Research Article

In Vivo Comparison of the Osteogenic Properties of Equine Mesenchymal Stem Cells From Periosteum, Muscle, and Bone Marrow with and without Demineralized Bone Matrix

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Abstract

Bone healing in horses is often problematic because the high forces associated with trauma and soft tissue disruption lead to poor healing. Limitations in this process have led to research of new methods to promote bone healing in horses. We evaluated the ability of equine MSCs with scaffolds to produce new bone in nude rats after intramuscular surgical implantation of a combination of mesenchymal stem cells and scaffolds. Bone production in the following groups of nude rats was compared by histology, histochemistry, and radiography after 8 weeks of the surgical implantation: 1) Periosteum [P] Mesenchymal stem cells [MSCs] in Fibrin glue [FG]; 2) Bone marrow [BM] MSCs in FG; 3) Muscle [M] MSCs in FG; 4) P MSCs in FG with Demineralized Bone Matrix [DBM]; 5) BM MSCs in FG with DBM; 6) M MSCs in FG with DBM; 7) DBM alone; 8) FG and DBM. MSCs were pre-induced with Osteogenic medium (OM) for 48 h prior to being implanted intramuscularly into nude rats. Bone formation was confirmed in Groups 2, 4, 5, and 6. Bone formation was evident in nude rats implanted with BM MSCs in combination with FG alone. However, bone production was increased when DBM was added as a carrier together with BM MSCs and FG. P MSCs and M MSCs were only able to produce bone when both FG and DBM were used as carriers. In this animal model, BM MSCs had the best osteogenic potential in vivo, followed by M MSCs when combined with FG and DBM.

Keywords: Horse; Nude rat, Mesenchymal Stem Cells; Bone Marrow; Fibrin Glue; Scaffolds.

Introduction

Bone healing in horses is often problematic because the high forces associated with trauma to the bone result in high energy fractures with extensive bone and soft tissue disruption [1]. Although improved stabilization techniques are continually being developed and implemented, the prognosis for fracture healing of many long bones in adult horses remains guarded due to complications [1-8] many of which are associated with slow bone healing. Methods that can be considered for promoting bone healing in the horse are similar to methods used in human orthopedic surgery, which include application of: autogenous cancellous bone graft, scaffolds, growth factors, and cell based therapies [6, 9, 10]. While the use of autogenous, cancellous bone graft is routinely used in clinical cases, many other methods to stimulate bone healing which are currently under investigation are not routinely used. The application of scaffolds and growth factors have been successful in promoting bone healing in research horses [6, 11]. However, these techniques are not consistently used in clinical equine fracture treatment.

Tissue engineering with the use of mesenchymal stem cells (MSCs) and progenitor cells combined with various scaffolds have shown promise in research models of bone healing in other species in vivo [12-20]. Few reports in bone fracture models in horse have shown different outcomes in bone repair. For instance no bone fracture repair was observed in horses when periosteal MSCs combined with fibrin glue were injected at the fracture site [21]. In another study bone repair was observed in horses treated with a combination of gelatine-tricalcium sponges, bone marrow MSCs and bone morphogenetic protein-2 [22, 23].

The success of cell based therapy in vivo depends on the optimum combination of cells, carrier, and growth factors [24, 25]. We have previously characterized and investigated equine MSCs from various sources, including bone marrow, periosteum, muscle tissue and fat in an attempt to determine an optimum source of MSCs for use in bone tissue engineering [26]. While in vitro results are similar for MSCs from several donor sources [26], potential for in vivo production of new bone varies greatly [27]. In a nude mouse model, pre-induced MSCs from bone marrow had the ability to produce new bone at a heterotopic site. In contrast, pre-induced MSCs from muscle and periosteum, which showed in vitro osteogenic potential similar to that of bone marrow derived MSCs, did not produce new bone in vivo [21, 27].

Fibrin is a natural biopolymer that has been used alone or in combination with other materials to successfully generate bone tissue [28]. Fibrin has been investigated, in the form of a fibrin hydrogel or fibrin glue (FG), which are injectable, as a carrier for cells in cell-based therapies [29-36]. Many researchers recognize fibrin glue as an excellent scaffold in tissue engineering [14]. However, the gold standard carrier for MSCs in osteogenesis has not yet been determined [13].

