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Translational Research

Local administration of allogeneic or autologous bone marrow-derived mesenchymal stromal cells enhances bone formation similarly in distraction osteogenesis

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ABSTRACT

Background aims: Distraction osteogenesis (DO) is a surgical technique to promote bone regeneration that requires a long time for bone healing. Bone marrow-derived mesenchymal stromal cells (MSCs) have been applied to accelerate bone formation in DO. Allogeneic MSCs are attractive, as they could be ready to use in clinics. Whether allogeneic MSCs would have an effect similar to autologous MSCs with regard to promoting bone formation in DO is still unknown. This study compares the effect of autologous MSCs versus allogeneic MSCs on bone formation in a rat DO model.

Methods: Rat bone marrow-derived MSCs were isolated, characterized and expanded *in vitro*. Adult rats were subjected to right tibia transverse osteotomy. On the third day of distraction, each rat received one injection of phosphate-buffered saline (PBS), autologous MSCs or allogeneic MSCs at the distraction site. Tibiae were harvested after 28 days of consolidation for micro-computed tomography examination, mechanical test and histological analysis.

Results: Results showed that treatment with both allogeneic and autologous MSCs promoted bone formation, with significantly higher bone mass, mechanical properties and mineral apposition rate as well as expression of angiogenic and bone formation markers at the regeneration sites compared with the PBS-treated group. No statistical difference in bone formation was found between the allogeneic and autologous MSC treatment groups.

Conclusions: This study indicates that allogeneic and autologous MSCs have a similar effect on promoting bone consolidation in DO. MSCs from an allogeneic source could be used off-the-shelf with DO to achieve early bone healing.

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Introduction

Distraction osteogenesis (DO) is a powerful technique for tissue regeneration that has been commonly applied to the treatment of limb discrepancy, bone defect, nonunion, infection and malformation [1,2]. Although tremendous achievements have been made, a major limitation for its clinical application is the long treatment duration, which places great physical and economic burdens on patients [3].

Multiple therapeutic strategies, such as gene therapy [4], small-molecule therapy [5], growth factor-based therapy [6], scaffold-based therapy [7,8] and stem cell-based therapy [1,9], have been developed to accelerate DO-mediated bone healing.

Mesenchymal stromal cells (MSCs) have been identified and applied in tissue regeneration for decades as a result of their special properties in producing regenerative cytokines, replicating and differentiating into connective tissue cells. The wide application of MSCs as immune regulatory cells represents a promising approach to modulating the micro-environment. Studies have shown that MSCs are effective in promoting bone healing and enhancing bone consolidation in DO animal models [1,7,10–13]. The authors' recent review article addressed the successful applications of MSCs in DO systematically in various animal species (mice, rabbits, dogs, sheep), bone sites (femur, tibia, skull), DO protocols and cell transplantation methods [14]. Systemic reviews from other groups have also addressed the efficacy of locally applied MSCs in promoting bone formation in mandible DO animal models [15,16]. In these reviews, most of the studies found that MSCs from autologous, allogeneic or xenogeneic sources were safe and effective in DO-mediated mandibular bone formation. However, identification of ideal MSC populations for DO-mediated bone formation remains largely elusive.

Regarding the safety and efficiency of clinical application of MSCs in DO, many factors, such as cell source, cell numbers, cell delivery and timing of administration, should be addressed at the pre-clinical stage [9]. Among these, an appropriate cell source is a practical issue that greatly impacts the success of cell therapy in DO. Autologous stem cell therapy uses the patient's own cells and is considered the current gold standard with regard to cell source, with no risk of disease transmission or ethical concerns. Recently, a multi-centric clinical trial revealed that *ex vivo*-expanded autologous bone marrow-derived MSCs with bioceramic granules were safe and effective for the treatment of long bone non-union [17]. Additionally, the efficacy of autologous stem cells in regenerating bone has been confirmed by many pre-clinical models [18,19], including a DO animal model [7]. Despite the favorable safety profile and efficacy, culturing autologous MSCs from individuals remains a clinical challenge. The isolation and *ex vivo* expansion of MSCs to achieve clinically relevant cell numbers are time-consuming [20]. In addition, donor site pain and age-associated dysfunction in proliferation and differentiation limit the broad application of autologous MSC therapy [21,22].

Allogeneic stem cell therapy uses stem cells harvested from a donor. These cells can be expanded and stored, providing an off-the-shelf choice for patients in need [23]. Therefore, allogeneic MSCs could be an interesting alternative, as they are anticipated to be immune-privileged and even immune-regulatory [24–26]. The safety and efficacy of allogeneic MSCs in fracture or bone defect healing have been verified in many pre-clinical animal models [27–29]. Clinically, allogeneic mesenchymal progenitor cells have also been successfully applied in children suffering from osteogenesis imperfecta through their effects on promoting osteogenesis and accelerating growth velocity [30,31].

