

Effects of equine bone marrow aspirate volume on isolation, proliferation, and differentiation potential of mesenchymal stem cells

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Objective—To compare the mesenchymal stem cell (MSC) yield and chondrogenic and osteogenic differentiation from 5- and 50-mL bone marrow aspirates from horses.

Animals—Six 2- to 5-year-old mixed-breed horses.

Procedures—2 sequential 5-mL aspirates were drawn from 1 ilium or sternebra. A single 50-mL aspirate was drawn from the contralateral ilium, and 2 sequential 50-mL aspirates were drawn from a second sternebra. The MSC yield was determined through the culture expansion process. Chondrogenesis and osteogenesis were evaluated by means of conventional laboratory methods.

Results—The second of the 2 sequential 50-mL sternal aspirates yielded few to no MSCs. Independent of location, the highest density of MSCs was in the first of the 2 sequential 5-mL fractions, although with subsequent culture expansion, the overall yield was not significantly different between the first 5-mL and first 50-mL fractions. Independent of location, chondrogenesis and osteogenesis were not significantly different among fractions. Independent of fraction, the overall cell yield and chondrogenesis from the ilium were significantly higher than that from the sternum.

Conclusions and Clinical Relevance—This study failed to detect an additional benefit of 50-mL aspirates over 5-mL aspirates for culture-expanding MSCs for equine clinical applications. Chondrogenesis was highest for MSCs from ilial aspirates, although it is not known whether chondrogenesis is indicative of activation of other proposed pathways by which MSCs heal tissues. (*Am J Vet Res* 2013;74:801–807)

Equine bone marrow–derived MSCs are considered a promising cell type for treating orthopedic injuries on the basis of their ability to differentiate among numerous connective tissue lineages.¹ Although MSC treatments are often investigated from a tissue engineering approach of combining cells with a scaffold for implantation into a defect,² equine practitioners have also explored the therapeutic potential of injections of undifferentiated autologous MSCs.³ This relatively simple approach to treatment with MSCs has resulted in positive return-to-work outcomes in clinical cases of tendon and ligament core lesions^{3–6} and joint disease³ and enhanced articular cartilage repair in a 12-month equine study.⁴ Such evidence of healing has sustained interest in the use of autologous MSCs in equine clinics

ABBREVIATIONS	
GAG	Glycosaminoglycan
MSC	Mesenchymal stem cell

and fostered the formation of commercial processing services to meet this demand.⁷

Mesenchymal stem cells represent a small percentage of the nucleated cell population in bone marrow^{8,9}; therefore, laboratory processing and culture expansion are necessary to obtain the cell numbers (millions) of MSCs that are currently used in equine clinics.³ The ideal protocol for isolating and expanding MSCs from bone marrow would minimize the time in culture to avoid senescence associated with extensive MSC growth, although a consensus on optimal techniques for equine MSCs has not been reached. One aspect of this process that has received little attention is the effect of the volume of bone marrow aspirate on the efficiency of MSC isolation, growth, and differentiation potential. The lack of consensus on optimal volumes of bone marrow aspirate is important for the equine industry because the numerous processing services provide different recommendations. Data from human literature suggest that small aspirates may be ideal for MSC cultures because the density of MSCs decreases with increasing

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volume of bone marrow owing to dilution with blood beyond the first 2 mL.⁹ Similarly, for equine subjects, an analysis of 4 sequential 5-mL bone marrow aspirates from the sternum revealed the greatest density of colony-forming MSCs in the first 5-mL fraction.¹⁰ Although both studies revealed the efficiency by which MSCs may be established from small aspirate volumes, the recommendations of commercial processing services are more consistent with basic science studies of equine MSCs^{11–16} that have used aspirates ranging from 10 to 60 mL. Despite the high concentration of MSCs in the initial volume of aspirate, it remains possible that larger aspirate volumes contain a greater total number of MSCs. The objectives of the study reported here were to determine the effect of aspirate volume on the MSC yield by comparing small (5-mL) and large (50-mL) aspirate fractions taken from the same animal and to evaluate chondrogenesis and osteogenesis as measures of the differentiation potential of the MSCs.

