Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue

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Objective—To determine the optimal osteogenic source of equine mesenchymal stem cells (eMSCs) and optimize collection of and expansion conditions for those cells.

Animals—10 adult Quarter Horses and 8 newborn Thoroughbred foals.

Procedures—eMSCs were isolated from bone marrow (BM), adipose tissue, and umbilical cord blood and tissue, and the osteogenic potential of each type was assessed. Effects of anatomic site, aspiration volume, and serum type on eMSC yield from BM were investigated.

Results—BM-eMSCs had the highest overall expression of the osteogenic genes *Cbfa1*, *Osx*, and *Omd* and staining for ALP activity and calcium deposition. There was no significant difference in BM-eMSC yield from the tuber coxae or sternum, but yield was significantly greater from the first 60-mL aspirate than from subsequent aspirates. The BM-eMSC expansion rate was significantly higher when cells were cultured in fetal bovine serum instead of autologous serum (AS).

Conclusions and Clinical Relevance—eMSCs from BM possessed the highest in vitro osteogenic potential; eMSCs from adipose tissue also had robust osteogenic potential. The tuber coxae and the sternum were viable sources of BM-eMSCs in yearlings, and 60 mL of BM aspirate was sufficient for culture and expansion. Expanding BM-eMSCs in AS to avoid potential immunologic reactions decreased the total yield because BM-eMSCs grew significantly slower in AS than in fetal bovine serum. Additional studies are needed to determine optimal ex vivo eMSC culture and expansion conditions, including the timing and use of growth factor–supplemented AS. (*Am J Vet Res* 2010;71:1237–1245)

In horses with limb fractures, delayed union due to implant failure before bony union is a common problem. In addition, when instability is present, the affected horse often develops laminitis in the support limb. Nonunions are more common in open and comminuted fractures and in insufficiently immobilized fractures or as a result of infection or disturbed blood supply.[1,2] In addition, the age and body weight of the patient,[3] diseases such as osteoporosis and diabetes, and NSAID use[4] may influence nonunion develop-

Abbreviations

ALP	Alkaline phosphatase
AS	Autologous serum
AT	Adipose tissue
AT-eMSCs	Adipose tissue–derived eMSCs
BM	Bone marrow
BM-eMSCs	Bone marrow–derived eMSCs
Cbfa1	Gene encoding core binding factor 1
eMSC	Equine mesenchymal stem cells
FBS	Fetal bovine serum
MSC	Mesenchymal stem cell
Omd	Gene encoding osteomodulin
Osx	Gene encoding osterix
Ppib	Gene encoding peptidyl-prolyl cistrans isomerase B
UCB	Umbilical cord blood
UCB-eMSCs	Umbilical cord blood–derived eMSCs
UCT	Umbilical cord tissue
UCT-eMSCs	Umbilical cord tissue–derived eMSCs

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ment. In adult equine fracture repair, particularly in fractures of the upper limb, low-grade instability often causes implant failure by cyclic loading before bony union. In conjunction, low-grade instability during the healing period results in lameness in the treated limb. If lameness persists, then overuse of the contralateral limb occurs, resulting in laminitis in the supporting limb. Therefore, an important goal in equine fracture repair is to expedite bony union to help prevent these fatal complications. The injection of adult-derived MSCs directly into the fracture site represents a promising mechanism to accelerate fracture healing and thereby decrease the risk of nonunion in humans and animals.[5] Although a patient's own stem cells will migrate to the fracture site to repair the skeletal defect, providing the injured area with an excess of cells may accelerate fracture healing[5] or initiate it in the situation of nonunions.

In equine as well as human medicine, adult-derived MSCs may be isolated from multiple tissue sources including BM,[6–9] AT,[10,11] UCT,[12] or UCB.[13–16] However, issues concerning ease of isolation, cell yield, and donor site complications suggest that certain sources may be more favorable than others. A concern specific to fracture healing is whether the stem cells have sufficient osteogenic potential. The differentiation of MSCs into the osteoblastic phenotype involves activation of the transcription factor genes *Cbfa1*[16,17] and *Osx*,[18] which are expressed relatively early and late, respectively, after osteogenic induction. Other markers of osteogenesis include gene expression of the matrix protein osteomodulin,[19] increased ALP activity,[9,14,15,20] and calcium deposition[9,12,14,15] into the extracellular matrix.

Because of differences in ease of isolation and, perhaps, the osteogenic potential of stem cells from various sources, it is important to identify the tissue source that would provide the optimal osteogenic stem cell for use within a clinical setting. Another clinical issue involves the collection and expansion of BM-eMSCs, which are widely used for equine stem cell therapies. Ideally, cell therapies are initiated as soon as possible after fracture to speed healing and to prevent the development of a nonunion defect. For this reason, it is necessary to expand isolated cells as rapidly as possible. Variables that affect both time of expansion and final cell yield, such as patient age,[21] are outside the clinician's control. However, the clinician can affect the time to reach clinically relevant cell numbers for transplantation through selection of the BM aspiration site, BM aspiration fraction, and source of serum used during culture expansion. Thus, it would be useful to determine the effect such variables have on eMSC expansion.

The purpose of the study reported here was to directly compare multiple tissue sources of adult-derived eMSCs to determine the source that would yield maximally osteogenic cells in vitro. Additionally, we sought to optimize isolation and postisolation expansion protocols to yield a maximal number of BM-eMSCs. In this way, we hoped to assist clinicians in choosing and preparing the ideal source of adult-derived eMSCs for equine fracture repair.