Recently, the authors' group reported that administration of allogeneic MSCs during the distraction phase, but not the consolidation phase, accelerated bone consolidation in a rat DO model, providing an optimized time point for stem cell therapy in DO [1]. Regarding stem cell therapy in bone healing, the literature shows various results, ranging from superior effect on bone formation to equally efficient bone regeneration using autologous MSCs compared with allogeneic MSCs [19,32]. Even though stem cell therapy in DO using either autologous or allogeneic MSCs has been reported to be effective in promoting early bone healing [1,7,10–13,33], there is still a lack of direct evidence to compare the safety and efficiency of allogeneic or autologous MSC treatment in the DO process. Moreover,

timing in DO procedures is a very important factor in influencing clinical outcomes, making cell therapy using autologous MSCs technically difficult to achieve.

In this study, the authors investigated whether allogeneic or autologous bone marrow-derived MSCs are equally safe and effective in bone consolidation in a rat DO model. This translational study also provides a clinical indication for stem cell-based therapy in patients subjected to DO procedures to achieve early bone consolidation.

Methods

Animals and ethics

Thirty 16-week-old male Sprague Dawley (SD) rats were used in this study. All rats were provided by the Laboratory Animal Services Centre of the Chinese University of Hong Kong. Ethics approval was obtained from the Animal Ethics Experimentation Committee of the Chinese University of Hong Kong. Surgeries were carried out under anesthesia, and efforts were made to minimize the animals' suffering.

Isolation and culture of bone marrow-derived MSCs (BMSCs)

Ten SD rats were anesthetized with a solution of 0.2% (v/v) xylazine and 1% (v/v) ketamine in phosphate-buffered saline (PBS) before surgery. Bone marrow was harvested from the left femur of animals ($n = 10$) in the autologous bone marrow-derived MSC treatment group using an 18-gauge needle after penetrating the cortex using a hand drill. Bone marrow was aspirated into a 3-mL syringe with 0.3 mL PBS. The bone marrow cells were plated and cultured in minimum essential medium alpha modification (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, Waltham, CA) and a 1% penicillin–streptomycin antibiotic mixture (Gibco, Waltham, CA) at 37°C with 5% carbon dioxide and 95% humidity. The culture medium was changed every 3 days. The cells were subcultured after reaching 80% confluence. Non-adherent cells were eliminated after subculture. Cells of passage three were used in the animal experiment. For the PBS treatment group and allogeneic bone marrow-derived MSC treatment group, the left femurs of the animals were subjected to sham operations. For the allogeneic bone marrow-derived MSC treatment group, the bone marrow-derived MSCs were pooled from the cells in the autologous bone marrow-derived MSC treatment group before cell injection.

Characterization of bone marrow-derived MSCs

Flow cytometry and multi-lineage differentiation assays were performed to identify bone marrow-derived MSCs as previously described [45,46]. The cells were rinsed with PBS after reaching 80% confluence and treated with 0.05% trypsin–ethylenediaminetetraacetic acid. The serum-containing medium was added to end trypsinization. All fluid was collected and centrifuged ($400 \times g$ for 5 min). The supernatant was discarded, and the precipitate was resuspended in staining buffer and incubated with fluorochrome-conjugated primary antibodies against CD29, CD34, CD45, CD90 and corresponding isotype controls (BD Biosciences, Franklin Lakes, NJ) at 4°C for 30 min in the dark. The stained cells were immediately detected using flow cytometry (BD Biosciences, Franklin Lakes, NJ). Data analysis was performed using FlowJo software (BD Biosciences, Franklin Lakes, NJ). The trilineage differentiation potential of the cells was determined by Alizarin Red S stain for osteogenesis, Oil Red O stain for adipogenesis and Safranin O stain for chondrogenesis as previously described [47].

DO animal model

Thirty 16-week-old male SD rats were used in the authors' study. Each rat was operated on under general anesthesia with a solution of

0.2% (v/v) xylazine and 1% (v/v) ketamine in saline. A transverse 5-mm osteotomy was performed on the mid-shaft of the right tibia as previously described [1,48,49]. A monolateral external fixator (Tianjin Xinzhang Medical Devices Co., Ltd., Tianjin, China) with four stainless steel pins was used to fix the bone segments. The DO procedure included three periods: a 5-day latency phase, a 5-day distraction phase with a lengthening rate of 0.5 mm/12 h and a 28-day consolidation phase. After the operations, the rats were locally injected with autologous bone marrow-derived MSCs (5×10^5 cells in 100 μ L PBS, $n = 10$), allogeneic bone marrow-derived MSCs (5×10^5 cells in 100 μ L PBS for each dose, $n = 10$) or PBS (100 μ L PBS, $n = 10$) on the third day of the distraction phase. Before the injections, MSCs were trypsinized and washed with PBS. The PBS with or without cells was injected immediately into the lengthening gap once the cells were prepared. To avoid any leakage, the injections were performed by gently inserting a 32-gauge needle into the defect site to a depth of around 10 mm, waiting for 5 seconds and then slowly withdrawing the needle. The procedures were performed under isoflurane anesthesia.

