JACOBS PUBLISHERS

OPEN ACCESS

Jacobs Journal of Veterinary Science and Research

Research Article

Evaluation of Two Populations of Equine Stem Cells in Promotion of Bone Healing in a Nude Rat Fracture Model

Rodolfo Nino-Fong[1*], Blanca P. Esparza Gonzalez[2], Enrique Aburto[3], Marion Desmarchelier[4], Juan Carlos Rodriguez-Lecompte[5],Laurie A. McDuffee[6]

[1]*Department of Biomedical Sciences, Ross University School of Veterinary Medicine, P. O. Box 334, Basseterre, St. Kitts, West Indies*

[2]*Comparative Orthopaedic Research Laboratory, Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown. Prince Edward Island, C1A4P3, Canada*

[3]*Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, C1A4P3, Canada*

[4]*Centre Hospitalier Universitaire Vetérinarie, Faculté de Médicine Vetérinaire, Université de Montreal. 3200, rue Sicotte, Saint Hyacinthe, Québec, J2S7C6, Canada.*

[5]*Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, C1A4P3, Canada*

[6]*Comparative Orthopaedic Research Laboratory, Department of Health Management.*

Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown. Prince Edward Island, C1A4P3, Canada

**Corresponding author: Dr. Rodolfo Nino-Fong, Department of Biomedical Sciences, Ross University School of Veterinary Medicine, P. O. Box 334, Basseterre, St. Kitts, West Indies, Tel: 869.664.2272; Email:Rninofong@rossvet.edu.kn*

Received: 06-21-2016

Accepted: 07-29-2016

Published: 09-20-2016

Abstract

Equine bone marrow (BM) derived mesenchymal stem cells (MSC) have been used as a potential cell-based therapy in bone healing due to their osteogenic differentiation capacity. In this tibia fracture model in nude rats, the new bone formation was evaluated using a cell-based treatment with either fractioned or heterogeneous population of equine BM MSC in combination with Fibrin Glue (FG). Sternal equine BM MSCs were obtained from a donor horse and expanded *in vitro*. Stimulation of osteogenic differentiation of equine BM MSC was performed *in vitro* after cell incubation with osteogenic medium. Osteotomized tibias were treated with FG (Group 1), fractioned equine BM MSC combined with FG (Group 2), and heterogeneous cell population of equine BM MSC combined with FG (Group 3). Bone density, area of new bone formation and histology were evaluated 4 weeks after the surgery and treatment. Tibia fractures treated with a heterogeneous cell population of equine BM MSC and FG (Group 3) showed better callus formation, higher bone density and histology score as compared with rats treated with fractioned equine BM MSC in FG (Group 2) and FG alone (Group 1). A heterogeneous cell population of equine BM MSC could be a better cell-based therapy choice for bone healing in horse fractures.

Keywords: Horse; Mesenchymal Stem Cells; Bone Marrow; Nude rat; Osteotomy

Introduction

Skeletal injuries in horses including bone fractures are a common clinical problem for equine orthopedic surgeons. Different surgical procedures have been used for improving bone fracture healing including autografts, allografts and implantation of implants [1]. The gold standard procedure is the implantation of autogenous bone graft which has osteogenic, osteoinductive and osteoconductive properties [2, 3]. However, limitations of this technique include the fact that harvest of bone graft may cause additional musculoskeletal pain, and the cost of such surgical procedures can be high with limited graft material availability [4, 5]. Tissue engineering approaches based in cell based therapy are an emerging option for delivering cells, growth factors, and extracellular matrix components or scaffolds in the site of bone fractures in order to improve and/or speed up the bone healing. Mesenchymal stem cells (MSC) from different types of tissues have shown the potential capability to differentiate into specific cell types under proper stimuli. It has been shown that bone marrow (BM) derived MSCs have the potential to differentiate into cells of the osteoblastic lineage and have been studied for use in regeneration of bone [6-14]. The osteogenic differentiation of BM derived MSCs has been shown *in vitro* with or without collagen scaffold [15]. In our laboratory, we have shown equine BM MSCs produce new bone after intramuscular implantation in nude mice [16].

While bone tissue engineering results have been promising, there is continued MSC characterization which focuses on identifying the best tissue source of MSCs for specific treatment based on the cellular morphology, presence of CD markers and stemness gene expression [17, 18]. A previous study has shown that equine muscle and BM MSCs have better osteogenic capacity compared with MSCs from periosteum and adipose tissue[19]. It is well known that as a primary culture, MSCs are a heterogeneous cell population [18, 20]. MSC subpopulations have shown different characteristics based on their morphology, proliferation, and differentiation abilities [21, 22]. In addition, the identification of a specific BM MSC subpopulation with the best osteogenic differentiation *in vitro* has been determined using a non-equilibrium gravitational field-flow fractionator (Flow s.r.l, Bologna, Italy). It has been suggested that an equine BM MSC fraction or subpopulation with ideal osteogenic differentiation may be valuable in cell-based therapy for bone healing in horses [23].