Materials and Methods

Animals—Ten adult Quarter Horses and 8 newborn Thoroughbred foals were used as sources of eMSCs. All Quarter Horses were property of the Center for Equine Health at the University of California-Davis. Consent of horse and farm owners was obtained for Thoroughbred mares housed at the Thoroughbred farm. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of California-Davis.

BM aspiration and eMSC isolation—Bone marrow aspirates were collected from 7 Quarter Horse geldings between 1 and 18 years of age (mean age, 4.1 years). Collection and isolation of eMSCs were performed by use of a modification of published protocols.[8,22] For collection, horses were moved to standing stocks and sedated with detomidine hydrochloride (0.01 mg/kg, IV) and butorphanol (0.01 mg/kg). Local anesthesia was provided with 10 mL of 1% lidocaine solution.

The aspiration site for the sternum was in the ventral midline at a position, in a cranial and caudal direction, that bisected the caudal edge of the point of the elbow joint. The aspiration site for the tuber coxae was located at the ventral third of the caudal aspect of the cortex of the right or left ileal wing, approximately 4 cm axial to the tuber coxae. This location allowed access to the tuber coxae from the caudal aspect of its cortex. Both sites were approached similarly. A skin incision was made with a No. 15 scalpel blade, and a 13-gauge, 6.35-cm (sternum) or 8.89-cm (tuber coxae) BM aspiration needle[a] was advanced by slow clockwise and counterclockwise rotation until the marrow cavity was penetrated and the stylet was removed. Two to 3 mL of heparin (1,000 U/mL), from a total of 10 mL within the syringe, was injected into the marrow space, from which 60 mL of BM was immediately aspirated. Another 60 mL of BM was aspirated with a second syringe containing 10 mL of heparin.

To isolate eMSCs, each BM aspirate was mixed with an equal volume of Hank balanced salt solution,[b] agitated gently, and centrifuged at 300 × *g* for 15 minutes. The supernatant was discarded, and the pellet was resuspended in balanced salt solution and centrifuged at 1,000 × *g* for 5 minutes. The pelleted cellular fraction was resuspended in 50 mL of 10% serum medium (RPMI,[b] 10% FBS,[b] and 1% antibiotic-antimycotic[b]) and plated into 75-cm^3 flasks.[c] The equivalent of 5 mL of unprocessed BM was added to each flask. Cells were incubated for 2 days at 37°C with 5% CO_2 in a standard humidified incubator,[d] after which nonadherent cells were washed away with PBS solution. Culture medium was changed every 2 days until colonies of ≥ 30 cells were observed (mean ± SD interval, 8.4 ± 0.4 days). After first passage with 0.25% trypsin-EDTA,[b] the medium was changed the next day to wash away any remaining nonadherent cells and every 4 days after this. Cells were expanded up to passage 3 and subsequently frozen in 5% DMSO and 95% culture medium for later characterization.

AT collection and eMSC isolation—Adipose tissue was collected from the tail head of 3 Quarter Horse

yearling geldings. Horses were sedated and anesthetized as described for BM collection. Local anesthesia was provided by means of a line nerve block with 10 to 20 mL of 1% lidocaine solution. A 10-cm incision was made to 1 side of the tail head (4 to 5 cm from midline) with a No. 10 scalpel blade. Sharp dissection with Metzenbaum scissors was used to remove 9 to 10 g of AT with tissue forceps. The AT was placed in a sterile tube, and the subcutaneous tissue was sutured with 2-0 polyglactin 910[e] in a simple continuous pattern, followed by skin closure with stainless steel staples.

The AT-eMSCs were isolated following a modification of published protocols.[23,24] Collected AT was minced with a razor blade and washed 4 to 5 times with PBS solution. An equal volume of a solution of collagenase (0.1% collagenase type I[f]) and 1% bovine serum albumin in PBS solution was added, and tissue samples were incubated at 37°C with 5% CO_2 in a standard humidified incubator for 1 hour and shaken every 15 minutes. Collagenase was neutralized with an equal volume of medium (Dulbecco modified Eagle medium F12, 10% FBS, and 1% penicillin-streptomycin). Tissue samples were centrifuged at 162 × *g*, floating adipocytes were removed, and the stromal vascular fraction was resuspended in complete medium. Cells were plated at 3,500 cells/cm^2 and incubated for 2 days, after which nonadherent cells were removed by washing in PBS solution. Cells were passaged and cryopreserved as described previously.

UCT collection and eMSC isolation—Umbilical cord tissue was collected from 5 Thoroughbred foals at birth. The UCT-eMSCs were isolated and cryopreserved as described elsewhere.[25] Briefly, each umbilical cord was tied at both ends with cable ties, separated from the foal and placental tissue, and disinfected with 0.05% chlorhexidine.[g] Then the umbilical cord was shipped from the farm to the laboratory overnight.