It is well recognized that the type of scaffold can influence cell behaviour [37-39] and osteoblasts prefer a rigid scaffold for optimum function [40]. Demineralized Bone Matrix (DBM) is a hard scaffold that is known to be osteoinductive [41-44], and the combination of DBM and fibrin glue has been used as a successful scaffold in bone tissue engineering [16, 18]. Therefore, we also investigated the addition of a hard scaffold, equine demineralized bone matrix, to the equine pre-induced MSCs and fibrin glue combination.

We hypothesized that the addition of DBM would enhance new bone production by MSCs from all tissue sources. The objective was to compare new bone production in vivo among implants containing different types of MSCs which included DBM and/or FG as a scaffold and those without DBM.

This study confirmed that equine pre-induced BM MSCs in FG with or without DBM can produce new bone at a heterotopic site when transplanted as a xenograft in nude rats, and the addition of DBM as a rigid scaffold promoted new bone production by pre-induced muscle and periosteal derived MSCs which otherwise did not produce new bone.

Materials and Methods

All animal study protocols were approved by the University of Prince Edward Island Animal Care Committee.

Tissue collection

Bone Marrow, Muscle and Periosteum samples were obtained from a horse, 3 years of age, immediately post-mortem. The horse was donated to the Atlantic Veterinary College for reasons other than this study and determined to be systemically healthy based on physical examination, complete blood count, and chemistry panel. All specimens were collected as described previously [26]. Briefly, a volume of 9.5 mL of BM was aspirated from the fourth and fifth sternebrae, and collected into a 12 mL syringe pre-loaded with 2.5 mL of 1000 IU/mL heparin. A section of muscle was dissected and harvested from the area of the left semitendinosus muscle and a section of periosteal tissue collected from the proximo-medial aspect of the left tibia. The tissues collected were placed in alpha minimal essential media (αMEM; Invitrogen, Toronto, ON, Canada) on ice.

Muscle and Periosteum Cryopreservation.

Muscle and periosteum tissues were processed within 24 hours; tissues that were not processed immediately were kept on ice and refrigerated at 4°C. Cold, sterile phosphate buffered saline (PBS, Invitrogen) was placed in petri dishes to keep a moist environment for the tissues while being cut into 1 cm segments. Tissue segments were placed into 2 mL cryovials and submerged in freezing media composed of 92.5% PBS and 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich. St. Louis, Mo., USA). The cryovials remained at room temperature for 30 minutes to allow for freezing media to penetrate the tissue. Samples were then placed at -80°C for a minimum of 24 hours. All samples were placed into a liquid nitrogen tank within 72 hours of processing. Muscle and periosteum tissues were kept frozen and digested a few

days later in order to start the experiments from passage one as well as BM cells [45, 46].

Cell Isolation

Cells were isolated from BM using a centrifuge gradient technique as described previously [26]. Briefly, the samples were centrifuged at 1500 g for 10 minutes, and the buffy coat was removed and placed into standard medium (SM) [(Dulbecco's Modified Eagle Medium (DMEM) Sigma Aldrich), 10% fetal bovine serum (FBS) (PAA, Laboratories Inc. Etobicoke ON, Canada), 100 µg/mL penicillin (Sigma Aldrich), 50 µg/mL gentamicin sulphate (Sigma Aldrich), and 0.3 µg/mL fungizone (Invitrogen)].

Cryopreserved muscle and periosteum was removed from liquid nitrogen, thawed and washed prior to isolation of MSCs. Muscle and periosteal cells were isolated using an enzyme digestion technique [26]. Briefly, muscle tissue was treated with Collagenase Type 1–2000 U/mL (Invitrogen). Then, tissue was placed in a 50 ml tube and incubated at 37°C and vortexed every 20 minutes for 1 minute. Once the tissue was digested, 10 mL of SM was added to the mixture to inhibit further enzyme digestion. The cell suspension was filtered through a 100 µm tissue strainer followed by a 70 µm tissue strainer and centrifuged at 377 g for 10 minutes. The supernatant was removed and the cell pellet was resuspended in SM. Viable cell number was determined by 0.4% Trypan blue dye exclusion and hemocytometer counts. Viable muscle cells were plated in T-75 flasks at a cell density of 33 x 10^3 cells/cm^2 in SM.