This study was intended to compare the osteogenic capacity and improvement in bone fracture healing amongst 3 groups (a heterogeneous cell population of equine BM MSCs; a purified, fractionated, equine BM MSC subpopulation; and control). Both BM MSC sources were combined with fibrin glue as a treatment in a rat tibia osteotomy experimental model. We hypothesized that a fractioned equine BM MSC subpopulation with optimum *in vitro* osteogenic differentiation capacity would improve the bone healing to a greater extent compared to a heterogeneous cell population of equine BM MSC and compared to a control.

Methodology

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

Animals

Rats

Twenty-four 9-week-old male NIH nude rats, with an average weight range of 233 +/- 4.16 grams were used in the study (Charles River Laboratories, Sherbrook, QC. Canada). Rats were housed as one rat per cage with a 12-hour light cycle. Food and water were provided *ad libitum*. Rats were randomly divided into two treatment groups (n=8 each) and one control group (n=8).

Horse

One horse was used for bone marrow collection. The horse was donated to the Atlantic Veterinary College for reasons independent to this study and determined to be systemically healthy based on physical examination, CBC, and chemistry panel. The horse was sedated with xylazine hydrochloride (1.1 mg/kg, IV) and euthanized with pentobarbital (108 mg/kg IV). Bone marrow aspirate was obtained aseptically immediately post mortem from the sternum with a bone marrow biopsy needle. The aspirate was collected into a 20ml syringe containing 2.5 ml heparin (1000 U/ml) and transported to the laboratory.

Cell Isolation

Cells were isolated from BM using a centrifuge gradient technique and maintained in standard medium, as previously described [19]. Briefly, the samples were centrifuged at 1500 g for 10 minutes, then buffy coat was collected and placed in 75 cm 2 flasks with standard medium (SM) [(Dulbecco's Modified Eagle Medium (DMEM) Sigma Aldrich. St. Louis, MO), 10% fetal bovine serum (FBS) (PAA, Laboratories Inc. Etobicoke ON, Canada), 100 µg/mL penicillin (Sigma Aldrich, St Louis, MO), 50 µg/mL gentamicin sulphate (Sigma Aldrich, St Louis, MO), and 0.3 µg/mL amphotericin B (Invitrogen, Toronto, ON, Canada)]. Cultures were maintained in a humidified 5% CO2 and 95% air atmosphere incubator at 37°C. Once cells adhered in 24-48 hours, unattached cells were washed off the flask with PBS. Medium was changed three times per week. Cells were maintained until 85% confluence. These cells were used as heterogeneous cell population of equine BM MSC treatment.

Preparation of Fractioned BM MSC for Treatment

Cell Fractionation-Gravitational Field-Flow Fractionation (Gr-FFF)

A heterogeneous cell population of equine BM MSCs was washed with PBS and detached with 1 part trypsin (Invitrogen, Toronto, ON) and 4 parts Cellstripper solution (Corning, Manassas, VA). After 10 minutes of incubation at 37°C, the reaction was stopped with SM. Cell suspension

was spun at 1500 g for 10 min and counted. BM MSCs were aliquoted in $5x10^5$ cells in 50 µl mobile solution (1g bovine serum albumin in PBS). Each aliquot was injected into a Gr-FFF system (Flow s.r.l, Bologna, Italy) with a 100 µl micro syringe (Hamilton, Com., Reno, NV.) and sorted in 5 fractions by changing the collection tube every 5 minutes (0.5 ml per minute). Equine BM MSC fractions were incubated in SM at 37°C in a humidified 5% CO_2 and 95% air atmosphere. Fraction 4 was cryopreserved for further propagation and used for cell-based treatment.

Fibrin Glue Preparation

Two donor horses, 5 and 15 years old, that were not enrolled in the study, were used for collection of blood which was used for making a fibrin glue (FG) scaffold. Fibrin glue was produced using a cryoprecipitation technique as descirbed by Dresdale [24]. Briefly, plasma was divided in 50 mL polypropylene centrifuge tubes (BD Falcon, Franklin Lakes, NJ) and placed in a freezer at -80°C for 12 hours. Then, tubes were slow thawed at 4°C. After thawing, tubes were centrifuged at 1000 g for 15 minutes, supernatant was decanted and saved for further use, leaving a yellowish precipitate of fibrinogen. Fibrinogen concentration was measured and diluted with the supernatant at a final concentration of 50 mg/mL.