Tissue was processed by removing a 5- to 8-cm section of UCT, removing the loose amnion from the exterior of the cord, then removing the large blood vessels from the interior. The UCT was soaked for 1 hour in Dulbecco PBS solution containing 3% penicillin-streptomycin and 0.1% amphotericin B[h] (antimycotic). In a laminar flow hood, tissue was removed from the soaking solution and minced finely by use of sterile scissors. Ten to 15 mL of minced UCT was transferred to a 50-mL polypropylene centrifuge tube, to which was added 25 mL of a digestion cocktail (0.2% [wt/vol] collagenase type I, 0.2% [wt/vol] collagenase type II, 0.004% [wt/vol] elastase, and 0.1% [wt/vol] hyaluronidase, dissolved in Dulbecco PBS solution and sterilely filtered). The tissue and digestion cocktail was mixed thoroughly, incubated in a 37°C water bath for 2 hours, and mixed every 15 minutes. After incubation, cells were pelleted at 100 × *g* for 10 minutes. Supernatant was removed and filtered through a 70-µm nylon filter. Thirty-six milliliters of Dulbecco PBS solution was added to the remaining tissue in the tube and mixed. Cells were then pelleted again at 100 × *g* for 10 minutes. Supernatant was filtered again through a 70-µm nylon filter. Dulbecco PBS solution was added to the supernatant to fill a conical tube to 50 mL, mixed thoroughly, and spun at 1,200 × *g* for 10 minutes to pellet cells. Supernatant was discarded, the pellet was resuspended in Dulbecco PBS solution, and the spin was repeated. Again, the supernatant was discarded and pellets were resuspended in 6 mL of 20% serum medium (Dulbecco modified Eagle medium, 10% FBS,[i] 10% equine serum,[i] and 1% penicillin-streptomycin) with 0.1% amphotericin B.[h] Cells were plated in a single 25-cm^2 flask and incubated in standard cell culture conditions of 21% O_2. After the first 2 medium changes, the amphotericin B was not included in the culture medium. Cells were expanded and cryopreserved at passage 2 to 3.

UCB collection and eMSC isolation—Umbilical cord blood was collected from the umbilical cords of 3 Thoroughbred foals at birth, and eMSCs were isolated and cryopreserved as described elsewhere.[15,25] Briefly, UCB samples were extracted into a syringe or a standard 250-mL collection bag containing citrate-phosphate-dextrose-adenine[j] and shipped overnight from farm to laboratory. The UCB was RBC and volume depleted[k] and then passed through a Ficoll gradient[l] and plated in 20% serum medium with 0.1% amphotericin B.[h] Cells were cryopreserved at passage 2 to 3.

Osteogenic differentiation—The eMSCs from each tissue source (BM, UCB, UCT, and AT) were plated at 5,000 cells/cm^2 and expanded to passage 4 to 5 for characterization of osteogenic potential. A minimum of 3 samples/tissue source was used, each of which was from a different donor horse. The donors for both BM- and AT-eMSC sources were matched. On day 0, expanded eMSCs from BM, UCT, and AT were plated on 10-cm plates (for RNA collection) and 35-mm plates (for staining) at a density of 3,000 cells/cm^2 in 10% serum medium.

After collection of samples (day 1), culture medium was replaced with 10% serum medium containing osteogenic supplements (50 µg of ascorbic acid[m]/mL and 5mM β-glycerophosphate[m]). Day 1 samples never came into contact with osteogenically supplemented media. Culture medium was changed on day 5 by replacing half the volume with fresh medium. Because UCB-eMSCs did not grow in 10% FBS medium and therefore required the use of 20% serum medium, the osteogenic potential of UCB-eMSCs was compared with that of UCT-, UCB-, and BM-eMSCs expanded and differentiated in 20% serum medium. This medium was initially used to isolate and expand the UCT- and UCB-eMSCs and has been shown to yield sufficient numbers of UCB-eMSC.[15]

RNA collection and PCR analysis—On days 1, 4, and 10, cells were briefly washed with PBS solution, and total RNA was collected from 10-cm dishes and purified by use of a commercial kit.[n] Fifty to 1,000 ng of total RNA was reverse-transcribed by use of a first-strand synthesis system for reverse transcriptase PCR assay[o] in an automatic cycler.[p] Primers were designed through use of equine gene sequences (**Appendix**; GenBank accession Nos. AF113507, XM_001494930, XM_001917971, and NM_001099761 for *Cbfa1*, *Osx*, *Omd*, and *Ppib*, respectively). Expression of RNA was determined by performance of real-time RT-PCR analysis. The PCR assay was carried out with DNA polymerase[q] in an automated cycler.[r] Samples were run in duplicate,

and fluorescence was monitored by SYBR green intercalation into amplicons at each cycle. Cycle conditions were as follows: denaturation at 95°C for 30 seconds, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and elongation at 72°C for 20 seconds. Data were normalized to the results for the *Ppib* gene. The PCR products were verified by sequencing (data not shown) and compared with known sequences in the National Center for Biotechnology Information database.

Stains—On days 1, 2, 4, 6, 8, and 10, 35-mm plates were rinsed 3 times with warm PBS and were either stained with a commercial ALP activity staining kit[m] in accordance with the manufacturer's instructions or fixed in a 5% formaldehyde solution for 15 minutes, rinsed with deionized water for 15 minutes, and stained with a 0.5% alizarin red[m] in water for 30 minutes. Plates were then rinsed with deionized water until the rinse water was clear. All plates were dried, and digital images were acquired by use of a flatbed scanner.

Aspiration site and volume experiment—Two sequential 60-mL BM aspirates were collected from the tuber coxae and sternum of 5 Quarter Horse yearling geldings following the BM collection protocol previously described. Each 60-mL aspirate was washed and seeded into four 75-cm^3 flasks (with the equivalent of 5 mL of raw BM in each) containing 50 mL of 10% serum medium and cultured as described previously. The BM-eMSC isolation protocol was also followed as described. At first passage (8.4 ± 0.4 days after seeding), cells were trypsinized, counted via hemocytometer, and cryopreserved. Cell counts from the first and second aspirates were combined to compare total cell yield from both the tuber coxae and sternum. Because there was no significant difference in total cell yield between the tuber coxae and sternum, cell counts from both sites were then combined to compare cell yield from the first and second aspirates.