Isolation of cells from the periosteum was achieved by placing the tissue in 2000 U/ml Collagenase Type 1 for 2 hours and 50 minutes at 37°C. For the first 10 minutes, the tissue and enzyme were placed in a 37°C water bath. At the end of 10 minutes, used collagenase was replaced with fresh collagenase, and the tissue was placed into a 5% CO_2 incubator at 37°C. The tissue and enzyme mixture was vortexed every 30 minutes for 1 minute. At the end of the enzyme digestion period, collagenase was neutralized with an equal amount of SM. The cell suspension was placed through a tissue strainer, as described above, to remove remaining undigested tissue and centrifuged at 377 g for 10 minutes. Viable periosteal cells were plated in T-75 flasks at density of 33x10^3 cells/cm^2 in SM.

All cell cultures were maintained in a humidified 5% CO_2 and 95% air atmosphere incubator at 37°C. Cells were allowed to adhere for 24-48 hours; unattached cells were washed off with 1X PBS. Medium was changed three times per week. Cells were passaged once they reached 85 % confluence.

Cell membrane fluorecent labeling

MSCs were harvested during the second passage and were labeled with two different cell membrane flourescent labels, PKH26 and PKH67 fluorescent linker dye (Sigma Aldrich) according to the manufacturer's instructions. In brief, after the removal of SM, the cells were immediately resuspended 1 ml of labeling vehicle and mixed with an equal volume of a 2 X PKH26 or PKH67 stock solution in labeling vehicle. After 5 minutes at room temperature an equal volume of FBS was added to stop the staining reaction. The cells were again pelleted, transferred to a fresh centrifuge tube and washed twice in DMEM with 10% FBS. Viability was over 95% as determined by 0.4% Trypan blue dye exclusion.

Fibrin glue preparation

Two donor horses, 5 and 15 years old, not enrolled in the this study were used for collection of blood to make the fibrin glue (FG) scaffold. FG was produced using a cryoprecipitation technique as descirbed by Dresdale [47]. Briefly, plasma was divided in 50 mL polypropylene centrifuge tubes (BD Falcon, Franklin Lakes, NJ, USA) and placed in a freezer at -80°C for 12 hours.After slow thawing at 4°C, tubes were centrifuged at 1000g for 15 minutes, supernatant was decanted and saved for further use, leaving a yellowish precipitate of fibrinogen. Fibrinogen was measured and diluted with the supernatant to a concentration of 50 mg/mL.

Treatment groups

Treatment groups (7) were as follows: *Group 1*. P MSCs in FG; *Group 2*. BM MSCs in FG; *Group 3*. M MSCs in FG; *Group 4*. P MSCs in FG with DBM; *Group 5*. BM MSCs in FG with DBM; *Group 6*. M MSCs in FG with DBM; *Group 7*. DBM alone; *Group 8*. FG and DBM (Table 1). Each group of treatment comprised seven hind limbs for implantation. All MSCs implanted into left leg muscles of rats were labelled with PKH26, and all MSCs implanted into right leg muscles of rats were labelled with PKH67. Labelling of implanted MSCs was performed in order to observe the localization of the cells *in situ*.

Group	Mesenchymal stem cells			Scaffold	
	Periosteum	Bone Marrow	Muscle	Fibrin glue	Demineralized bone matrix
1	✓			✓	
2		✓		✓	
3			✓	✓	
4	✓			✓	✓
5		✓		✓	✓
6			✓	✓	✓
7					✓
8				✓	✓

Table 1. Groups of nude rats and implant treatments.

Scaffold Preparation

For Groups 1, 2, and 3, five million labelled MSCs were mixed with 75 µL of fibrin (50mg/mL) and 75µL of thrombin (Sigma Aldrich), diluted in 40nM $CaCl_2$ (Sigma Aldrich), to obtain a final thrombin concentration of 250 U/mL. For Groups 4, 5, and 6, five million labelled MSCs were mixed with 75 µL of fibrin and 25 mg of equine DBM. The equine DBM was commercially available as Equine Osteoallograft Orthomix ® (Veterinary Transplant Services, Kent, WA, USA) which was purchased for use in this study. Then, 75µL of thrombin was added as descibed previously. MSCs and scaffolds were supplemented with osteogenic media (OM) [α-MEM, 5% FBS, 100 µg/mL penicillin, 50 µg/mL gentamicin sulfate, 0.3 µg/mL fungizone, 50 µg/mL ascorbic acid (Sigma Aldrich), 10^{-8}M dexamethasone (Sigma

Aldrich), and 10 mM, β-glycerophosphate (Sigma Aldrich)] for 48 hours to stimulate osteogenic differentiation prior to surgical implantation. Control groups 7 and 8 included 25 mg of DBM placed in PBS for 48 hrs or 25 mg of DBM combined with 75 μL of fibrin and 75μL of thrombin, diluted in 40nM $CaCl_2$ and also placed in OM for 48 hours.