Scaffold Preparation and Osteogenic Differentiation

Five million equine BM MSCs from the heteroenous cell population were mixed with 75 µL of fibrin and 75µL of thrombin, diluted in 40nM CaCl2 (Sigma Aldrich, Canada Ltd., Oakville, ON, Canada), to obtain a final concentration of 250 U/mL. Five million of fractioned equine BM MSC was mixed with FG as previously described. Fibrin glue without MSCs was prepared mixing fibrin and thrombin only. Mesenchymal stem cells and FG scaffolds were supplemented and incubated with osteogenic media (OM) [α-MEM, 5% FBS, 100 µg/mL penicillin, 50 µg/mL gentamicin sulfate, 0.3 µg/mL amphotericin B, 50 µg/mL ascorbic acid (Sigma Aldrich, St. Louis, MO), 10^{-8} M dexamethasone (Sigma Aldrich, St Louis, MO), and 10 mM, β-glycerophosphate (Sigma Aldrich, St Louis, MO)] to stimulate osteogenic differentiation for 48 hours prior to surgical implantation.

***In Vivo* Experiment**

Surgical Procedure

Rats were maintained in a sterile enviroment in the laboratory animal facility for the duration of the study. Rats were anaesthetized with 2% isoflourane (Pharmaceutica Partners of Canada Inc. ON, Canada), placed in dorsal recumbency, and aseptically prepped. Pre-operative treatment for each rat consisted of 3 mL of subcutaneous (SC) fluids (Lactate Ringers solution Baxter, Mississauga On), buprenorphine 0.05mg/kg SC (Vetergesic, Alstoe Ltd., Sheriff Hutton, York, UK), enrofloxacin (10 mg/kg SC) and eyes were covered with sterile ophthalmic liquid gel (Tear-gel Novartis, Dorval, QC, Canada).

Surgery was conducted using routine aseptic techniques. The left tibia and stifle were clipped and aseptically prepped. The rat was draped and the distal aspect of the tibia tuberosity was palpated as a landmark. A 1-cm incision was made through the skin and periosteum starting at the distal aspect of the tibia tuberosity and extending distally over the medial aspect of the left tibia. One centimeter of periosteum was elevated off the tibia diaphysis. The osteotomy was made in the center of this area (5 mm distal to the distal end of the tibia tuberosity). The bone was lavaged with saline, and the bone was cut with an oscillating saw using a small sagittal blade (ConMed Linvatec., Largo, FL). A 0.035-inch diameter K-Wire (IMEX Veterinary Inc., Longview, TX.) was used to stabilize the fracture. The pin was placed retrograde. One centimetre of pin was placed in the distal aspect of the tibia beyond the osteotomy site. The pin was cut at the proximal end. Rats were randomly assigned to a treatment group. Treatment groups were as follows (n=8): *Group 1*. FG; *Group 2*. Fractioned equine BM MSCs in FG; *Group 3*. Heterogeneous cell population of equine BM MSCs in FG. The randomly assigned treatment (group 1, 2, and 3) was implanted at the osteotomy site after stabilization of the fracture. Muscles were sutured with 4(0) polydiaxone (PDS II, Ethicon Inc., Somerville, NJ). The skin incision was glued using GLUture (Abbott laboratories Ltd., Berksire, UK). Rats were maintained with food and water *ad libitum*, and monitored daily for the remaining 4 weeks of the study. Rats were administrated meloxicam per os 2mg/kg (Metacam, Boehringer Ingelheim, Burlington, ON, Canada) daily for one week. Enrofloxacin (0.1 mg/ml) and buprenorphine B (0.0006 mg/ml) were dissolved in water and provided *ad libitum* for one week. Post-operative monitoring of the rats included evaluation of the attitude and use of the limb each day when medication was administered, as well as weight assessment once a week. Rats were euthanized with CO_2 4 weeks post-surgery.

Radiography

Radiographic images of the hind limbs were taken immediately post mortem after 4 weeks from treatment and surgery using Provectra V digital imaging (AllPro Imaging, Melville, NY). Objective bone density data was obtained for each hind limb using an image analysis system (Image J, National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/, 1997–2011).

Histology

At the 4-week endpoint, all left tibias were dissected and fixed in buffered formalin for 24 hours. Then tibias were demineralized in constant stir for 24 hours in a solution containing 120ml formic acid, 24g sodium formate in one liter of deionized water. After demineralization, tibias were placed in 70% ethanol for 48 hours. Tibias were paraffin-embedded and cut into 5 µm sections and stained with H & E. Three of the authors evaluated the tissue sections in a blinded manner. Presence or absence of cartilage, bone, condensed mesenchyme, or normal muscle tissues were recorded for H and E stained sections (two sections per

sample) [25-27] (Table 1). When new bone was identified, image analysis was used to quantify the area of new bone for comparison among groups using an image analysis system (Image J, National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/, 1997–2011).