AS collection and serum type experiment—Autologous serum was collected from 3 Quarter Horse yearling geldings, a 6-year-old Quarter Horse gelding, and an 18-year-old Quarter Horse gelding. Blood collection was performed at the time of BM or AT collection, and as such, horses were sedated as described for BM collection. The jugular groove of each horse was clipped and surgically prepared, and a 7.62-cm, 10-gauge catheter was inserted into the jugular vein. The catheter was attached to tubing that emptied into a 500-mL glass bottle. The jugular vein was occluded below the distal end of the catheter, and a blood sample was extracted with the aid of a vacuum pump. A total of approximately 1 L of blood was collected from each donor horse. Blood samples were coagulated at room temperature (approx 25°C) for 45 minutes, and 20 mL of sterile glass beads was added to each 500-mL bottle. Bottles were centrifuged at 1,328 × *g* for 45 minutes. In a sterile cell culture hood, serum was aspirated, sterile filtered, and frozen at –20°C until used.

The BM-eMSCs from the same 5 horses were seeded at a density of 5,000 cells/cm^2 into 6 × 10-cm plates. Three plates contained 10% serum medium, and the remaining 3 plates contained 10% AS medium (RPMI,[b] 10% AS, and 1% antibiotic-antimycotic[b]). After 4 days in culture, cells within each experimental group were trypsinized and combined. The total cell count for each experimental group was determined by use of a digital automated cell-counting system.[o]

Statistical analysis—For PCR-assay data, the expression of genes associated with MSC differentiation relative to that of *Ppib* ($2^{-\Delta Ct}$)[26] was calculated and averaged for each tissue source and then compared among eMSC collection sites by use of an unpaired, 2-tailed Student *t* test. For the aspiration volume, aspiration site, and serum type experiments, paired 2-tailed Student *t* tests were performed to determine whether the difference between groups was significant. Significance for all tests was set at a value of $P < 0.05$.

Results

Characterization of eMSCs—All eMSCs were isolated on the basis of plastic adherence. Isolated cells had a fibroblastic appearance characteristic of MSCs[11,12,15,27] (**Figure 1**). The eMSCs isolated from BM and AT had the most spindle-like morphology, whereas eMSCs isolated from UCT and UCB were more rounded. In all situations, the cellular population was mostly homogeneous in cellular morphology. Populations became more homogeneous with subsequent passages, as was expected.[27]

Comparison of osteogenic potential of eMSCs—The osteogenic potential of eMSCs was determined by use of quantitative PCR assay, enzymatic assays, and staining of matrix deposition. In the 10% serum experiment, eMSCs derived from BM, AT, and UCT were compared (**Figure 2**). At day 1, prior to induction of the eMSCs with osteogenic medium, gene expression of *Cbfa1* was not significantly different among the 3 tissues. However, gene expression of *Osx* and *Omd* was significantly higher in BM than in AT and UCT. At day 4, BM-eMSCs had significantly higher expression of *Cbfa1*, *Osx*, and *Omd* than did UCT- and AT-eMSCs. The AT-eMSCs also had significantly higher gene expression of *Osx* than did UCT-eMSCs. At day 10, BM-eMSCs had significantly higher expression of all 3 genes than did UCT-eMSCs and significantly higher expression of *Omd* than did AT-eMSCs. There was no significant difference in the gene expression of *Cbfa1* and *Osx* between BM- and AT-eMSCs. Gene expression of *Cbfa1* in AT-eMSCs was significantly higher than that in UCT-eMSCs.

The 20% serum experiment was designed to compare osteogenic induction of BM-, UCT-, and UCB-eMSCs. At day 1, BM-eMSCs had significantly higher gene expression of *Cbfa1* than did UCT- and UCB-eMSCs, as well as significantly higher expression of *Osx* than did UCT-eMSCs. There was no significant difference in gene expression of *Omd* among these 3 tissues at day 1. At day 4, BM-eMSCs had significantly higher expression of all 3 genes than did UCT- and UCB-eMSCs (with the exception of *Osx* with UCB). In addition, there was significantly higher gene expression of *Cbfa1* in UCT-eMSCs than in UCB-eMSCs. At day 10, *Osx* expression was significantly higher in BM-eMSCs than in