In Vivo experiment

NIH female nude rats (n=28) (Charles River, Wilmington, MA, USA) eight weeks of age were used in this experiment. Treatment groups were randomly assigned to be implanted into the muscle of both hind legs. Rats were maintained in a sterile enviroment in the laboratory animal facility for the duration of the study. During the surgical procedure, rats were maintained under general anesthesia with 2% isoflourane (Pharmaceutica Partners of Canada Inc. Richmond Hill, ON, Canada), placed in sternal recumbency, and aseptically prepped. A 5 mm incision was made through the skin and muscle in the hamstring region of both the left and right hind legs. The implant of each assigned treatment group (7 hind limbs per group/treatment) was introduced into the muscle which was sutured with 4(0) polydiaxone (PDS II, Ethicon, Inc Markham, ON, Canada). The skin incision was glued using GLUture (Abbott Laboratories Ltd. Chicago, IL, USA). Rats were given Buprenorphine in water for 24 hrs post-op and maintained with food and water *ad libitum*, and monitored daily for the remaining 8 weeks of the study. Rats were euthanized with CO_2 at 8 weeks post implantation.

Radiography

Radiographic images of the hind limbs were taken at 8 weeks post-implantation with a Provectra V digital imaging station (AllPro Imaging, Melville, NY, USA). Grayscale data was measured for the implant within the muscle in each hind limb using an image analysis system (Image J, National Institutes of Health, Bethesda,MD; http://imagej.nih.gov/ij/, 1997–2011). The tibia of the same hind limb was used as control. The mean of the gray scale of the normal tibia bone was calculated and compared with the mean of the implant gray scale. This comparison is referred as the gray scale ratio. Gray scale was used as a proxy of bone density.

Histology and histochemistry

Following euthanasia of the rats at 8 weeks post implantation, muscle tissue samples from the surgery sites were collected, fixed in 10% neutral buffered formalin, and paraffin-embedded. Tissues were cut into 5 μm sections and stained with H & E or von Kossa using routine methods. Three of the authors (EA, BE, LM) evaluated the two tissue sections in a blinded manner. Presence or absence of cartilage, bone, condensed mesenchyme, or normal muscle tissues were recorded in H & E stained sections, Image J analysis was used to quantify the area of new bone formation.

Statistical analysis

The results of quantitative analysis are reported as the mean (±SD). Kruskal-Wallis test was used to compare the differences between groups, and a post-hoc analysis was performed with Mann-Whitney U test. Statistical analyses were performed using IBM SPPS Statistics 21 software. Statistical significance was set at $P < 0.05$.
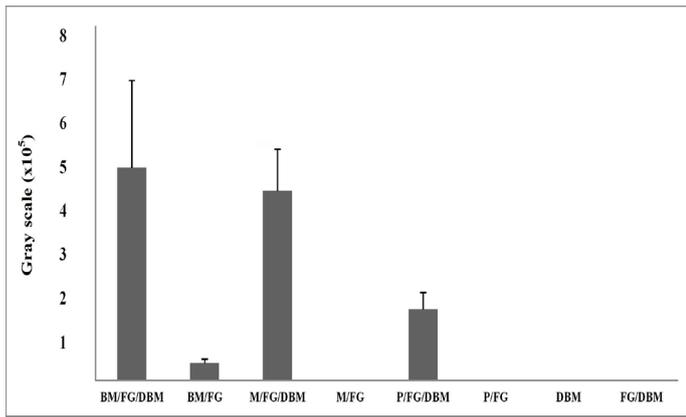
Results

