone. On day 2, (ie, 2 days after initial exposure to the induction medium), the medium was changed to DMEM, 1% 100mM minimum essential medium, sodium pyruvate, 1% 100X penicillin-streptomycin-glutamine, and 0.01% insulin. Treated cells were maintained in this medium until full differentiation was achieved. Full differentiation was achieved by day 8, and the wells were stained with oil red O stain to enable us to examine cells for lipid droplets. Oil red O was extracted from samples by the addition of isopropanol to wells and incubation for 15 minutes; lipid droplets were quantified by use of a spectrophotometer at 540 nm.

Chondrogenic differentiation—Cells were washed in PBS solution, trypsinized, counted, and resuspended at 100,000 cells/20 µL. Cells (volume, 20 µL) were seeded dropwise onto plates, which were incubated at 37°C for 30 to 90 minutes. Cells were then pelleted, and the pellets were covered with chondrogenic-induction medium (DMEM, 1% insulin-transferrin-selenium plus linoleic acid [1 mg of insulin from bovine pancreas/mL, 0.55 mg of human transferrin/mL, 0.5 µg of sodium selenite/mL, 5 mg of bovine serum albumin/mL, and 470 µg of linoleic acid/mL], 0.1mM l-ascorbate 2–phosphate, 100mM dexamethasone, and transforming-growth factor-β3 [5 ng/mL]) or with control medium (same as induction medium, except it did not contain transforming-growth factor-β3). Plates were incubated for 3 weeks, and medium was changed twice weekly. After incubation, cells were stained with alcian blue to enable us to evaluate GAG content. Alcian blue was extracted from samples by the addition of 6M guanidine hydrochloride to wells and incubation overnight. The GAG content was quantified by use of a spectrophotometer at 650 nm.

Osteoblastogenic differentiation—Cells were seeded in 60-cm² well plates (in triplicate) at a density of 4 X 10⁶ cells/well in base medium and grown until confluence. Cells were induced with osteoblastogenic medium that contained DMEM, 10% FBS, 1% penicillin-streptomycin, 5mM β-glycerophosphate, and 0.1mM ascorbate 2–phosphate; cells were incubated for 3 weeks, with medium changed twice weekly. Cultured cells were subsequently stained with alizarin red S to enable us to detect mineral formation. Alizarin red S was extracted from samples by addition of 10% cetylpyridinium chloride in 10mM sodium phosphate (pH, 7.0 [wt/vol]) to wells and incubation for 60 minutes. Mineral content was quantitated by use of a spectrophotometer at 562 nm.

Figure 1—Comparison of the number of RBCs (A), platelets (B), and total mononuclear cells (C) and the percentage of mononuclear cells (D) in a sample of equine blood obtained from a peripheral vein (white bars) and an apheresis product (gray bars). Values reported are mean ± SEM for 6 peripheral blood samples and 6 apheresis products. In panel D, the value reported was calculated as follows: (number of mononuclear cells/total number of WBCs) X 100. *Value differs significantly (P < 0.05) from the value for the peripheral blood sample.
Statistical analysis—Data were expressed as mean ± SEM. Data were tested for a normal distribution by use of statistical software program. All data were normally distributed, and 1-way and 2-way ANOVAs were used to detect significant differences between control and treated samples. Values of $P < 0.05$ were considered significant.

Results

MSC isolation—Processing via apheresis required 2 to 3 hours for each 4-L sample and yielded 200 to 220 mL of product. The RBC count was significantly decreased in the apheresis product (by 96.2%) when compared with the RBC count in the peripheral blood sample (Figure 1). The platelet count was significantly increased in the apheresis product when compared with that in the peripheral blood sample. The apheresis product had similar total mononuclear cell counts when compared with those in the peripheral blood sample; however, there was a significant increase in the percentage of total mononuclear cells in the total apheresis product, compared with the percentage for the peripheral blood sample. Despite this increase in mononuclear cells, adherence to tissue culture plates at low levels was evident in only one of the horse-derived samples, and these cells did not proliferate. Adipose tissue and BM were readily obtained from all horses, and adherent cells were obtained in all 6 horses for adipose tissue and in 4 horses for the BM.