Subcutaneous injection of calcein 10 mg/kg (Sigma–Aldrich, St Louis, MO, USA) was performed 13 days before termination, and subcutaneous injection of Xylenol Orange 30 mg/kg (Sigma–Aldrich, St. Louis, MI) was performed 3 days before termination. Bilateral tibiae were harvested after 28 days of consolidation for further analysis.

Digital radiographs

X-ray images were taken after 5 and 28 days of consolidation to monitor bone healing at the distraction site using an MX-20 digital x-ray machine (Faxitron X-Ray Corporation, Wheeling, IL), an exposure time of 6000 ms and a voltage of 32 kV.

Micro-computed tomography

Micro-structural change within the distraction regeneration area in the rat was quantitatively assessed using micro-computed tomography (micro-CT) after 28 days of consolidation as previously described [1,50]. Briefly, all specimens ($n = 10$ per group) were imaged using a high-resolution micro-CT (Scanco Medical, Bassersdorf, Switzerland) at a custom isotropic resolution of 8 μ m isometric voxel size with a voltage of 70 kV and a current of 114 μ A. Three-dimensional (3D) reconstructions of the mineralized callus were performed using a global threshold (hydroxyapatite 158 mg/cm³), and a Gaussian filter (sigma = 0.8, support = 2) was applied to suppress noise. Cross-sectional images of the distraction zone were used to perform 3D reconstruction analysis. The region of interest was defined as the distraction zone (regenerate) between the two closest proximal and distal half-pins. Bone mineral density (BMD) and bone volume/total tissue volume (BV/TV) of each specimen were recorded with the built-in software for analysis.

Mechanical testing

To evaluate the mechanical properties of specimens, four-point bending tests were performed within 24 h of sample collection after micro-CT analysis. A material testing system (H25KS; Hounsfield Test Equipment Ltd., UK) was applied with a 250 N load cell to test the mechanical properties of the tibiae by load-to-failure test. The tibiae ($n = 10$ per group) were placed on the blades with inner and outer spans set as 2 cm and 6 cm. Maximum load, energy to failure and stiffness were recorded and calculated using the built-in software (QMat Professional; Tinius Olsen, Inc., Horsham, PA) for further analysis.

Decalcified and undecalcified histological assessments

All specimens were fixed in 10% formalin buffer for 48 h immediately after mechanical test and then transferred to 70% ethanol. Tibiae ($n = 10$ per group) were cut into two equal parts through the sagittal plane using a low-speed bone saw (Buehler, Lake Bluff, IL). Half of the samples ($n = 10$ per group) were decalcified in 10% ethylenediamine-tetraacetic acid solution for 4 weeks and then embedded in paraffin. Sections (5 μ m) were cut using an HM 355S microtome (Thermo Fisher Scientific, Walldorf, Germany) in the sagittal plane of the tibiae along the long axis. After deparaffinization, immunohistochemistry staining and hematoxylin and eosin staining were performed. Immunohistochemistry staining was performed following a standard protocol. Sections were incubated with anti-osteocalcin (anti-OCN) (ab13418; Abcam, Cambridge, UK), anti-runt-related transcription factor 2 (anti-RUNX2) (ab76956; Abcam, Cambridge, UK), anti-platelet endothelial cell adhesion molecule 1 (anti-PECAM-1)/CD31 (AF3628; R&D Systems, Minneapolis, MN) and anti-vascular endothelial growth factor A (anti-VEGFA) (ab1316; Abcam, Cambridge, UK) overnight at 4°C. A horseradish peroxidase–streptavidin system (Dako, Santa Clara, CA) was used for signal detection, followed by counterstaining with hematoxylin. Semi-quantitative analysis was performed in two sequential sections (100 μ m and 200 μ m apart from the center) per specimen ($n = 10$). Under a magnification of $\times 100$, five fields in the distraction regeneration area were randomly selected for evaluation. The mean value of all fields measured in each animal was taken for statistical analysis.

The other half of the samples ($n = 10$ per group) were treated by gradient alcohol dehydration and xylene defatting and then embedded in methyl methacrylate. Sections (10 μ m) were cut using an RM2155 hard tissue microtome (Leica, Wetzlar, Germany) in the sagittal plane of the tibiae along the long axis. The sections underwent von Kossa and Goldner trichrome staining for histomorphometric analysis. Quantitative analysis was performed in one section per specimen ($n = 10$ per group). Mineral apposition rate (MAR) was calculated based on dynamic histomorphometric measurement principles using the OsteoMeasure system (OsteoMetrics, Atlanta, GA) as described previously [1].