Radiographic evaluation

The capacity of periosteum, bone marrow and muscle MSCs to generate new bone using FG and DBM as a scaffolds in intramuscular implants in nude rats was first evaluated using radiography. Radiographic images from 4 out of 7 hind limbs implanted with BM MSCs and FG (Group 2) revealed a radiopaque area in the implant site after 8 weeks (Figure 1a, b). As well as 7 out of 7 hind limbs implanted with BM MSCs in FG with DBM (Group 5) (Figure1c). In control groups containing scaffolds without MCSs the observed radiodensity was much decreased (Figure 1d). The gray scale ratio determined from the radiographs was similar between BM MSCs combined with FG and DBM (Group 5) and M MSCs combined with FG and DBM (Group 6), and there was a significantly greater gray scale ratio for Groups 5 and 6 compared with all other groups. BM MSCs combined with FG (Group 2) was the only treatment group which included MSCs and FG alone that showed radiodensity compared with other MSCs combined with FG only (Groups 1 and 3). The gray scale ratio was zero in nude rats implanted with P and M MSCs combined with FG (Groups 1 and 3) and in scaffold control groups without cells (Groups 7 and 8) (Figure 2).
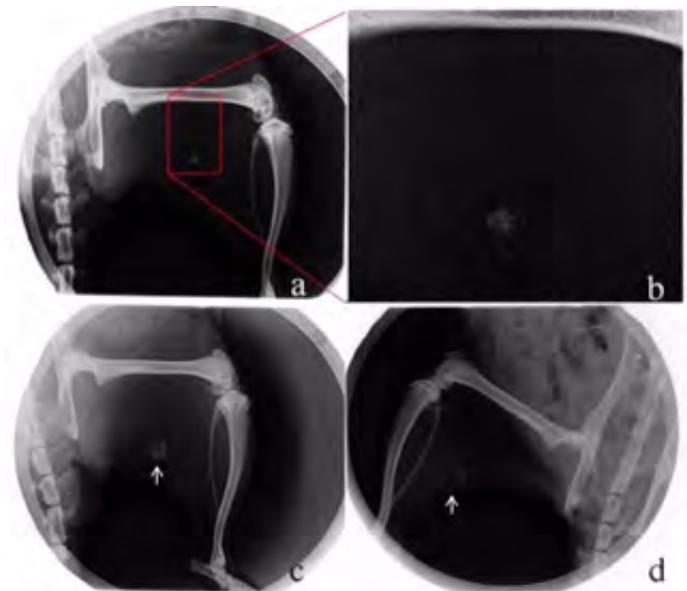
Figure 1. Representative radiographic images of new bone production.

New bone formation was observed radiographically at 8 weeks in legs implanted with BM and FG (Group 2) (a). The image on the top right square is a higher-magnification view of the density of new bone (b). All MSCs combined with FG and DBM showed a more visible radiopaque area (arrow) (c) compared with DBM alone (arrow) (d).

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Figure 2. Significant difference was observed in the gray scale ratio in rats implanted with BM MSCs combined with FG and DBM and M MSCs FG and DBM as compared with all treatments. No significant difference was observed between BM MSCs FG and DBM and M MSCs FG and DBM. (P <0.05).

New Bone Production

Skeletal muscle sections from implant sites were stained with H & E, and photomicrographs were taken for quantitative measurement of the total area (μm^2) of new bone formation. BM and M MSCs were most consistent in producing new bone (Groups 5 and 6) when DBM was added as a rigid scaffold (Table 2). Groups 5 and 6 showed significantly greater new bone formation compared with other groups (Figure 3). When P and M MSCs were implanted with FG alone (Groups 1 and 3), no new bone was formed. Similar results were observed in control groups 7 and 8. The addition of DBM to P MSCs (Group 4) resulted in new bone production similar to Groups 5 and 6 (Table 2) (Figure 3). BM MSCs combined with FG (Group 2) was the only treatment group which included MSCs and FG alone where new bone was evident.

Morphology	Group 1 P/FG	Group 2 BM/FG	Group 3 M/FG	Group 4 P/FG/DBM	Group 5 BM/FG/DBM	Group 6 M/GF/DBM	Group 7 DBM	Group8 FG/DBM
Cartilage	0	3	0	1	3	1	0	0
Endocondrial Ossification	0	3	0	1	4	1	0	0
Woven Bone	0	3	0	0	5	1	0	0
Osteoblast	0	3	0	2	3	4	0	0
Mature Bone	0	0	0	3	5	3	0	0

Table 2. Histological findings description for bone formation in muscle tissue from treated nude rats. Numbers 0-5 indicate the number of nude rats per group where histological findings were observed.