Materials and Methods

Bone marrow harvest—Bone marrow aspirate procedures were approved and performed according to guidelines set forth by the Colorado State University Animal Care and Use Committee. Bone marrow aspirates were taken from the ilium and sternum of six 2- to 5-year-old standing mixed-breed horses in a manner that mimics the typical clinical approach. Bone marrow was aspirated with an 8-gauge trephine bone marrow aspiration needle into syringes that were preloaded with 0.5 mL (for 5-mL aspirates) or 5 mL (for 50-mL aspirates) of heparin solution (2,000 U/mL). The depth of the needle placement was 4 to 6 cm. All samples were aspirated into 60-mL syringes. For ilial aspirates, 2 sequential 4.5-mL aspirates, for a total volume of 5 mL, were taken from 1 ilium. A single 45-mL aspirate, for a total volume of 50 mL, was taken from the contralateral ilium. For each horse, the first aspirate was taken from the left side, with small and large fractions taken in alternating fashion. Sternal samples were taken in a similar manner from 2 sternbrae, with the initial aspirate taken from the sternbra located approximately 9 cm cranial to the xyphoid process and the second taken from the sternbra cranially adjacent to the first. Two sequential 4.5-mL fractions were drawn from one sternbra, and 2 sequential 45-mL fractions were drawn from the second. Small and large fractions were taken from the 2 sternbrae in alternating fashion. For each horse, 9 mL of blood was collected into 1 mL of sodium citrate anticoagulant. The nucleated cell density was determined by diluting bone marrow and blood samples 10-fold in 0.8% ammonium chloride solution and counting with a hemocytometer.

Isolation and expansion of MSCs—Bone marrow aspirates were divided into aliquots in centrifuge tubes and spun at $100 \times g$ for 5 minutes. The bone marrow plasma was collected down to the interface of the RBC pellet to collect approximately 50% to 75% of the nucleated cell population, and the number of nucleated cells in the supernatant was counted. The bone marrow plasma supernatant was centrifuged at $1,000 \times g$ for 10 minutes to pellet the nucleated cells, which were then

resuspended in low-glucose Dulbecco modified Eagle medium^a containing 10% fetal bovine serum^b plus an antimicrobial-antimycotic.^a Mesenchymal stem cell colony-forming cultures were established by seeding the nucleated cells into tissue culture flasks^c at a concentration of 0.267×10^6 cells/cm². The medium was changed after 24 hours to remove nonadherent cells. The cultures were maintained for an additional 7 days to allow colonies to form and grow until the middle of the colonies were nearly confluent. The cells were harvested with 0.25% trypsin and EDTA,^a pooled if multiple flasks were used, reseeded into monolayer cultures at 5×10^3 cells/cm² in proliferation medium (α minimal essential medium^a plus 10% fetal bovine serum) and 2 ng of fibroblast growth factor 2^d/mL, and cultured for 3 days. The MSCs were collected with 0.25% trypsin and EDTA, counted, reseeded into monolayer cultures at 0.8×10^3 cells/cm² in proliferation medium, and expanded for 4 days to $\leq 80\%$ confluence. Following this passage, all cultures were seeded into chondrogenic or osteogenic cultures.

Reported data from MSC isolation and expansion cultures—The isolation and culture-expansion of MSCs was conducted in a manner that mimics the process used to generate clinical treatments. Colony-forming cultures often contain a substantial population of adherent cells that do not morphologically resemble MSCs and are not associated with MSC colonies. Such cells are rapidly eliminated with monolayer expansion; therefore, the MSC yield following colony-forming culture and a single passage over 3 days of expansion culture was estimated to reflect the MSC density in the aspirates. At this point in the expansion process, the MSC yield is typically less than is currently used in equine clinical cases; therefore, MSC proliferation was evaluated through a second passage. To better estimate the maximum potential yield for each aspirate after passages 1 and 2, the MSC population calculated was the population that would have been realized had all of the nucleated cells been seeded into colony-forming culture and all MSCs been carried through monolayer expansion.