Statistical analysis

Results were presented individually in scatter plots with means and standard deviations. All quantitative parameters were analyzed using one-way analysis of variance and post-hoc Turkey's test. Statistical analysis was performed using SPSS 16.0 (IBM, Armonk, NY), and the level of significance was set as $P < 0.05$.

Results

Study design and characterization of bone marrow-derived MSCs

As shown in Figure 1A, rats were treated with PBS, autologous bone marrow-derived MSCs or allogeneic bone marrow-derived MSCs during the DO procedure. The DO procedure was performed at the right tibia. For the animals treated with autologous bone marrow-derived MSCs, bone marrow was harvested from the same animals before the DO procedure at the left femur using a syringe (Figure 1). Sham operation was performed at the left femur in PBS- or allogeneic MSC-treated groups (Figure 1). After subculturing three times, the cells were characterized by flow cytometry through the surface markers. Results showed that most of the bone marrow cells were positive for CD90 and CD29, the surface markers of MSCs, and negative for CD45 and CD34 (Figure 1B). Results of the trilineage differentiation assays suggested that they had adipogenic, osteogenic and chondrogenic differentiation potentials (Figure 1C). In order to verify the

Figure 1. Study design and characterization of BMSCs. (A) Animal study design. (B) Flow cytometric analysis showing the expression level of CD90+, CD29+, CD45– and CD34– in BMSCs (passage three) isolated from rats in the autologous BMSC group. (C) Trilineage differentiation of BMSCs (passage three), including the potency of adipogenic, osteogenic and chondrogenic differentiation. BMSCs, bone marrow-derived MSCs. (Color version of figure is available online.)

consistency in cell proliferation, cell doubling time ($22.03\text{h} \pm 3.32$) at passage 3 and culture duration ($15\text{d} \pm 1\text{d}$) from passage 0 to 3 were also determined (Supplemental Table 1).

Radiographic and micro-CT assessment

Rats were subjected to 5 days of latency, 5 days of distraction and 28 days of consolidation. Bone marrow-derived MSCs were expanded *ex vivo* before the DO procedure, and then the cells were injected into the regeneration site of the rats during the distraction phase (Figure 1A). After 5 days and 28 days of consolidation, the outcome of bone healing was monitored by x-ray imaging. As shown by the representative x-ray images, the gaps between bone segments in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group were filled with visible newly formed bone after 5 days of distraction, and the density became much higher after 28 days of consolidation (Figure 2A). These changes in the control group were not as obvious as that seen in the cell treatment groups. The representative 3D micro-CT images in Figure 2B show that the bone defect gap was still visible in the 0.5-cm lengthening regenerate in the control group. Interestingly, the 0.5-cm lengthening regenerates in the autologous bone marrow-derived MSC group and allogeneic bone marrow-derived MSC group were filled with comparable newly formed bone. The results were further confirmed by quantitative micro-CT results, showing significantly higher BV/TV fraction ($+273.25\%$, $P < 0.05$ and $+279.759\%$, $P < 0.05$) and BMD ($+333.38\%$, $P < 0.05$ and $+334.76\%$, $P < 0.05$) in the

regenerate of the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group compared with the control group after 28 days (Figure 2B–D). BV/TV and BMD showed no significant difference between the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group ($P > 0.05$) (Figure 2C,D).

Mechanical properties

Quality of the regenerates was assessed by four-point bending test. As shown in Figure 3, the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group showed significantly higher maximum load ($+2.94\text{-fold}$, $P < 0.01$ and $+2.19\text{-fold}$, $P < 0.01$), energy to failure ($+1.63\text{-fold}$, $P < 0.05$ and $+2.04\text{-fold}$, $P < 0.05$) and stiffness ($+3.36\text{-fold}$, $P < 0.01$ and $+1.19\text{-fold}$, $P < 0.05$) compared with the control group. Results also showed that maximum load, energy to failure and stiffness in the autologous bone marrow-derived MSC group were not statistically different from those in the allogeneic bone marrow-derived MSC group.

Decalcified and undecalcified histological assessments

After mechanical tests, the bone samples were cut longitudinally in half for decalcified bone histology or undecalcified bone histomorphometry. Results of hematoxylin and eosin staining showed that the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group contained a considerable

Figure 2. The 2D and 3D radiographic imaging results of DO animals treated with PBS, Auto-BMSCs or Allo-BMSCs. (A) Representative x-ray images taken after 5 days and 28 days of consolidation. (B) Representative micro-CT 3D images of samples harvested after 28 days of consolidation. Quantitative micro-CT data showing BV/TV fraction (C) and BMD (D) in the 0.5-cm lengthening regenerates. Data are shown individually in scatter plots with means and standard deviations ($n = 10$ per group). Two red lines indicate the regeneration site in between. * $P < 0.05$ versus control group, ns = $P > 0.05$ when Auto-BMSC group versus Allo-BMSC group analyzed by one-way ANOVA and post-hoc Turkey's test. Allo, allogeneic; ANOVA, analysis of variance; Auto, autologous; BMSCs, bone marrow-derived MSCs; d, day; ns, not significant; 2D, two-dimensional. (Color version of figure is available online.)

amount of newly formed bone bridging the whole regenerate. However, in the control group, the defects were still visible in the regenerate after 28 days of consolidation (Figure 4A). Results of von Kossa and Goldner trichrome staining in the undecalcified samples (Figure 4B,C) verified the findings of hematoxylin and eosin staining, showing more mineral deposition (black color after von Kossa staining or green color after Goldner trichrome staining) in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group compared with the control group. Moreover, mineral deposition was comparable in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group (Figure 4B,C).