Score/Grade	Bone Healing Stage
0	Non union (fibrous tissues)
1	Incomplete cartilage union (cartilage with some fibrous tissue)
2	Complete cartilage union (entirely cartilage)
3	Incomplete bone union with early ossification phase (predominantly cartilage with some trabecular bone)
4	Incomplete bony union with late ossification phase (equal amounts of cartilage and trabecular bone)
5	Incomplete bony union with late ossification phase (predominantly trabecular bone with some cartilage)
6	Complete bony union (entirely bone)

Table 1. Scale Grading for Bone Healing

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

Area of New Bone Formation

The area of the endochondral ossification in the tibia osteotomies was measured from H & E histological sections performed 4 weeks after surgery and treatment. Tibial fractures treated with fractioned equine BM MSCs and heterogeneous cell population of equine BM MSCs (Groups 2 and 3, respectively) showed a significant increased area of endochondral ossification (P<0.05) in the osteotomy sites, as compared with tibia osteotomies treated with FG only (Group 1). No statistical difference was observed between tibia fractures treated with fractioned equine BM MSCs and heterogeneous cell population of equine BM MSCs (Groups 2 and 3) (Figure 1).

Radiographic Analysis and Bone Density

New bone or callus formation was evident in rats 4 weeks after surgery and treatment. Control group rats showed less callus formation at the tibia osteotomy treated with FG only (Group 1) (Figure 2A).

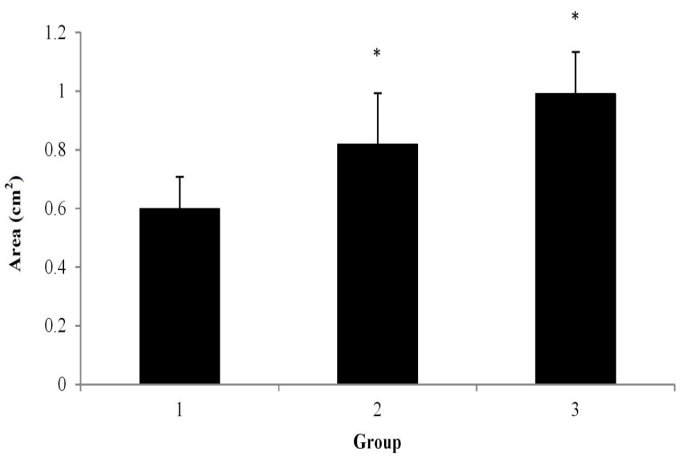

Figure 1. An increased area of new bone formation was evident in rats treated with fractioned equine BM MSCs (Group 2) and heterogeneous cell population of equine BM MSCs (Group 3). Compared to the control group (P<0.05) (Group 1).

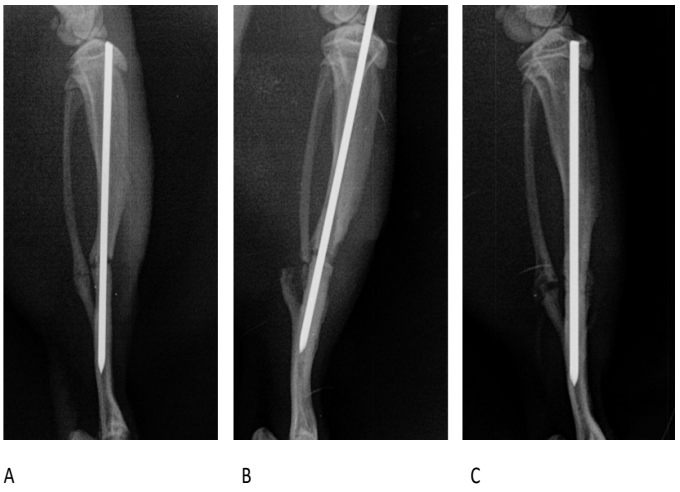

Figure 2. Representative radiographic images of rat tibias osteotomies treated with FG (A), fractioned equine BM MSCs (B) and heterogeneous cell population of equine BM MSCs (C).

Therefore, no established bone union was observed in this group. In the group of rats treated with fractioned equine BM MSCs (Group 2) more callus formation was observed at the tibia osteotomy. This space was partially filled with callus formation (Figure 2B). In the group of rats treated with heterogeneous cell population of equine BM MSCs (Group 3), the osteotomy site was covered by callus formation, representing bone union (Figure 2C). Results from radiological density at 4 weeks showed higher bone density in rats treated with heterogeneous cell population of equine BM MSC (P <0.05) (Group 3), as compared with rats treated with fractioned equine BM MSCs (Group 2) and control group treated with FG only (Group 1) (Figure 3).