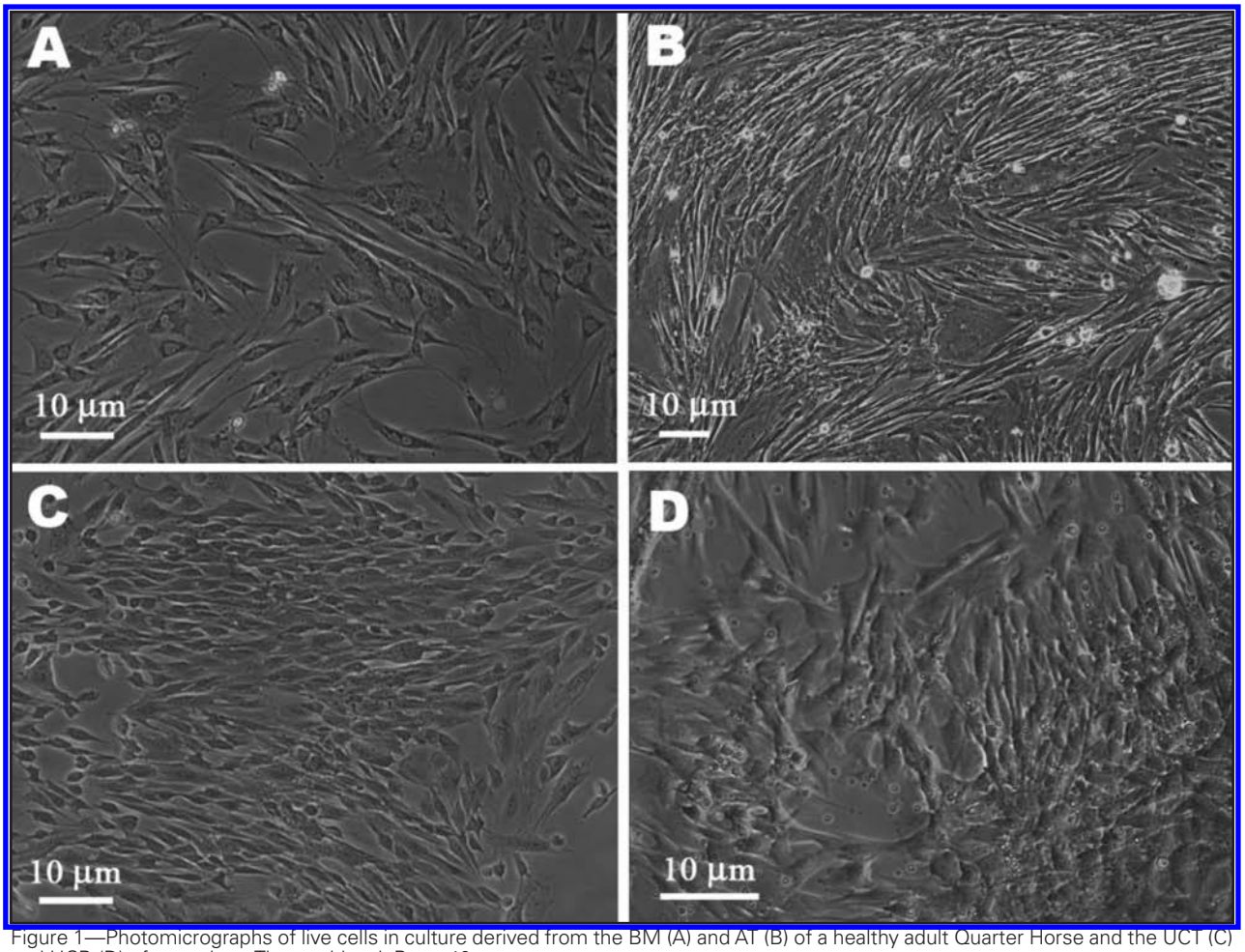

Figure 1—Photomicrographs of live cells in culture derived from the BM (A) and AT (B) of a healthy adult Quarter Horse and the UCT (C) and UCB (D) of a newborn Thoroughbred. Bar = 10 µm.

UCT- and UCB-eMSCs. For *Cbfa1* and *Omd*, there appeared to be increased expression by BM-eMSCs, compared with expression in UCT- or UCB-eMSCs, but significance was not achieved. Thus, BM-eMSCs had the greatest expression of osteoblast differentiation marker genes, compared with other tissue sources in both serum conditions.

Calcium deposition and ALP activity, as analyzed through histologic staining, were used to qualitatively assess the osteogenic potential of eMSCs from the various anatomic locations. Whereas all sources had some osteogenic potential as evidenced by these stains, BM-eMSCs in the 10% serum experiment stained most intensely for ALP activity and calcium deposition, compared with staining in UCT- and AT-eMSCs (**Figure 3**), although AT-eMSCs stained nearly as strongly as BM-eMSCs at culture days 8 and 10. In addition, UCT-eMSCs stained minimally for ALP activity throughout the duration of the study. These data indicated that BM-eMSCs differentiated into osteoblasts more quickly and efficiently than eMSCs from the other tissue sources.

For BM-, UCT-, and UCB-eMSCs cultured in 20% serum, all sources had some degree of osteogenic potential as indicated by ALP activity and calcium deposition stains. The BM-eMSCs again stained most strongly for ALP activity and calcium deposition, similar to results in the 10% serum study. The UCT-eMSCs stained almost as strongly for calcium as the BM-eMSCs, but again stained minimally for ALP activity. Culture in 20% serum containing osteogenic supplements enhanced osteogenic differentiation in BM-eMSCs and UCT-eMSCs, compared with differentiation in 10% serum medium, as indicated by stronger calcium staining. Together, these data suggested that BM-eMSCs differentiated toward the osteoblastic lineage more rapidly and efficiently than eMSCs derived from other tissue sources.