Results of immunohistochemistry showed that a larger number of PECAM-1/CD31-positive endothelial cells [34] were found in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group compared with the control group (+63.94%, $P < 0.01$ and +45.87%, $P < 0.05$) (Figure 5A). In addition, results showed that higher expression of VEGFA, an important angiogenic marker that promotes coupling of angiogenesis and osteogenesis [35], was found in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group compared

with the control group (+156.20%, $P < 0.01$ and +114.13%, $P < 0.01$) (Figure 5B). Moreover, expression of the bone formation marker OCN was significantly increased in the regenerates in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group compared with the control group (+77.87%, $P < 0.01$ and +86.01%, $P < 0.01$) (Figure 5C). Furthermore, data showed significantly higher expression of the bone formation marker RUNX2 in the regenerates in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group compared with the control group (+55.41%, $P < 0.01$ and +38.88%, $P < 0.05$) (Figure 5D). However, expression of PECAM-1/CD31, VEGFA, OCN and RUNX2 showed no significant difference between the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group ($P > 0.05$).

MAR was analyzed using the *in vivo* double fluorescence labeling method. Representative images in Figure 6A show that the distance between the green and red fluorescence labels was comparable in the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group and was wider than that seen in the control group. The quantitative results (Figure 6B) revealed that MARs in the autologous bone marrow-derived MSC group ($1.12 \pm$

Figure 3. Four-point bending mechanical test for the evaluation of bone healing quality in DO animals treated with PBS, Auto-BMSCs or Allo-BMSCs. Mechanical properties included maximum load, energy to failure and stiffness. Data are shown individually in scatter plots with means and standard deviations ($n = 10$ per group). * $P < 0.05$ and ** $P < 0.01$ versus control group, ns = $P > 0.05$ when Auto-BMSC group versus Allo-BMSC group analyzed by one-way ANOVA and post-hoc Turkey's test. Allo, allogeneic; ANOVA, analysis of variance; Auto, autologous; BMSCs, bone marrow-derived MSCs.

Figure 4. Representative images of H-E, von Kossa or Goldner trichrome staining of distraction regenerates in the tibiae of DO animals after 28 days of consolidation. Scale bar = 500 μm . (A) Representative images of H-E staining of the regenerates. (B) Representative images of von Kossa staining of the regenerates. Black areas represent bone mineral deposition. (C) Representative images of Goldner trichrome staining of the regenerates. Green areas represent mineralized bone tissue. Allo, allogeneic; Auto, autologous; BMSCs, bone marrow-derived MSCs; NB, new bone; OB, old bone. (Color version of figure is available online.)

0.19 $\mu\text{m}/\text{day}$) and the allogeneic bone marrow-derived MSC group ($1.15 \pm 0.17 \mu\text{m}/\text{day}$) were significantly higher than that of the control group ($0.63 \pm 0.08 \mu\text{m}/\text{day}$) (+77.08.41%, $P < 0.01$ and +81.19%, $P < 0.01$).

Discussion

The long treatment duration of DO leads to frequent complications; therefore, shortening the treatment period may provide significant clinical benefits. Transplantation of MSCs could accelerate new bone formation, which represents a potential strategy for shortening

DO treatment time [14]. Although autologous MSC transplantation has advantages with regard to safety and efficiency in tissue regeneration, the procedure has several inevitable disadvantages, including source limits, risk of donor site infection and age-related impairment of MSC function [21]. By contrast, allogeneic MSC transplantation has many advantages, including off-the-shelf use, selective high quality of cells, easy access and reduced costs. It has been reported that MSCs do not express major histocompatibility class II and co-stimulatory molecules [36], suggesting an immune-privileged property, there is still controversy regarding the potential immunogenicity of allogeneic bone marrow-derived MSCs, which may diminish the effect of

Figure 5. Results of immunohistochemistry of distraction regenerates in the tibiae of DO animals after 28 days of consolidation. Expression of angiogenic markers CD31 and VEGFA and osteogenic markers OCN and RUNX2. Data are shown individually in scatter plots with means and standard deviations ($n = 10$ per group). * $P < 0.05$ and ** $P < 0.01$ versus control group, ns = $P > 0.05$ when Auto-BMSC group versus Allo-BMSC group analyzed by one-way ANOVA and post-hoc Turkey's test. Allo, allogeneic; ANOVA, analysis of variance; Auto, autologous; BMSCs, bone marrow-derived MSCs. (Color version of figure is available online.)

transplanted cells [37]. Fortunately, no inflammatory callus or inflammatory cell infiltration was observed in the authors' study, indicating no immunological rejection in the animals subjected to allogeneic bone marrow-derived MSCs or autologous bone marrow-derived MSCs.