Histology Score

Histology assessment of tibia osteotomy of rats treated with FG (Group 1) showed nonunion bone, or incomplete cartilage union with fibrous tissue (Figure 4A). Tibia osteotomy sections from rats treated with fractioned equine BM MSCs (Group 2) showed incomplete bone union

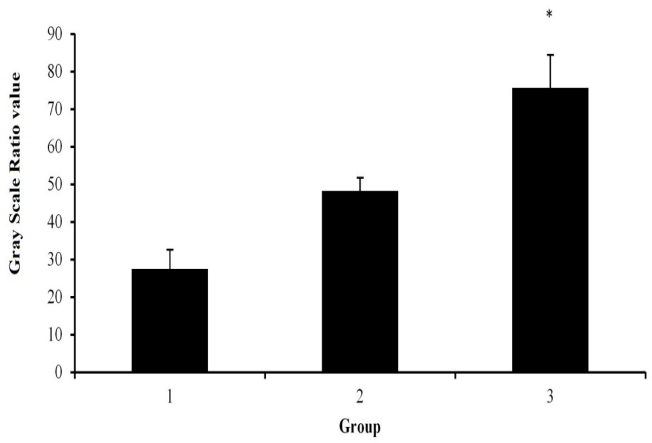

Figure 3. Gray scale ratio values representing bone density was significantly greater (P<0.05) in rats treated with a heterogeneous cell population of equine BM MSCs (Group 3) as compared with rats treated with fractioned equine BM MSCs (Group 2) and control group (FG) (Group 1).

with similar amounts of cartilage and trabecular bone (Figure 4B). Histology sections from rats treated with a heterogeneous cell population of equine BM MSCs (Group 3) showed incomplete or complete bony union predominantly composed of trabecular bone and small amounts of cartilage (Figure 4C). Histology score analysis from the three groups showed a significantly higher score of new bone formation in rats treated with heterogeneous cell population of equine BM MSCs (Group 3) as compared with rats treated with FG and fractioned equine BM MSCs (Group 1 and 2 respectively) (Figure 5).

Discussion

The present study demonstrated that a locally applied osteogenic, primed, heterogeneous cell population of equine BM MSCs mixed in a fibrin glue scaffold improved the bone healing in a tibia fracture model in nude rats compared with fractioned equine BM MSCs in FG and control group (FG). Cell based treatment in this experiment was selected based on previous *in vitro* and *in vivo* characterization of equine

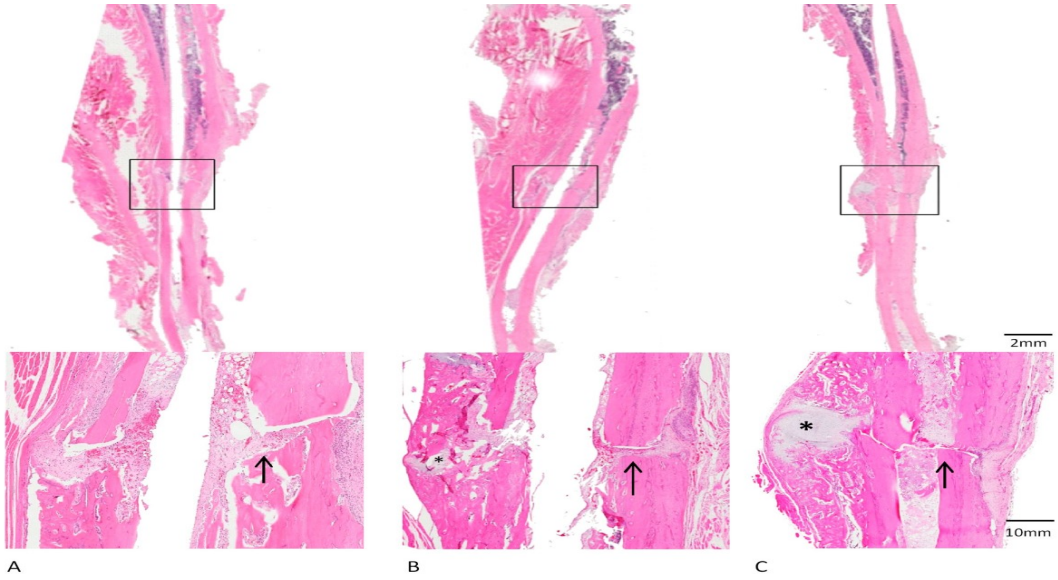

Figure 4. Representative histology micro-sections stained with H & E from rat tibias treated with FG (Group 1), fractioned equine BM MSC (Group 2), and heterogeneous cell population of equine BM MSC (Group 3). Rectangle in top images is showed in amplified bottom images. Arrows indicate the bony union site. Asterisks indicate presence of newly cartilage.

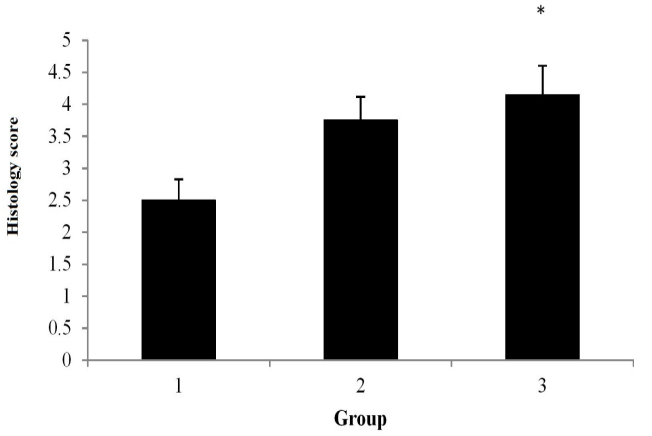

Figure 5. Histology score from rat tibia osteotomies treated with a heterogeneous cell population of equine BM MSCs (Group 3) showed significantly higher (P<0.05) new bone formation compared with rats treated with fractioned equine BM MSCs (Group 2) and FG (Group 1).