Four million cells were seeded for each horse from either adipose tissue or BM and allowed to adhere and proliferate in 10-cm plates. Adipose-derived cells reached confluence much faster than did BM-derived cells. All adherent cells from each horse were passaged 2 times at a plating density of 1:2, which resulted in four 15-cm plates. On the basis of the initial plating density of 4 million cells, adipose-derived cells had much greater expansion efficiency (Figure 2), which suggested that more MSCs can be obtained from equivalent starting cell numbers in adipose tissue than in BM.

Adipogenesis—Adipose-derived adipogenic-induced cells had higher amounts of oil red O staining than did those treated with control medium (Figure 3). When oil red O was extracted and the lipid quantified, there were significantly higher amounts in the treated cells. In contrast, although there were low amounts of oil red O staining in the adipogenic-treated BM cells, we detected no significant difference in oil red O–extracted cells between the control and treated samples. Additionally, adipogenesis was reduced in the BM-derived cells when compared with that in the adipose-derived treated cells.
Quantification of GAG produced by treated adipose-derived cells was superior for adipogenesis. More GAG when induced, compared with the amount of mineral produced by cells from different sources revealed that BM-derived cells had a significantly higher value than BM-derived cells. Considered together with the preceding data, these results indicated that there are differences in the efficiencies with which adipose- and BM-derived cells undergo adipogenesis, osteoblastogenesis, and chondrogenesis.

**Discussion**

In the study reported here, we attempted to concentrate multipotent cells from peripheral blood by use of apheresis. Apheresis was useful for concentrating mononuclear cells and platelets and for reducing RBC contamination. However, the mononuclear cells collected were not able to adhere to tissue culture plates or differentiate into cell lineages under the conditions of this study. Adipose tissue and BM were sources capable of yielding adherent cells, with adipose tissue having higher adherence efficiency. In matched horses, adipose- and BM-derived MSCs were capable of becoming adipocytes, osteoblasts, and chondrocytes. Treated BM cells were superior to untreated BM cells and both treated and untreated adipose cells for staining of mineral in matched horses.

The ability to isolate and differentiate fibroblast-like cells from equine peripheral blood has been reported. The cells used in that study were obtained by use of a polysaccharide gradient sedimentation technique, and cells were found to be capable of osteoblastogenic and adipogenic differentiation but not chondrogenic differentiation. In a subsequent study, investigators attempted to optimize the differentiation protocol for horses and were able to detect adipogenic, osteoblastogenic, and chondrogenic potential. The mononuclear cell fraction of peripheral blood in all species contains lymphocytes, monocytes, and hematopoietic stem cells. In humans, mice, rabbits, guinea pigs, dogs, and horses, the concurrent presence of multipotent MSCs has also been confirmed. In studies in humans, investigators have isolated MSCs from peripheral blood but have observed the frequency of these cells to be extremely low without the addition of granulocyte-monocyte colony-stimulating factor.

It is likely that low MSC concentrations within the mononuclear cell fraction contributed strongly to the lack of adherent cells in the study reported here. Despite concentrating mononuclear cells with apheresis, it is possible that the apheresis resulted in less concentration of mononuclear cells relative to that achieved with a polysaccharide gradient. The use of a polysaccharide gradient technique in conjunction with the apheresis-derived product to isolate PBPs, evaluation of the cell types obtained, and the subsequent adherence potential of cells obtained by use of these different isolation techniques are potential areas for future research. Additional studies in horses should focus on determining the concentration of normally circulating MSCs as well as the exact fraction of peripheral blood to which they belong, which may allow for apheresis to become useful in isolation of high concentrations of MSCs. Additionally, the use of granulocyte-monocyte colony-stimulating factor to increase the proportion of cells may be effective at improving results.
Delay of sample processing because of transportation and the relatively long processing times may also have been a contributing factor to the lack of adherence. Most researchers obtain tissue samples and then immediately process them. Studies focused on the effect of transportation and the resultant delay between sample acquisition and processing have not been performed to the authors’ knowledge, and this should also be a focus of future experiments. Similarly, although we used established growth protocols for MSCs, it is possible that the growth conditions (media formulations, type of serum, and type of tissue culture plates) used in the present study may not have been ideal for the cultivation of equine MSCs from blood. Finally, it is possible that the apheresis process removes MSCs or accessory cells required for growth of MSCs in culture. These factors will require further verification.