Influence of aspiration volume, anatomic site, and serum source on BM-eMSC yield—No significant (P = 0.17) difference in BM-eMSC yield was detected for BM aspirates from the tuber coxae and sternum of yearling horses (mean ± SD yield, 2.23×10^6 cells ± 0.96×10^6 cells vs 1.30×10^6 cells ± 0.56×10^6 cells, respectively). In contrast, there was a significantly (P = 0.03) greater eMSC yield from the first 60-mL aspiration (2.56×10^6 cells ± 0.97×10^6 cells) than in the second 60-mL aspiration (9.76×10^5 cells ± 0.50×10^5 cells). Significantly (P = 0.005) more BM-eMSCs were obtained from BM aspirates cultured with medium containing 10% FBS than were those cultured in medium containing 10% AS (1.87×10^6 cells ± 0.44×10^6 cells vs 1.05×10^6 cells ± 0.26×10^6 cells.

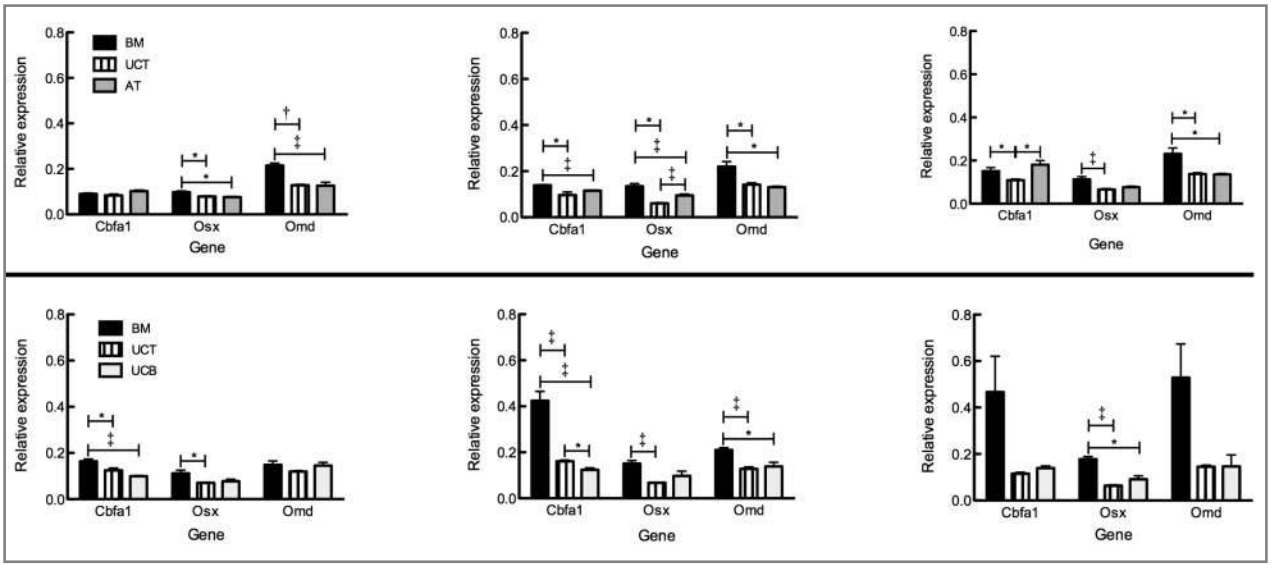

Figure 2—Mean ± SEM relative gene expression of *Cbfa1* (CBFA1), *Osx* (OSX), and *Omd* (OM) in eMSCs cultured in 10% (top panels) and 20% (bottom panels) serum medium from BM and AT from healthy adult Quarter Horses (n = 3, for all except AT [2]) and UCT and UCB from newborn Thoroughbreds (3). Total RNA was collected at day 1 (before induction of osteogenesis; left panels), day 4 (middle panels), and day 10 (right panels). The 10% serum medium contained FBS. The 20% serum medium contained FBS (10%) and equine serum (10%). *,†,‡Indicated values differ significantly (*$P < 0.05$, †$P < 0.001$, and ‡$P < 0.01$).

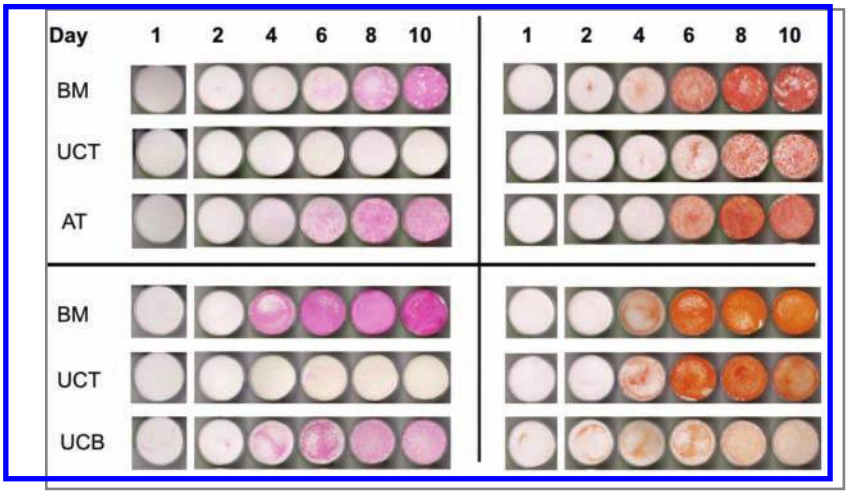

Figure 3—Photographs of results of staining for ALP activity (left panels) and alizarin red staining for calcium deposition (right panels) in eMSCs derived from BM and AT from healthy adult Quarter Horses and UCT and UCB from newborn Thoroughbreds. The eMSCs were induced with osteogenic medium containing 10% (top panel) or 20% (bottom panels) serum on day 1. Plates were stained at days 1 (before osteogenic medium was added), 2, 4, 6, 8, and 10. Images are representative of 3 separate experiments.