Chondrogenic differentiation—Culture-expanded MSCs were encapsulated in 2% (wt/vol) low-melting temperature agarose^a hydrogel at a concentration of 10^7 cells/mL. Bead samples were created by resuspending MSCs in warm agarose solution, pipetting the cell-agarose solution onto the surface of a petri dish, and placing the petri dish on a freezer pack for several minutes. The MSC-seeded hydrogels were cultured in high-glucose Dulbecco modified Eagle medium^a supplemented with 1% insulin-transferrin-selenium+1,^c 100nM dexamethasone,^e ascorbate-2-phosphate^f (37.5 μ g/mL), and recombinant human transforming growth factor β -3 (10 ng/mL).^{17,d} Cultures were maintained for 15 days to allow accumulation of cartilage-like extracellular matrix that is indicative of chondrogenic differentiation in vitro.

Accumulation of GAG-rich extracellular matrix—Ten- to 20-mg samples were cut from the cell-seeded agarose hydrogel and digested in proteinase K^e in Tris solution overnight at 60°C. From the digests, the total

GAG content that was associated with the accumulation of large proteoglycans in chondrogenic equine MSC cultures¹⁸ was measured via the dimethylmethylene blue dye binding assay.¹⁹ These data were normalized to the wet weight of the samples.

Osteogenic differentiation—Mesenchymal stem cells were seeded in monolayer cultures at a concentration of 30×10^3 cells/cm² in 48-well plates. The cells were maintained in high-glucose Dulbecco modified Eagle medium^a supplemented with 10% fetal bovine serum, 5mM β -glycerol phosphate,^c 0.01 μ M dexamethasone,^e and ascorbate-2-phosphate (37.5 μ g/mL).^f Cultures were maintained for 15 days to allow mineralization and synthesis of alkaline phosphatase that is indicative of osteogenic differentiation in vitro. Half the cultures were fixed in neutral-buffered 2% formalin and stained with 0.5% alizarin red to identify mineralization. Alkaline phosphatase activity was determined in the remaining cultures with a commercial colorimetric assay kit.^g Each well was incubated in cell lysis buffer for 5 minutes. The cell lysate was diluted 250-fold, and the alkaline phosphatase activity was quantified per the manufacturer's instructions. Alkaline phosphatase activity was normalized to DNA in the cell lysate as determined by Hoescht dye binding,²⁰ which was normalized to a standard curve created with bovine thymus DNA.

Data analysis—Experiments were repeated for samples from 6 donor horses collected over 2 days. Because of lack of MSC growth in colony-forming cultures, the sample sizes for the second sequential 5-mL ilial fraction (aspirate) and the single 50-mL fraction taken from the contralateral ilium were 3 and 5, respectively, for measures of proliferation and differentiation. Data were log-transformed to meet the assumption of normality as determined by residual plots and analyzed via a mixed model ANOVA, with the donor animal used as a random effect. Individual comparisons were made via the least squares means procedure.^{21,h} For all outcomes except the nucleated cell counts, the harvest location and volume fraction were considered main effects. Individual comparisons of main effects and their interactions were performed on the basis of a protected *F* test, with values of *P* < 0.05 considered significant. For individual comparisons, values of *P* < 0.05 were considered significant. Data are reported as mean \pm SEM.

Results

Aspiration of bone marrow—The ease of aspiration varied among horses. For the ilium, 5-mL fractions were collected during several minutes of aspiration. For 50-mL fractions, the rate of aspiration was not greater than with a 5-mL syringe, and at least 10 minutes of aspiration was necessary to collect 50 mL. For the sternum, 5-mL fractions were readily collected in < 1 minute. For the first 50-mL aspirate, the initial 30 to 35 mL was rapidly aspirated as for 5-mL fractions. At this point, the aspiration rate slowed and several additional minutes were necessary to collect the full 50 mL. The flow rate remained slow for the second 50-mL fraction, requiring 5 to 10 minutes to complete.

Nucleated cells in bone marrow aspirates—In aspirates from the ilium, the first sequential 5-mL ilial aspirate contained a higher nucleated cell density than the second sequential 5-mL ilial aspirate and the single 50-mL aspirate taken from the contralateral ilium (Figure 1). In sternal aspirates, the cell density in the first sequential 5-mL fraction was not significantly different from the second sequential 5-mL fraction, and both were significantly greater than the first and second sequential 50-mL fractions. When comparing fractions between locations, the cell density in the first sequential 5-mL ilial aspirate was not significantly different than the first sequential 5-mL fraction drawn from the sternum. The cell density in the second sequential 5-mL ilial aspirate was less than that in the second sequential 5-mL fraction drawn from the sternum. Cell densities among the single 50-mL aspirate taken from the contralateral ilium, the first sequential 50-mL fraction drawn from the sternum, and the second sequential 50-mL fraction drawn from the sternum were not significantly different. Of the marrow aspirates, the only fraction that did not contain a significantly higher nucleated cell density than blood was the second sequential 50-mL fraction drawn from the sternum.