In this study, the authors found that the outcome of bone healing was similar in both the allogeneic and autologous MSC treatment groups. Both the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group showed significantly higher bone mass in the regenerates than the control group. More

Figure 6. Results of undecalcified histomorphometry of distraction regenerates in the tibiae of DO animals after 28 days of consolidation. Representative images of double fluorescent *in vivo* labeling (A) and quantitative analysis of MAR (B) after 28 days of consolidation. Data are shown individually in scatter plots with means and standard deviations ($n = 10$ per group). * $P < 0.05$ and ** $P < 0.01$ versus control group, ns = $P > 0.05$ when Auto-BMSC group versus Allo-BMSC group analyzed by ANOVA and post-hoc Turkey's test. Allo, allogeneic; ANOVA, analysis of variance; Auto, autologous; BMSCs, bone marrow-derived MSCs. (Color version of figure is available online.)

interestingly, there was no significant difference between the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group, indicating an equivalent beneficial effect on enhancing new bone formation during DO. A previous study showed that there were variations in the osteogenic differentiation potential of bone marrow-derived MSCs obtained from rabbits, leading to various bone healing outcomes in the rabbit DO model [38]. In this study, the osteogenic differentiation potential of bone marrow-derived MSCs isolated from rats proved to be consistent and was further verified by *in vivo* outcomes in the rat DO model.

As a gold standard of bone quality assessment, mechanical properties measured by four-point bending tests, including maximum load, energy to failure and stiffness, showed no statistical difference between the autologous and allogeneic bone marrow-derived MSC groups. Mechanical properties in both cell treatment groups were higher than those in the untreated control group. Furthermore, as a dynamic bone formation marker, MAR of the callus was found to be significantly higher in both the autologous bone marrow-derived MSC group and the allogeneic bone marrow-derived MSC group compared with the non-treated group, suggesting both autologous and allogeneic MSCs may enhance bone formation during bone consolidation.

MSCs play various roles in tissue regeneration via self-renewal, differentiation into mature cells and secretion of anabolic cytokines [1,7,10–13]. However, it is reported that engrafted stem cells usually present poor viability and differentiation *in vivo*, indicating that they may promote tissue regeneration through paracrine mechanisms during DO [33]. A recent study found that exosomes from young MSCs (2 weeks) promoted DO-mediated bone regeneration in older rats (15 months) by enhancing the proliferation and osteogenic differentiation capacity of older bone marrow-derived MSCs [39]. Previous studies also reveal that MSCs may be involved in DO-mediated bone regeneration through the regulatory effect of their exosomes [1,14,48]. However, the underlying mechanisms behind this stem cell- or exosome-based therapy have yet to be elucidated. The authors' results showed that both autologous and allogeneic groups had significantly higher expression of angiogenic factors CD31 and VEGFA in callus, suggesting that MSC therapy, regardless of whether it derived from an autologous or allogeneic source, effectively promoted angiogenesis in distraction regenerates. Both autologous and allogeneic MSCs upregulated the expression of osteogenic factors RUNX2 and OCN, indicating that autologous and allogeneic MSCs promote osteogenic differentiation. Among the factors increased in both autologous and allogeneic MSC treatment groups, VEGFA signaling may be one of the key pathways that accounted for the enhancement of angiogenesis and bone consolidation in DO. During the distraction phase of DO, the VEGF-related family of proteins is upregulated and involved in new vessel formation. However, these angiogenic growth factors gradually diminish at the bone consolidation phase [40]. MSC therapy has been demonstrated to effectively promote angiogenesis and vasculogenesis and regenerate a new, robust vascular network during DO [41]. VEGFA is an important angiogenic factor that regulates the growth of the vascular network in the mammalian skeletal system and has multiple functions, including maintenance of vascular permeability, pro-angiogenic activity, endothelial cell stimulation and macrophage migration [42]. Type H vessels, with high expression of CD31, have been reported to be involved in bone formation and bone remodeling [43], and VEGFA has been reported to promote type H vessel formation and coupling of angiogenesis and osteogenesis [44]. Hence, MSC therapy might provide an additional, sustainable angiogenic and osteogenic micro-environment for regenerates during bone consolidation.