BM MSCs in our laboratory [15, 19, 23, 28]. Those results showed a higher: osteogenic gene expression in equine BM MSC stimulated with osteogenic medium; osteogenic differentiation in different types of scaffolds; and heterotopic new bone formation in nude mice and rats compared to MSCs derived from other equine tissues. In addition, we evaluated the osteogenic differentiation of equine BM MSCs sorted into subpopulations by gravitational field-flow fractionation. Equine subpopulations of BM MSCs showed different osteogenic gene expression *in vitro*, between sorted fractions. Of cell subpopulations, fraction 4 showed the highest osteogenic gene expression [23, 29]. Thus, this cell fraction was selected as a cell-based treatment in this animal fracture model experiment. Equine MSC fractionation has been reported using FACS as a lab technique to identify stemness characteristics [18] as well as sieved-size subpopulations from equine umbilical cord [20]. In addition, enriched and purified human BM MSC CD34+

showed increased vascularisation and bone formation in a femur fracture model in nude rats [27].

Results from this experiment demonstrated that a heterogeneous cell population of equine BM MSCs combined with FG resulted in better bone healing of the fractured tibias compared with fractioned equine BM MSCs in FG. Scaffolds and cell carriers are one of the most important issues in tissue engineering. Particularly, FG has been shown to be an excellent carrier/scaffold [30] which allows cell survival and differentiation [15]. Overall, the benefit of FG in bone healing has been reported in human and animal models through the induced new bone formation when combined with stem cells and demineralized bone [30]. In addition, FG has shown osteoinductive properties when implanted in the extra skeletal site of mice and human as a potential biologic tissue adhesive [33, 34]. Previous results from *in vivo* experiments in our laboratory demonstrated new bone formation with heterotropic injection of equine BM MSCs in FG. New bone formation did not occur when equine muscle derived MSCs in FG were injected in the same model [16]. Similar results were observed in other laboratories. In nude mice subcutaneously implanted with equine BM MSCs and FG, there was increased vascularisation and tissue repair compared with FG alone [31, 32]. In addition, *in vitro* experiments have shown that equine BM MSCs had better osteogenic differentiation in FG and in collagen type I carriers as compared with monomeric collagen and rat tail collagen [15].

Our histologic evaluation from tibia fractures treated with the heterogeneous cell population of equine BM MSCs combined with FG showed better histology score, presence of callus, and new bone formation compared with fractioned equine BM MSCs combined with FG. Equine BM MSCs have been shown to have better osteogenic capacity *in vitro* when combined with FG and collagen type I scaffolds [15] compared to equine MSCs derived from other tissues. In spite of few reports using equine BM MSCs in bone repair in live animal models, there are publications regarding the potential use of BM MSCs from different animal species in calvarial defects, experimental polytrauma and hemorrhagic shock, femur fracture, mandible and alveolar defects, and osteoporosis [15, 35-40].

There are few reports regarding the use of equine MSCs in equine fractures, and these reports showed variable results [41, 42]. Periosteal-derived stem cells did not improve the bone healing in a horse osteotomy model [41]. Whereas, in a mouse heterotopic model, of BM derived equine MSCs showed new bone formation and mineralization [16]. In an equine metatarsal bone defect model, equine BM MSCs stimulated the bone formation only with the addition of bone marrow morphogenetic protein-2 [42].

It has been shown that osteogenic gene expression is different in fractioned equine BM MSC [23]. However, fractioned equine BM MSCs with the highest osteogenic gene expression [fraction 4, 23] did not result in better bone repair in this tibia fracture model. Those fractioned equine BM MSCs with lower *in vitro* osteogenic gene expression may have a role in the extracellular matrix production and/or in the production of growth factors which are required for bone remodeling. Further studies, such as proteomic profile are necessary to understand the molecular mechanisms of osteogenic differentiation of equine MSCs.

In conclusion, tibia fractures treated with a whole population of equine BM MSCs showed better bone healing compared with a subpopulation of equine BM MSCs. Although there is evidence of the successful use of MSCs in bone repair of animal models, more studies are necessary to identify the best source of MSCs with osteogenic capacity, as well as the best *in vitro* culture conditions. As there are only few reports using MSCs in equine bone repair, more clinical studies are required to demonstrate the feasibility of the MSC based therapy as a potential choice in the treatment of bone fractures in horses.