Total cell counts—The total nucleated cell counts in the single 50-mL aspirate taken from the ilium, the first sequential 50-mL fraction drawn from the sternum, and the second sequential 50-mL fraction drawn from the sternum were not significantly different (Figure 1). The total nucleated cell counts in the first sequential 5-mL ilial aspirate, the first sequential 5-mL fraction drawn from the sternum, and the second sequential 5-mL fraction drawn from the sternum were not significantly different and were less than the 50-mL aspirates. The total nucleated cell count in the second sequential 5-mL ilial aspirate was less than all other fractions.

MSC yield through passage 1—The centrifugation technique used to process the bone marrow as-

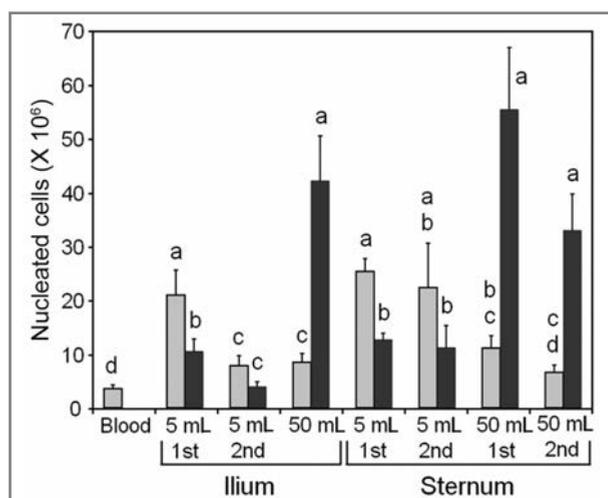


Figure 1—Mean \pm SEM nucleated cell density (cells/mL [gray bars]) and total nucleated cell count ($\times 10$ [black bars]) in sequential bone marrow aspirates of 5- or 50-mL volumes from the ilium and sternum of 6 horses. ^{a-d}Different lowercase letters indicate significant (*P* < 0.05) differences within and between aspirates from the sternum and ilium.

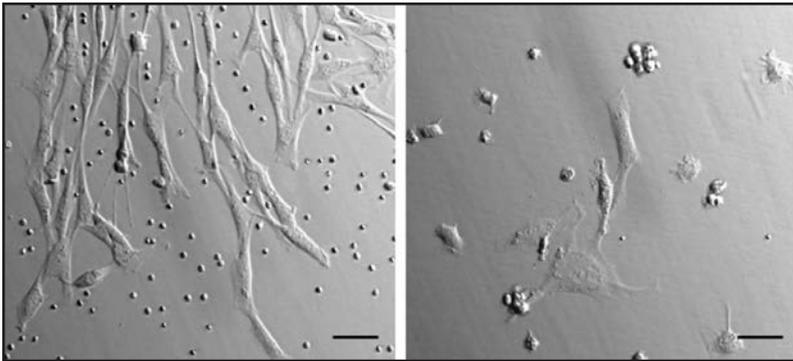


Figure 2—Photomicrographs of equine cells in monolayer culture. Left image—Spindle-shaped MSCs in colony-forming cultures after 8 days of culture. Right image—Non-colony-forming cells. Bar = 100 μ m.