Histological Evaluation

Early bone formation was indicated by evidence of focal endochondral ossification surrounded dense fibrous tissue (Figure 4 a, d), and areas of cartilage and immature (woven) bone (Figure 4 e). Deposits of homogeneous eosinophilic material (osteoid matrix) containing small numbers of osteocytes were also observed. 4 out of 7 hind limbs implanted with BM MSCs with FG (Group 2)

showed new bone formation in contrast with the histological sections from hind limbs implanted with P and M MSCs and FG (Groups 1 and 3) which showed no evidence of new bone formation (Figure 4 b, c). 7 out of 7 hind limbs implanted with BM MSCs in FG with DBM (Group 5); 6 out of 7 hind limbs implanted with M MSCs in FG with DBM (Group 6); and 3 out of 7 hind limbs implanted with P MSCs in FG with DBM (Group 4) had evidence of bone formation indicated by small trabeculae of viable bone, often surrounded by osteoblasts (Figure 4f) (Table 2). In addition, presence of fluorescent labelled MSCs were observed in histology samples (Figure 5).
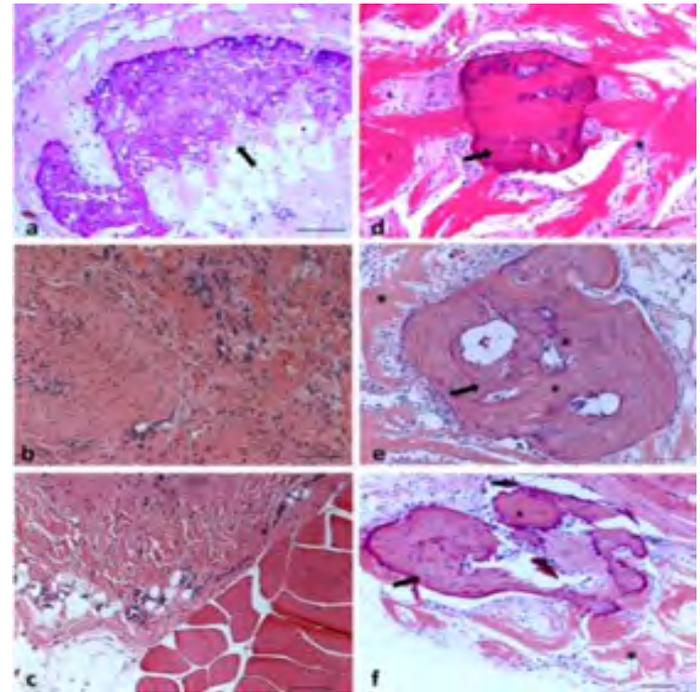
Figure 3. Total area (μm^2) of new bone formation was significantly different between BM MSCs combined with FG and DBM and M MSCs FG and DBM as compared with all treatments. No significant difference was observed between BM MSCs FG and DBM and M MSCs FG and DBM. (*P <0.05).
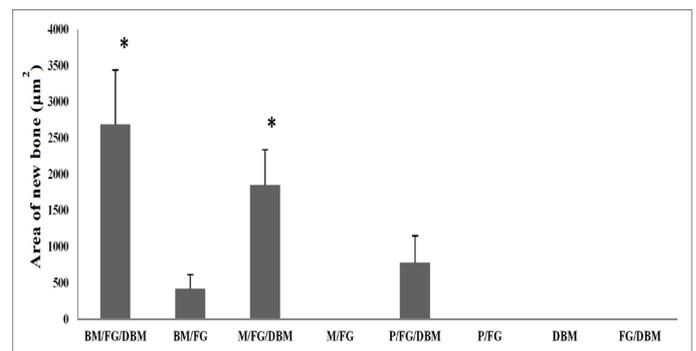
Figure 4. Representative histologic staining showed early bone formation (arrow) in rats' legs implanted with BM MCSs combined with FG (a). No evidence of bone formation was observed in rats legs implanted with M and P MSCs combined with FG (b,c) All groups with MCSs, FG, and DBM showed new bone (arrow) formation which could be distinguished from DBM (*) (d,e,f). *Bar* 100 µm.
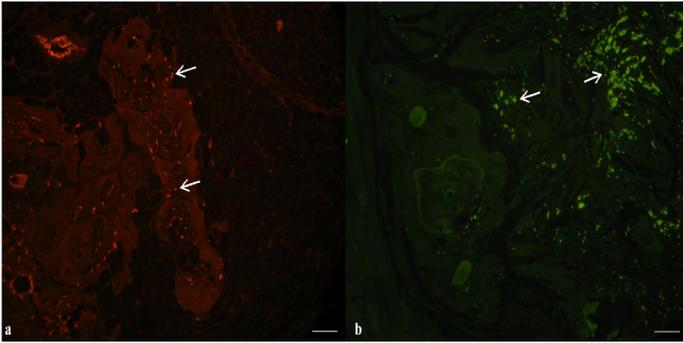
Figure 5. Representative fluorescent images of MSCs labelled with PKH26 (red)(a) and PKH67 (green)(b) were abundant at all sites of new bone production. Labelled cells (arrows) remained localized at the muscle implant site. *Bar* 100 μm.