Discussion

Mesenchymal stem cells are an adult-derived stem cell population that can be obtained from various body tissues. These multipotent cells are capable of differentiating into tissues of the mesenchymal lineage including bone, cartilage, and fat[9,27] and may also provide trophic mediators that attenuate the inflammatory response.[28] In equine clinical practice, BM-eMSCs are used to treat lesions of bone, cartilage, ligament, and tendon. For example, stem cells have reportedly been used to repair soft palate defects[29] and superficial digital flexor tendon lesions.[30] Bone marrow is commonly chosen as a source of eMSCs because of the high yield of cells and ease of their attainment. Although many studies[27,31] have confirmed the osteogenic potential of BM-MSCs, other studies have highlighted the osteogenic potential of MSCs from alternate sources. For example, MSCs with osteogenic capacity have been isolated from AT,[10,11,32] UCB,[15] and UCT.[12,33] To our knowledge, the study reported here is the first in which the osteogenic potential of stem cells derived from equine BM, AT, UCT, and UCB was compared.

Our data suggested that BM-eMSCs possess the highest in vitro osteogenic potential, compared with eMSCs derived from other sources, as indicated by osteogenic gene expression and mineral deposition. The AT-MSCs were also capable of robust osteogenic differentiation, even though expression of osteogenic genes was significantly lower than that in BM-eMSCs at most time points. Our findings were consistent with those reported for other species in which the osteogenic potential of MSCs from various tissue sources was compared.[10,11,32] For example, human BM-MSCs deposit more calcified matrix than do human AT-MSCs, although human AT-MSCs have greater ALP activity.[10] Although the osteogenic capability of AT-eMSCs may be slightly lower than that of BM-MSCs, AT reportedly yields higher numbers of MSCs per unit volume than does BM,[34] which will enable clinicians to reach clinically relevant cell numbers for transplantation with reduced expansion times. Furthermore, the collection of AT may be a safer alternative to BM collection, which is associated with the risk of compromising the thoracic cavity and potentially puncturing the heart

when aspirating from the sternum. However, it may be difficult to obtain fat from highly fit athletes, there may be disease at the collection site, and obtaining a sterile sample from AT is more difficult than from BM. Overall, in some circumstances, AT may provide a good alternative clinical source of eMSCs. Regarding the expression of osteogenic genes, BM-eMSCs generally have the highest osteogenic capacity even before induction with osteogenic medium, indicating the presence of a relatively high number of osteoprogenitor cells at the time of collection. It is likely that the cells derived from these tissues are a mixture of stem cells, partially differentiated cells, and fully differentiated cells.[35–37]

Compared with BM- and AT-eMSCs, UCB- and UCT-eMSCs had poor osteogenic differentiation capacity. However, in another study[15] by our group, UCB-eMSCs had osteogenic capacity in the presence of the osteogenic supplements dexamethasone and recombinant human bone morphogenetic protein-6. In the present study, increasing the serum concentration in culture medium enhanced osteogenesis in UCT-eMSCs. The UCB- and UCT-eMSCs also required a medium with a large amount of serum for efficient expansion; UCB-eMSCs cells did not proliferate in the 10% serum medium. Taken together, these data suggested that the osteogenic capacity of UCT- and UCB-eMSCs may be enhanced by the presence of growth factors.[38,39] One advantage of placental tissue–derived MSCs may be their longevity. For example, a study[34] of human BM-, AT-, and UCB-MSCs revealed that UCB-MSCs were capable of sustained culture through at least passage 10, and senescence was never observed, whereas senescence occurred in BM-MSCs and AT-MSCs at passage 7 and 8, respectively. In addition, placental-derived MSCs may be more committed to promoting angiogenesis,[33] which is a critical step in the bone-healing cascade and may be more flexible in other types of tissue regeneration.[40,41] Although not robustly osteogenic in our experimental conditions, UCB- and UCT-eMSCs may provide a useful alternative source of eMSCs if maintained in appropriate growth and differentiation conditions.

Large numbers of MSCs are needed for clinical therapeutics. Because the yield of MSCs from raw BM is low, we investigated potential ways to maximize cell yield. For these experiments, we chose to use BM because it was the source of MSCs that had the greatest osteogenic potential. Specifically, we determined the influence of aspiration site, aspiration volume, and serum source on cell yield and growth. Although there was no difference between MSC yield from the tuber coxae and sternum, significantly more cells were derived from a first 60-mL aspirate than from a subsequent 60-mL aspirate. This is consistent with other findings that the first aliquot of equine BM from the sternum contains a significantly greater number of eMSCs than the subsequent 4 aliquots of the same volume.[30] In a study[42] of BM aspiration from the human iliac crest, it was determined that although nucleated cell number increased with increasing aspiration volume, the fraction of BM that was composed of peripheral blood increased. Furthermore, increasing aspiration volume may not significantly increase MSC yield and may simply dilute the sample[42] with peripheral-source blood that has few or no isolatable eMSCs.[30,31] In addition, in a comparison[31] of MSCs derived from equine peripheral blood and BM, it was found that MSCs from peripheral blood senesced early and had limited multilineage ability when compared with BM-eMSCs. To confirm this, additional studies are needed to compare the osteogenic potential of eMSCs from a 60-mL aspiration of BM with that of eMSCs from a 120-mL aspiration.