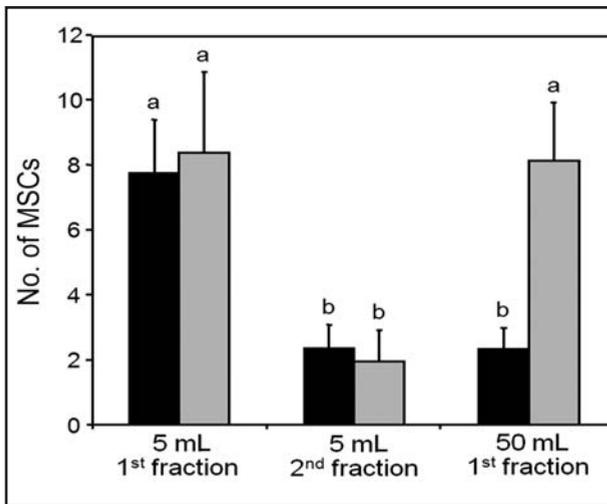


Figure 3—Mean \pm SEM MSC yield from 6 horses at passage 1 in first and second sequential aspirates (fractions) from the ilium and sternum. Data for each culture were first calculated by normalizing the number of MSCs obtained after colony-forming culture and passage 1 expansion culture to the number of nucleated cells seeded into colony-forming culture, yielding the number of MSCs/100 nucleated cells (black bars). The maximum potential MSC yield ($\times 10^6$) that would have been realized had all of the nucleated cells been seeded into colony-forming culture is indicated by gray bars. ^{a,b}Different lowercase letters indicate significant ($P < 0.05$) differences identified as a function of fraction independent of location (ilium or sternum).

pirates collected approximately 50% to 75% of the nucleated cell population and eliminated $> 95\%$ of the RBCs. At the end of the 8-day colony-forming culture, visual inspection by use of a light microscope identified MSC colonies in all the first sequential 5-mL ilial aspirates, the first sequential 5-mL fraction drawn from the sternebra, the second sequential 5-mL fraction drawn from the sternebra, and the first sequential 50-mL fraction drawn from the sternebra cultures. Few to no colonies were observed for 3 of the second sequential 5-mL ilial aspirates, 1 of the single 50-mL aspirates taken from the ilium, and all 6 of the second sequential 50-mL fractions drawn from the sternebra cultures. Cultures that did not appear to contain MSC colonies were trypsinized and reseeded, without counting, into a single well of a 6-well plate. Most reseeded cells did not mor-

phologically resemble colony-forming MSCs after 24 hours, and little proliferation was observed during the 3-day post-colony expansion period (Figure 2). Therefore, these cultures were terminated, and the yield was taken to be zero for analysis. Data for each culture were first calculated by normalizing the number of MSCs obtained after colony-forming culture and passage 1 expansion culture to the number of nucleated cells seeded into colony-forming culture (Figure 3). Comparisons of the individual samples are not reported because the interactions between location and fraction were not significantly ($P = 0.53$) different. Similarly, there was

no difference between ilium and sternal aspirates because the main effect of location independent of fraction was not significant ($P = 0.36$). When considering the main effect of fraction independent of location, the cell yield from the first 5-mL fraction was greater than that from the second 5-mL and first 50-mL fractions. The cell yield was not significantly different between the second 5-mL and first 50-mL fractions. The maximum potential yield was calculated for each sample by multiplying the MSC yield as a function of nucleated cells by the total number of nucleated cells in the aspirate. Comparisons of the individual samples are not reported because the interactions between location and fraction were not significantly ($P = 0.21$) different. Similarly, there was no difference between ilium and sternal aspirates because the main effect of location independent of fraction was not significant ($P = 0.29$). When considering the main effect of fraction independent of location, the maximum cell yield from the first 5-mL fraction and 50-mL fraction was greater than that from the second 5-mL fraction. The maximum cell yield was not significantly different between the first 5-mL and first 50-mL fractions.

MSC proliferation in passage 2—Because of lack of MSC growth in colony-forming cultures, the sample sizes for the second sequential 5-mL ilial aspirate and the single 50-mL aspirate taken from the contralateral ilium were 3 and 5, respectively. Similarly, the second sequential 50-mL fractions drawn from the sternebra samples were not evaluated for the remainder of the study owing to the low yield from colony-forming culture. Mean proliferation among groups ranged from 1.12 to 1.35 population doublings/24 h. Comparisons of the individual samples are not reported because the interactions between location and fraction were not significant ($P = 0.55$). There was no difference among the first and second 5- and 50-mL aspirates because the main effect of fraction independent of location was not significant ($P = 0.46$). When considering the main effect of location independent of fraction, cultures from the ilium had 0.13 more population doublings/24 h than did sternal cultures ($P < 0.05$).