Discussion

Successful bone tissue engineering by MSCs depends on several key factors: use of the appropriate cells, carrier and growth factors [24, 48]. This study focused on the evaluation of the source of MSCs and carrier for production of new bone in a heterotopic animal model. Because MSCs are known to produce substantial growth factors [24, 49, 50], addition of exogenous growth factors were not considered here.

Although the materials used to retain cells play a central role in the quality of the constructs, the source of cells is key for bone regeneration[37, 51]. Bone marrow is the most studied cell source, but other tissues are now being explored, such as periosteum, fat, muscle, cord blood, and embryonic or induced pluripotent stem cells [10, 16, 49-56]. Though MSCs from various donor tissues have shown great promise in bone tissue engineering because of their osteogenic differentiation in vitro [14, 16, 57-61], the osteogenic potential has not been widely confirmed in vivo, or in clinical use. This is the case in horses [10, 19, 51, 62] as well as in other animal species. In vivo bone production by MSCs has been confirmed by some studies, [14, 15, 20, 57, 58, 60, 61, 63, 64] but there is still controversy as to the efficacy of MSCs based therapy in clinical bone healing [10, 16, 19, 59, 65]. The in vitro osteogenic potential of various MSCs and skeletal progenitors do not necessarily predict their differentiation potential in vivo [59, 66].

In the field of human and veterinary medicine the most common sources of MSCs used clinically are bone marrow and adipose tissue [8, 10, 15, 39, 67, 68]. Other sources of equine MSCs, including those from umbilical cord elements, blood, and tendon, have been and continue to be investigated for use in musculoskeletal tissue regeneration in the horse [10, 51, 53, 65, 69].

MSCs from periosteum and skeletal muscle are known to be sources of MSCs in vivo and have been shown to be sources of MSCs in other species[26, 56, 70, 71]. Because characterization of a cell as an MSC typically requires proof that the cells can undergo tri-lineage differentiation into adipogenic, chondrogenic and osteogenic cells, cells characterized as MSCs are known to have osteogenic potential in vitro [56] and this is true of equine MSCs from muscle and periosteum [26, 36]. While there is much evidence that equine MSCs from several sources have osteogenic potential in vitro [26, 51, 53, 55] there is little evidence that the same cells can produce bone in vivo [10, 27].

Here we show that pre-induced equine bone marrow, muscle, and periosteal derived cells previously characterized as MSCs in vitro (26) had the ability to produce new bone in vivo at a heterotopic site when combined with the appropriate scaffold.

MSCs were pre-induced using osteogenic media which included dexamethasone. Pilot studies indicated pre-induction with osteogenic media was essential for the ability of cells to produce new bone in vivo (unpublished data). It is well recognized that dexamethasone is important for in vitro differentiation of MSCs into osteogenic cells, [72-76] and it has been shown that pretreatment of MSCs with dexamethasone, results in greater bone formation in vivo compared to untreated controls [18, 37, 48, 57, 58, 63, 77, 78].

One of the goals of bone tissue engineering is to design delivery methods for skeletal stem cells and progenitor cells for promotion of bone healing. Scaffolds play a critical role in supporting MSCs at the transplantation site [15, 37, 40] MSCs were here combined with FG as a carrier in an attempt to develop an injectable cell based therapy. However, in implant treatment where DBM was added, injection of cells and scaffold was difficult to perform because of the needle size recommended for rats. We have previously shown that the FG produced for use as a carrier was not detrimental to the ability of the MSCs to remain viable, proliferate, or differentiate to osteogenic lineage [36]. Based on fluorescent evaluation of labelled BM MSCs in an in vivo pilot study, the FG carrier also appeared to maintain the majority of transplanted MSCs at the region of implantation where new bone was produced (unpublished data). Similar fluorescent observation of labelled MSCs was evident in implant samples in this study. Other researchers have also concluded that FG can be a good carrier for equine MSCs [34, 79].