Conclusions

In summary, the authors' data show that local injection of either autologous or allogeneic MSCs into distraction regenerates enhances new bone formation and bone consolidation in the rat DO model,

leading to a shorter duration of the consolidation phase. The authors' data also show that allogeneic MSCs present a beneficial effect similar to that of autologous MSCs. Taken together, this study's data provide evidence that allogeneic bone marrow-derived MSC therapy may be a promising approach and an alternative to autologous MSCs in future clinical applications owing to its off-the-shelf potential. However, there is still a lack of proper clinical trials comparing the use of autologous versus allogeneic MSCs, and this is worth investigating in the future.

There are also some limitations in the current study. First, the underlying mechanisms that account for bone formation without immune rejection have not been addressed. Second, this study is only a proof-of-concept study in a rat DO model, which is far different from human patients. With regard to the translational aspect, further studies are needed to verify these findings in large animals before clinical trials are undertaken.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: QP, SL and GL. Acquisition of data: QP, YL, HW, YL, LK, XZ, SB and ZY. Analysis and interpretation of data: QP, YL, ZY and GC. Drafting or revising the manuscript: QP, HW, ZZ and SL. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.jcyt.2020.12.005](https://doi.org/10.1016/j.jcyt.2020.12.005).

References

- [1] Yang Y, Pan Q, Zou K, Wang H, Zhang X, Yang Z, Lee WY, Wei B, Gu W, Yang YP, Lin S, Li G. Administration of allogeneic mesenchymal stem cells in lengthening phase accelerates early bone consolidation in rat distraction osteogenesis model. *Stem Cell Res Ther* 2020;11(1):129.
- [2] Tsuchiya H, Tomita K. Distraction osteogenesis for treatment of bone loss in the lower extremity. *J Orthop Sci* 2003;8(1):116–24.
- [3] Liantis P, Mavrogenis AF, Stavropoulos NA, Kanellopoulos AD, Papagelopoulos PJ, Soucacos PN, Babis GC. Risk factors for and complications of distraction osteogenesis. *Eur J Orthop Surg Traumatol* 2014;24(5):693–8.
- [4] Jiang X, Chen J, Huang H, Chen Y, Peng H. Bone regeneration and mineralization was promoted during distraction osteogenesis by human periostin gene in rabbit mandibular model. *Journal of Stomatology, Oral and Maxillofacial Surgery* 2020;121(5):506–11.
- [5] Yang ZH, Wu BL, Ye C, Jia S, Yang XJ, Hou R, Lei DL, Wang L. Targeting P38 Pathway Regulates Bony Formation via MSC Recruitment during Mandibular Distraction Osteogenesis in Rats. *Int J Med Sci* 2016;13(10):783–9.
- [6] Makhdom AM, Hamdy RC. The role of growth factors on acceleration of bone regeneration during distraction osteogenesis. *Tissue Eng Part B Rev* 2013;19(5):442–53.