Overall maximum potential yield—The overall maximum potential yield for each aspirate was calculated on the basis of the yield after passage 1 and the

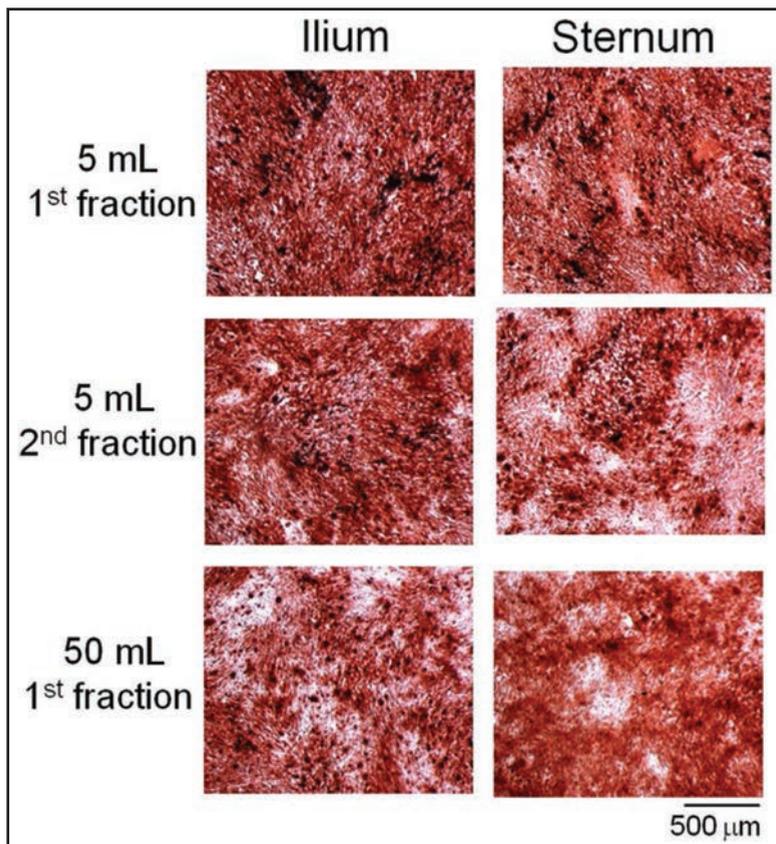


Figure 4—Photomicrographs of equine MSCs undergoing osteogenesis; cells were obtained in 5- or 50-mL aspirates (fractions) from the sternum or ilium. Alizirin red stain; bar = 500 μ m (applies to all panels).

population doubling rate during passage 2. Maximum potential yields for samples that did not produce MSCs from colony-forming culture were considered zero for this analysis. Comparisons of the individual samples are not reported because the interactions between location and fraction were not significant ($P = 0.09$). When considering the main effect of location independent of fraction, cultures established from the ilium (275×10^6 cells $\pm 79 \times 10^6$ cells) resulted in a cell yield 2.1 times that of sternal cultures ($P < 0.05$). When considering the main effect of fraction independent of location, the cell yield from the second 5-mL fractions (53×10^6 cells $\pm 27 \times 10^6$ cells) was 23% and 19% of the first 5-mL (249×10^6 cells $\pm 84 \times 10^6$ cells) and first 50-mL (304×10^6 cells $\pm 82 \times 10^6$ cells) fractions, respectively ($P < 0.05$). The cell yield was not significantly ($P = 0.49$) different between the first 5-mL and first 50-mL fractions.

Chondrogenesis—Mean GAG accumulation among groups ranged from 2.1 to 3.0 μ g/mg wet weight. Comparisons of the individual samples are not reported because the interactions between location and fraction were not significant ($P = 0.66$). There was no difference among the first and second 5-mL and 50-mL aspirates because the main effect of fraction independent of location was not significant ($P = 0.62$). When considering the main effect of location, cultures established from the ilium accumulated 29% more GAG than did sternal cultures ($P < 0.05$).

Osteogenesis and alizarin red staining—For alkaline phosphatase activity, interactions between location and fraction were not significant ($P = 0.66$). Differences due to the main effect of location ($P = 0.93$) and fraction ($P = 0.45$) were not significant. All cultures were positive for deposition of calcium via alizarin red staining (Figure 4).