Fibrin has also been paired with more mechanically stable materials such as bioceramics in bone tissue engineering with positive results [80-83]. While a soft carrier/hydrogel provides the advantage of administration of MSCs by injection, and have been determined to maintain MSCs at the site of implantation it may not be an optimum scaffold for stimulating osteogenic differentiation and new bone production by cells [34, 40]. Mechanical properties of biomaterial scaffolds used for stem cell delivery in tissue engineering are known to modify cell behaviour. In this study we tested the cell biocompatibility of DBM with equine MSC and FG. Observation of new bone formation in these implants are in agreement with the use of rigid matrixes that shown to stimulate osteogenic differentiation of MSCs [84, 85]. The use of suitable biomaterials as scaffolds

which provides a specific microenvironment for the cells is essential in achieving the desired engineered tissue [37, 38].

Decellularised allografts, in the form of demineralized bone matrix (DBM), also known as demineralized freeze-dried bone allograft (DFDBA), is a type of rigid matrix and currently is the most frequently used osteoinductive allograft tissue available on the market in human orthopedics [76]. Such allografts are also available in the veterinary field as demineralized cancellous bone chips (Equine Osteoallograft Orthomix®). DBM is known to have osteoinductive and osteoconductive properties and can be used to enhance bone healing in place of autograft [14, 41, 86]. The primary osteoinductive component of DBM is a series of low-molecular-weight glycoproteins that includes the bone morphogenetic proteins (BMPs) [14, 42, 44]. The decalcification of cortical bone exposes these osteoinductive growth factors buried within the mineralized matrix, thereby enhancing the bone formation process [44, 87]. Although the identification or addition of osteogenic factors were not included in this study, these characteristics of the DBM previously described, may develop the new bone formation in implants containing BM and M MSC's.

It is well accepted that allografts have osteoinductive properties due to the chemical nature (and BMPs) but it is important to note that other material properties including the porosity and surface properties may also play an important role in osteoinduction [57].

DBM in combination with MSCs or osteoprogenitors has also been shown to promote new bone formation in some animal models [12, 14, 25].

In the current study, addition of DBM to pre-induced equine MSCs and FG did promote new bone production. Therefore, the combination of exogenous equine pre-induced MSCs with FG and equine DBM appears to have potential as a bone graft substitute in equine bone healing. This combination could possibly be used as an injectable method of cell based therapy based on the fact that the Equine Osteoallograft Orthomix ® DBM particles are less than 2.3 mm in size and could fit through a large bore needle such as an 11 gauge needle. Although DBM could stimulate endogenous MSCs to produce new bone, it appeared that the new bone produced by P, BM, and M MSCs combined with FG and DBM (Groups 4, 5, and 6) was a result of exogenous transplanted MSCs due to the fact that control groups with DBM did not have new bone production and the labelled MSCs were evident in all specimens where new bone was formed .

Characterization of equine MSCs has been reported elsewhere. We have characterized the stemness, osteogenic differentiation, and cell viability in different types of scaffolds of horse derived muscle MSCs in vitro[26, 36, 88, 89]. However, to our understanding, this is the first report of new bone formation in a heterotopic animal model implanted with equine muscle MSCs in FG with DBM scaffold.

In conclusion, equine BM MSCs were most consistently able to produce new bone at a heterotopic site in vivo followed by M MSCs. DBM appeared to have an osteogenic stimulus on equine MSCs based on the fact that the amount of new bone produced by BM MSCs was increased and P MSCS and M MSCs were able to produce new bone when DBM was added as a rigid scaffold but were not able to produce new bone when combined with FG alone. The easy procedure of obtaining, the osteogenic capacity, the abundance of M MSCs in cell cultures, and its cell survival in different scaffolds make very attractive the potential use of horse M MSCs in cell-based therapy for bone healing.

Further studies involving the optimal in vitro culture of equine MSCs from bone marrow, muscle, and periosteum combined with FG and DBM in animal models of bone fracture are warranted.

Acknowledgements

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