As mentioned, the large MSC numbers required clinically mean that MSCs must be culture expanded ex vivo. Typically, expansion media include FBS as a serum source to provide plasma proteins that promote cellular adhesion and endogenous growth factors that stimulate proliferation. For human MSCs grown in 20% fluorescein isothiocyanate–labeled FBS, up to 30 mg of fluorescein isothiocyanate–labeled FBS protein was associated with 100 million MSCs.[43] In addition, extensive washing did not remove the contamination.[43] The presence of FBS proteins on MSCs raises the possibility of immune responses in patients. For example, it has been suggested that malignant ventricular arrhythmias and sudden death in patients that received autologous myoblast transplants were attributable to culture of these cells in bovine calf serum.[44] On the other hand, patients treated with cells cultured in autologous human serum had no complications.[44] However, in similar studies,[45,46] there were no adverse effects of transplantation of MSCs cultured in FBS medium.

Use of FBS for ex vivo cell expansion is controversial with regard to potential immunologic reactions, and other considerations include a potential for contamination with prions, viruses, and zoonotic agents.[46–48] For these reasons, the use of AS might be considered advantageous. To address this issue, we determined the influence of AS on MSC yield from BM. Cell yield from BM grown in AS was significantly lower than the yield from growth in FBS. Interestingly, this contrasts with findings in humans that suggest that both MSC proliferation and osteogenic differentiation are greater in autologous human serum than in FBS.[49,50] The importance of this species difference is unclear, but our data suggested that BM-eMSC expansion in AS may not be a viable alternative to expansion in FBS under our experimental conditions. Therefore, future studies should be conducted to determine a method through which the immunogenicity of MSCs grown in this manner can be reduced. For example, 10% AS supplemented with epidermal growth factor and basic fibroblast growth factor reportedly increases cell yield, compared with results in 20% AS alone and 20% FBS.[43] In addition, growth of FBS-contaminated MSCs in AS (with both supplemental compounds) significantly reduces FBS protein contamination by 99.99% after 42 hours.[43] Hence, BM-eMSCs could be culture expanded in FBS to increase cell yield and then subsequently grown in AS to reduce immunogenicity. Alternatively, because the preparation of large volumes of AS from horses is fairly simple, unlike in the human, an investigation of growth factors that promote cell proliferation may also be viable. We believe that BM may be the optimal source of eMSCs for clinical use of fracture repair because of the high osteogenic capacity of BM-eMSCs and ease of isolation and expansion.

In the study reported here, BM-eMSCs possessed the highest in vitro osteogenic potential when compared with MSCs derived from other sources, as indicated by osteogenic gene expression and mineral deposition. Adipose tissue–derived MSCs provided an excellent alternative source of MSCs, although their acquisition may be considered more invasive than BM aspiration. A BM aspiration of 60 mL was sufficient to yield a considerable number of eMSCs for expansion. The eMSC yield from the sternum and tuber coxae did not differ in yearling horses. However, given the anatomic location of the sternum, the tuber coxae may provide a safer collection site for the horse and practitioner. Another consideration regarding the age of the horse is that it is known that the composition of BM shifts from MSC-rich red marrow to fatty, yellow marrow with age and at different rates dependent upon anatomic location. For example, the marrow at the distal end of the human femur begins to convert to yellow marrow before puberty, whereas the marrow at the proximal end of the femur remains red throughout adulthood.[51] The influence of age on MSC yield from the sternum and tuber coxae is unknown. Future studies should be conducted to optimize ex vivo MSC culture and expansion conditions, including the timing and use of growth factor–supplemented AS, and in vivo studies should be performed to confirm our in vitro results.

a. Tyco Healthcare, Mansfield, Mass.
b. Invitrogen, Carlsbad, Calif.
c. Corning Inc, Corning, NY.
d. HeraCell 150, Thermo Scientific, Walton, Mass.
e. Vicryl, Ethicon Inc, Somerville, NJ.
f. Worthington Biochemical Corp, Lakewood, NJ.
g. Chlorhexidine, Nolvasan, Fort Dodge Animal Health, Overland Park, Kan.
h. Fungizone, Invitrogen, Carlsbad, Calif.
i. HyClone, Logan, Utah.
j. Baxter Corp, Deerfield, Ill.
k. AXP, Thermogenesis, Rancho Cordova, Calif.
l. GE Healthcare, Piscataway, NJ.
m. Sigma-Aldrich, St Louis, Mo.
n. RNeasy Mini-kit, Qiagen, Germantown, Md.
o. Countess, Invitrogen, Carlsbad, Calif.
p. BioRad iCycler, BioRad, Hercules, Calif.
q. Platinum Taq DNA Polymerase, Invitrogen, Carlsbad, Calif.
r. Mastercycler Realplex^2, Eppendorf, Westbury, NY.

Appendix

Equine primer sequences used for PCR assays.

Target gene	Sequence	Amplicon size
Cbfa1	Forward: GACAGAGTCAGATTACAGACC Reverse: GGCCCAGTTCTGAAGCAC	204 bp
Osx	Forward: GGCTATGCCAATGACTACCC Reverse: GGTGAGATGCCTGCATGGA	187 bp
Omd	Forward: GAGCCAGATGAAGTCTACC Reverse: CATTGATGAGTGAATCTGCGG	250 bp
Ppib	Forward: GCTCTGTCTTCTTCCTGCTGTTG Reverse: CCAATTCGCAGGTCAAAGTACA	100 bp

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