Discussion

The objective of commercial services that culture-expand autologous MSCs for equine application is to obtain millions of cells in the quickest and most efficient manner. As a first step in comparing small and large aspirates in the present study, the total nucleated cell density was considered, given that in human bone marrow aspirates, the nucleated cell density decreases with aspiration beyond 1 mL due to dilution of bone marrow by blood.⁹ Although cytologic examination was not performed to identify blood cells, the low nucleated cell density in blood suggests that dilution of bone marrow with blood is consistent with a decrease in nucleated cell density. In ilial bone marrow aspirates, the 63% decrease in nucleated cell density between the first and second sequential 5-mL fractions and the 60% decrease in nucleated cell density in IL-50 relative to the first sequential 5-mL ilial aspirate may be indicative of blood contamination with increasing aspirate volume. In sternal aspirates, nucleated cell counts did not suggest blood contamination in the first 10 mL of aspirate because the nucleated cell density in the first sequential 5-mL fraction drawn from the sternebra and the second sequential 5-mL fraction drawn from the sternebra samples was not significantly different. However, the 53% decrease in nucleated cell density from the first sequential 5-mL fraction drawn from the sternebra and the first sequential 50-mL fraction drawn from the sternebra may be indicative of blood contamination with increasing aspirate volume. Finally, the lack of significant differences in nucleated cell density between blood and the second sequential 50-mL fraction drawn from the sternebra would be consistent with substantial blood contamination beyond 50 mL of sternal aspirate.

In human bone marrow samples, increases in bone marrow aspirate volume coincide with decreases in the MSC density as determined by quantification of colony-forming units.⁹ In the present study, considering the yield of MSCs after passage 1 relative to the number of nucleated cells seeded into colony-forming culture, a similar overall pattern of decreasing MSC density between the first and second 5-mL fractions was apparent, which is consistent with the findings for equine sternal bone marrow aspirates.^{10,i} Unlike that in human aspirates, the reduction in MSC yield with volume did not necessarily coincide with a reduction in nucleated cell density because nucleated cell counts for the first sequential 5-mL fraction drawn from the sternebra and the second sequential 5-mL fraction drawn from the

sternebra were not significantly different. This result suggested that, in addition to bone marrow, the sternum contains an additional source of cells that is present in high density yet is not rich in MSCs. In the single 50-mL aspirate taken from the contralateral ilium and the first sequential 50-mL fraction drawn from the sternbra, the MSC yield relative to the number of nucleated cells seeded into colony-forming culture suggested that large aspirate volumes do not capture MSC-rich reservoirs of bone marrow beyond the initial 5-mL fraction. Finally, the lack of significant MSC growth in the second sequential 50-mL fraction drawn from the sternbra further supported the possibility that aspiration beyond 50 mL retrieves mostly blood.

After passage 1, calculations of the maximum potential MSC yield revealed that the low MSC density in 50-mL fractions relative to the first 5-mL fractions was compensated by the higher total nucleated cell count in 50-mL fractions. These data further suggested that the MSC populations in 50-mL samples were largely derived from the initial 5 mL of aspirate.

Following passage 1, proliferation in passage 2 and chondrogenic and osteogenic differentiation were not significantly different as a function of fraction. Given that MSCs are in part defined by the ability to undergo chondrogenesis and osteogenesis, these data suggested that aspirate volume does not affect the therapeutic potential of equine MSCs. However, the healing potential of MSCs is not necessarily based on the ability to differentiate into the phenotype of cells that reside in target tissues.²² A broader characterization that includes other MSC behaviors, such as synthesis of growth factors or anti-inflammatory cytokines, may be necessary to more fully elucidate the effect of aspirate volume on MSC therapeutic potential.

When considering the overall maximum potential MSC yield from sequential 5-mL fractions independent of location, the processing of both 5-mL fractions would result in only a modest increase (22%) over the initial 5-mL aspirate. Given that all cultures resulted in at least a population doubling per day in post-colony expansion culture, the benefit of processing both sequential 5-mL fractions could be equaled by the first 5-mL fraction alone with only several additional hours of post-colony expansion culture. When considering 5- and 50-mL fractions independent of location, the lack of significant difference in cell yields indicated that MSC treatments may be obtained in the same timeframe from small or large fractions. However, the initial 5 mL of aspirate alone may be considered advantageous over 50-mL fractions for 2 reasons. The yield of MSCs from the nucleated cell population in colony-forming cultures was 3 times as great for initial 5-mL fractions, compared with 50-mL aspirates. More specifically, given that colony-forming cultures are seeded by nucleated cell density, the 5-mL aspirates require less than one-third the culture materials and colony-harvesting manipulation than do 50-mL aspirates, both of which affect the costs of the processing. The 5-mL aspirates were obtained from the donor in seconds (sternum) to minutes (ilium), whereas the 50-mL fractions required approximately 10 times as long to collect. The time required to harvest large fractions may be particularly relevant to ilial aspirations for which extensive effort