References

1. Mastrogiacomo M, Muraglia A, Komlev V, et al. Tissue engineering of bone: search for a better scaffold. Orthod. Craniofac.Res. 2005,8(4):277-84.

2. Sen MK, Miclau T. Autologous iliac crest bone graft: should it still be the gold standard for treating nonunions? Injury 2007,38 Suppl 1:S75-S80

3. Khojasteh A, Behnia H, Dashti SG, Stevens M. Current trends in mesenchymal stem cell application in bone augmentation: a review of the literature. J.Oral Maxillofac. Surg. 2012,70(4):972-82.

4. Lopez MJ, Jarazo J. State of the art: stem cells in equine regenerative medicine. Equine Vet.J. 2015,47(2):145-54.

5. Perren SM. Fracture healing. The evolution of our understanding. Acta Chir Orthop. Traumatol. Cech. 2008,75(4):241-6.

6. Centeno CJ, Busse D, Kisiday J, Keohan C, Freeman M, Karli D. Regeneration of meniscus cartilage in a knee treated with percutaneously implanted autologous mesenchymal stem cells. Med.Hypotheses 2008,71(6):900-908.

7. Grigolo B, Lisignoli G, Desando G, et al. Osteoarthritis treated with mesenchymal stem cells on hyaluronan-based scaffold in rabbit. Tissue Eng Part C.Methods 2009,15(4):647-58.

8. Guillot PV, Abass O, Bassett JH, et al. Intrauterine transplantation of human fetal mesenchymal stem cells from first-trimester blood repairs bone and reduces fractures in osteogenesis imperfecta mice. Blood 2008,111(3):1717-25.

9. Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc.Natl.Acad.Sci.U.S.A 2002,99(13):8932-7.

10. Jager M, Hernigou P, Zilkens C, et al. Cell therapy in bone healing disorders. Orthop.Rev.(Pavia) 2010;2(2):e20

11. Jones E, Yang X. Mesenchymal stem cells and bone regeneration: current status. Injury 2011,42(6):562-8.

12. Kon E, Filardo G, Roffi A, et al. Bone regeneration with mesenchymal stem cells. Clin.Cases.Miner.Bone Metab 2012,9(1):24-7.

13. Kuroda Y, Kitada M, Wakao S, Dezawa M. Bone marrow mesenchymal cells: how do they contribute to tissue repair and are they really stem cells? Arch.Immunol.Ther.Exp. (Warsz.) 2011,59(5):369-78.

14. Zaky SH, Ottonello A, Strada P, Cancedda R, Mastrogiacomo M. Platelet lysate favours *in vitro* expansion of human bone marrow stromal cells for bone and cartilage engineering. J.Tissue Eng Regen.Med. 2008,2(8):472-81.

15. Nino-Fong R, McDuffee LA, Esparza Gonzalez BP, Kumar MR, Merschrod S EF, Poduska KM. Scaffold effects on osteogenic differentiation of equine mesenchymal stem cells: an *in vitro* comparative study. Macromol.Biosci. 2013,13(3):348-55.

16. McDuffee LA, Esparza Gonzalez BP, Nino-Fong R, Aburto E. Evaluation of an *in vivo* heterotopic model of osteogenic differentiation of equine bone marrow and muscle mesenchymal stem cells in fibrin glue scaffold. Cell Tissue Res. 2014,355(2):327-35.

17. De Schauwer C, Meyer E, Van de Walle GR, Van Soom A. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 2011,75(8):1431-43.

18. Pascucci L, Curina G, Mercati F, et al. Flow cytometric characterization of culture expanded multipotent mesenchymal stromal cells (MSCs) from horse adipose tissue: towards the definition of minimal stemness criteria. Vet.Immunol.Immunopathol. 2011,144(3-4):499-506.

19. Radtke CL, Nino-Fong R, Esparza Gonzalez BP, Stryhn H, McDuffee LA. Characterization and osteogenic potential of equine muscle tissue- and periosteal tissue-derived mesenchymal stem cells in comparison with bone marrow- and adipose tissue-derived mesenchymal stem cells. Am.J.Vet.Res. 2013,74(5):790-800.

20. Corradetti B, Lange-Consiglio A, Barucca M, Cremonesi F, Bizzaro D. Size-sieved subpopulations of mesenchymal stem cells from intervascular and perivascular equine umbilical cord matrix. Cell Prolif. 2011,44(4):330-42.

21. Zhang L, Chan C. Isolation and enrichment of rat mesenchymal stem cells (MSCs) and separation of single-colony derived MSCs. J.Vis.Exp. 2010,(37)

22. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002,418(6893):41-9.

23. Radtke CL, Nino-Fong R, Rodriguez-Lecompte JC, Esparza Gonzalez BP, Stryhn H, McDuffee LA. Osteogenic potential of sorted equine mesenchymal stem cell subpopulations. Can.J.Vet.Res. 2015,79(2):101-108.