In the study reported here, we used various concentrations of apheresis product and BM aspirate to maximize the probability of adherence. Direct plating of BM aspirate has been reported for various tissue sources. In the present study, we found higher numbers of CFUs for direct plating of BM aspirate with reduced variability, compared with numbers for polysaccharide and lysis isolation techniques. More commonly used techniques reported in the literature involve polysaccharide or lysis isolation techniques. The use of direct plating may explain the reason that only 4 of 6 horses had adherence of cells in the BMA group. However, in our experience, it is more difficult to achieve adherence with BMA-derived cells than with adipose-derived cells by use of a variety of isolation techniques, which reflects the percentages achieved in the present study. Because of the different isolation technique used for BM, it is difficult to compare the absolute numbers for isolation of samples obtained via BMA in the study reported here with results published for studies that evaluated MSC isolation and differentiation in horses. However, the present study indicated differences in cell adherence and differentiation potential within matched horses for different tissue sources. To our knowledge, no other study has been conducted to make comparisons among tissue sources within a horse. The large variability in age, breed, and sex of the horses used in other studies adds substantial variability and makes it difficult to compare results among studies. Additional studies to evaluate different cell sources and different isolation and differentiation protocols are warranted to standardize the methods for effective and efficient MSC isolation.

Evaluation of multipotency of BM- and adipose-derived MSCs has been reported. However, to the authors’ knowledge, no studies have been conducted to compare the differentiation of BM- and adipose-derived cells into adipogenic, osteoblastogenic, and chondrogenic lineages in matched horses. The chondrogenic potential of BM- and adipose-derived cells has been evaluated, and the authors in that study concluded that some variation in their data may have been attributable to the fact that tissues were isolated from 2 populations of horses. Investigators in another study evaluated the chondrogenic potential of BM- and adipose-derived cells in hydrogel culture in matched horses and found that BM-derived cells were superior to adipose-derived cells. We found that adipose tissue in matched horses was a more reliable source of adherent cells than was BM, as determined by use of the reported protocols. Adipose-derived cells also produced slightly more (but not significantly more) GAG, compared with GAG production for BM-derived cells. The BM-derived cells in matched horses were significantly more capable of differentiation via the osteoblastogenic pathway than were adipose-derived cells. The BM-derived cells treated with induction medium were significantly more likely to produce mineral, compared with results for untreated cells. This may have important implications for future orthopedic research in horses and could potentially...
result in improvements in techniques for stem cell-assisted fracture repair.

In the study reported here, we determined that apheresis processing was able to concentrate mononuclear cells and reduce RBC contamination in samples obtained from horses, however, the apheresis product was not able to adhere to tissue culture plates or differentiate into cell lineages under the conditions of this study. In the matched horses used in this study, adipose tissue and BM were sources capable of yielding adherent cells, with adipose tissue being the more effective of the 2 sources. Adipose- and BM-derived MSCs were capable of becoming adipocytes, osteoblasts, and chondrocytes. The BM was vastly superior to adipose tissue as a source of MSCs with osteoblastogenic potential in matched horses. Additional studies will be necessary to optimize apheresis techniques for horses before peripheral blood can be considered a suitable source for multipotential cells used for stem cell treatments.

References