was needed to obtain full 50-mL aspirates. The primary objective of this study was to evaluate the effect of aspirate volume on MSC yield and differentiation, but the collection of ilial and sternal bone marrow allowed for comparisons between these sites. From a harvesting standpoint, we noted that bone marrow was aspirated from the sternum more rapidly than from the ilium. Although the ease of aspiration in the sternbrae may be considered advantageous, it is also important to recognize that the present authors have not reported morbidity associated with ilial aspirates, whereas cardiac puncture may occur during improper insertion of the aspiration needle because of the close proximity of the heart to the sternbrae. With regard to the MSC population obtained from each site, the analysis of the effect of location independent of fraction indicated more rapid MSC proliferation in ilial aspirates relative to sternal samples. These data differ from a recent studyⁱ from the authors' laboratory on small-volume aspirates that detected no differences in MSC growth between the ilium and sternum samples, although the present study had greater sample numbers that allowed for greater distinction between locations. The difference in proliferation between ilial and sternal MSCs resulted in a 2-fold difference in the maximum potential yield, a result that predicts that MSC treatments from ilial aspirates may be obtained 1 day sooner than would be needed for sternal aspirates. Of greater consequence may be the difference in chondrogenesis between ilial and sternal samples because the ability to undergo chondrogenesis is a hallmark of the MSC phenotype, although it is not known whether chondrogenic potential is indicative of the potency of other proposed pathways by which MSCs heal tissues.

In this study, bone marrow was processed by use of a single centrifugation that pelleted most of the RBCs, and at least half the nucleated cell population was harvested in the plasma supernatant. Additional steps may be performed to harvest the maximum number of nucleated cells that accumulate in the RBC pellet with centrifugation, such as ammonium chloride lysis of the RBCs or repeated centrifugation, although for this study, these steps were not taken so that each sample could be processed in a timely manner. Protocols for preparing bone marrow aspirates for colony-forming culture can vary among laboratories. For example, in addition to centrifugation, bone marrow has been prepared by separating nucleated cells from the RBCs via density gradient or by simply diluting the bone marrow aspirate in culture medium. Although it is not fully clear whether centrifugation, density gradients, or dilution offers advantages over one another,^{23,24} it is possible that the technique used to prepare the bone marrow aspirates for colony-forming culture could influence the MSC yield.

In this study, we evaluated bone marrow from young adult (2- to 5-year-old) horses that were typical of equine patients that have received stem cell therapies from the authors. Furthermore, this age group is commonly used for live animal studies of equine musculoskeletal diseases^{25,26} and has been used by the authors to evaluate stem cell therapies in 2 studies.^{27,28} A limitation of this study was that the effect of age was

not explored, and it is not known how well the results could be extrapolated to older horses. Furthermore, a better understanding of the specific mechanisms by which MSCs heal tissues that have responded favorably in clinical cases would allow for the evaluation of the most clinically relevant MSC properties. Although additional study is needed to more fully characterize undifferentiated autologous MSCs for this emerging treatment modality, the present data indicated that small-volume aspirates from the ilium allow for the most efficient processing of MSCs with the highest proliferation and chondrogenic potential.

- a. Invitrogen, Carlsbad, Calif.
- b. Hyclone, Logan, Utah.
- c. Corning, Lowell, Mass.
- d. R&D Systems, Minneapolis, Minn.
- e. Sigma-Aldrich, St Louis, Mo.
- f. Wako Chemicals, Richmond, Va.
- g. Cell Biolabs, San Diego, Calif.
- h. Proc GLIMMIX, SAS, SAS Institute Inc, Cary, NC.
- i. Adams MK, Goodrich LG, Rao S, et al. Equine bone marrow-derived stem cells: comparing the ilium and the sternum (abstr), in *Proceedings. 57th Annu Meet Am Assoc Equine Pract* 2011;77.

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