24. Dresdale A,Rose EA,Jeevanandam V,Reemtsma K,Bowman FO,Malm JR.Preparation of fibrin glue from single-donor fresh-frozen plasma. Surgery 1985,97(6):750-5.

25. Allen HL, Wase A, Bear WT. Indomethacin and aspirin: effect of nonsteroidal anti-inflammatory agents on the rate of fracture repair in the rat. Acta Orthop.Scand. 1980,51(4):595-600.

26. Estai MA, Soelaiman IN, Shuid AN, Das S, Ali AM, Suhaimi FH. Histological changes in the fracture callus following the administration of water extract of piper sarmentosum (daun kadok) in estrogen-deficient rats. Iran J.Med.Sci. 2011,36(4):281-8.

27. Kawakami Y, Ii M, Alev C, et al. Local transplantation of ex vivo expanded bone marrow-derived CD34-positive cells accelerates fracture healing. Cell Transplant. 2012,21(12):2689- 709.

28. Nino-Fong R, Esparza Gonzalez BP, Aburto E, McDuffee LA. *in vivo* Compaison of the Osteogenic Properties of Equine Mesenchymal Stem Cells from Periosteum, Muscle, and Bone Marrow with and without Demineralized Bone Matrix. J.J.Vet Sci Res 2015,1(5):25

29. Radtke CL, Nino-Fong R, Esparza Gonzalez BP, McDuffee LA. Application of a novel sorting system for equine mesenchymal stem cells (MSCs). Can.J.Vet.Res. 2014,78(4):290-6.

30. Kang EJ, Byun JH, Choi YJ, et al. *in vitro* and *in vivo* osteogenesis of porcine skin-derived mesenchymal stem cell-like cells with a demineralized bone and fibrin glue scaffold. Tissue Eng Part A 2010,16(3):815-27.

31. Ferris D, Frisbie D, Kisiday J, McIlwraith CW. *in vivo* healing of meniscal lacerations using bone marrow-derived mesenchymal stem cells and fibrin glue. Stem Cells Int. 2012, :691605

32. Ferris DJ, Frisbie DD, Kisiday JD, et al. Clinical outcome after intra-articular administration of bone marrow derived mesenchymal stem cells in 33 horses with stifle injury. Vet. Surg. 2014,43(3):255-265.

33. Abiraman S, Varma HK, Umashankar PR, John A. Fibrin glue as an osteoinductive protein in a mouse model. Biomaterials.2002,23(14):3023-31.

34. Faverani LP, Ferreira S, Momesso GA, et al. Surgical treatment of severe frontal bone fracture. J.Craniofac.Surg. 2015,26(3):e229-e231

35. Kretlow JD, Spicer PP, Jansen JA, Vacanti CA, Kasper FK, Mikos AG. Uncultured marrow mononuclear cells delivered within fibrin glue hydrogels to porous scaffolds enhance bone regeneration within critical-sized rat cranial defects. Tissue Eng Part A 2010,16(12):3555-68.

36. Krumina G, Babarykin D, Krumina Z, et al. Effects of systemically transplanted allogeneic bone marrow multipotent mesenchymal stromal cells on rats' recovery after experimental polytrauma. J.Trauma Acute.Care Surg. 2013,74(3):785-91.

37. Schantz JT, Hutmacher DW, Lam CX, et al. Repair of calvarial defects with customised tissue-engineered bone grafts II. Evaluation of cellular efficiency and efficacy *in vivo*. Tissue Eng 2003,9 Suppl 1:S127-S139

38. Sun Y, Xu L, Huang S, et al. mir-21 overexpressing mesenchymal stem cells accelerate fracture healing in a rat closed femur fracture model. Biomed.Res.Int. 2015,(2015):412327

39. Yu Z, Zhu T, Li C, et al. Improvement of intertrochanteric bone quality in osteoporotic female rats after injection of polylactic acid-polyglycolic acid copolymer/collagen type I microspheres combined with bone mesenchymal stem cells. Int.Orthop. 2012,36(10):2163-71.

40. Zhang L, Wang P, Mei S, Li C, Cai C, Ding Y. *in vivo* alveolar bone regeneration by bone marrow stem cells/fibrin glue composition. Arch.Oral Biol. 2012,57(3):238-44.

41. McDuffee LA, Pack L, Lores M, Wright GM, Esparza-Gonzalez B, Masaoud E. Osteoprogenitor cell therapy in an equine fracture model. Vet.Surg. 2012,41(7):773-783.

42. Seo JP, Tsuzuki N, Haneda S, et al. Osteoinductivity of gelatin/beta-tricalcium phosphate sponges loaded with different concentrations of mesenchymal stem cells and bone morphogenetic protein-2 in an equine bone defect model. Vet.Res.Commun. 2014,38(1):73-80.