- [7] Sun Z, Tee BC, Kennedy KS, Kennedy PM, Kim DG, Mallery SR, Fields HW. Scaffold-based delivery of autologous mesenchymal stem cells for mandibular distraction osteogenesis: preliminary studies in a porcine model. *PLoS One* 2013;8(9):e74672.
- [8] Wang Y, Ni M, Tang PF, Li G. Novel application of HA-TCP biomaterials in distraction osteogenesis shortened the lengthening time and promoted bone consolidation. *J Orthop Res* 2009;27(4):477–82.
- [9] Drosse I, Volkmer E, Capanna R, De Biase P, Mutschler W, Schieker M. Tissue engineering for bone defect healing: an update on a multi-component approach. *Injury* 2008;39(Suppl 2):S9–20.
- [10] Kitoh H, Kitakoji T, Tsuchiya H, Mitsuyama H, Nakamura H, Katoh M, Ishiguro N. Transplantation of marrow-derived mesenchymal stem cells and platelet-rich plasma during distraction osteogenesis—a preliminary result of three cases. *Bone* 2004;35(4):892–8.
- [11] Sunay O, Can G, Cakir Z, Denek Z, Kozanoglu I, Erbil G, Yilmaz M, Baran Y. Autologous rabbit adipose tissue-derived mesenchymal stromal cells for the treatment of bone injuries with distraction osteogenesis. *Cytotherapy* 2013;15(6):690–702.
- [12] Aykan A, Ozturk S, Sahin I, Gurses S, Ural AU, Oren NC, Isik S. Biomechanical analysis of the effect of mesenchymal stem cells on mandibular distraction osteogenesis. *J Craniofac Surg* 2013;24(2):e169–75.
- [13] Dehghan MM, Baghaban Eslaminejad M, Motalebzadeh N, Ashrafi Halan J, Tagiyar L, Soroori S, Nikmahzar A, Pedram M, Shahverdi A, Kazemi Mehrjerdi H, Izadi S. Transplantation of Autologous Bone Marrow Mesenchymal Stem Cells with Platelet-Rich Plasma Accelerate Distraction Osteogenesis in A Canine Model. *Cell J* 2015;17(2):243–52.
- [14] Yang Y, Lin S, Wang B, Gu W, Li G. Stem cell therapy for enhancement of bone consolidation in distraction osteogenesis: a contemporary review of experimental studies. *Bone Joint Res* 2017;6(6):385–90.
- [15] Morillo CMR, Sloniak MC, Gonçalves F, Villar CC. Efficacy of stem cells on bone consolidation of distraction osteogenesis in animal models: a systematic review. *Braz Oral Res* 2018;32:e83.
- [16] Tee BC, Sun Z. Mandibular distraction osteogenesis assisted by cell-based tissue engineering: a systematic review. *Orthod Craniofac Res* 2015;18(Suppl 1(0 1)):39–49.
- [17] Gómez-Barrena E, Rosset P, Gebhard F, Hernigou P, Baldini N, Rouard H, Sensebé L, Gonzalo-Daganzo RM, Giordano R, Padilla-Eguiluz N, García-Rey E, Cordero-Ampuero J, Rubio-Suárez JC, Stanovici J, Ehrnthaller C, Huber-Lang M, Flouzat-Lachaniette CH, Chevallier N, Donati DM, Ciapetti G, Fleury S, Fernandez MN, Cabrera JR, Avendaño-Solá C, Montemurro T, Panaitescu C, Veronesi E, Rojewski MT, Lotfi R, Dominici M, Schrezenmeier H, Layrolle P. Feasibility and safety of treating non-unions in tibia, femur and humerus with autologous, expanded, bone marrow-derived mesenchymal stromal cells associated with biphasic calcium phosphate biomaterials in a multicentric, non-comparative trial. *Biomaterials* 2019;196:100–8.
- [18] de Girolamo L, Arrigoni E, Stanco D, Lopa S, Di Giancamillo A, Addis A, Borronovo S, Dellavia C, Domeneghini C, Brini AT. Role of autologous rabbit adipose-derived stem cells in the early phases of the repairing process of critical bone defects. *J Orthop Res* 2011;29(1):100–8.
- [19] Rapp AE, Bindl R, Erbacher A, Kruchen A, Rojewski M, Schrezenmeier H, et al. Autologous Mesenchymal Stroma Cells Are Superior to Allogeneic Ones in Bone Defect Regeneration. *Int J Mol Sci* 2018;19(9):2526.
- [20] Kaushik AP, Das A, Cui Q. Stem Cells in Osteonecrosis: Cons. Osteonecrosis. Berlin, Heidelberg: Springer; 2014. p. 285–9.
- [21] Zhuo Y, Li SH, Chen MS, Wu J, Kinkaid HY, Fazal S, Weisel RD, Li RK. Aging impairs the angiogenic response to ischemic injury and the activity of implanted cells: combined consequences for cell therapy in older recipients. *J Thorac Cardiovasc Surg* 2010;139(5):1286–94, 1294.e1–2.
- [22] Ahmed AS, Sheng MH, Wasnik S, Baylink DJ, Lau KW. Effect of aging on stem cells. *World J Exp Med* 2017;7(1):1–10.
- [23] Krampera M, Pizzolo G, Aprili G, Franchini M. Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. *Bone* 2006;39(4):678–83.
- [24] Xu L, Li G. Circulating mesenchymal stem cells and their clinical implications. *Journal of Orthopaedic Translation* 2014;2(1):1–7.
- [25] Bunnell BA, Betancourt AM, Sullivan DE. New concepts on the immune modulation mediated by mesenchymal stem cells. *Stem Cell Res Ther* 2010;1(5):34.
- [26] Chen X, Armstrong MA, Li G. Mesenchymal stem cells in immunoregulation. *Immunol Cell Biol* 2006;84(5):413–21.
- [27] Wan C, He Q, Li G. Allogenic peripheral blood derived mesenchymal stem cells (MSCs) enhance bone regeneration in rabbit ulna critical-sized bone defect model. *J Orthop Res* 2006;24(4):610–8.
- [28] Huang S, Xu L, Zhang Y, Sun Y, Li G. Systemic and Local Administration of Allogeneic Bone Marrow-Derived Mesenchymal Stem Cells Promotes Fracture Healing in Rats. *Cell Transplant* 2015;24(12):2643–55.
- [29] Liu G, Zhang Y, Liu B, Sun J, Li W, Cui L. Bone regeneration in a canine cranial model using allogeneic adipose derived stem cells and coral scaffold. *Biomaterials* 2013;34(11):2655–64.
- [30] Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T. 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.
- [31] Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999;5(3):309–13.
- [32] Coathup MJ, Kalia P, Konan S, Mirza K, Blunn GW. A comparison of allogeneic and autologous mesenchymal stromal cells and osteoprogenitor cells in augmenting bone formation around massive bone tumor prostheses. *J Biomed Mater Res A* 2013;101(8):2210–8.
- [33] Ando Y, Matsubara K, Ishikawa J, Fujio M, Shohara R, Hibi H, Ueda M, Yamamoto A. Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms. *Bone* 2014;61:82–90.
- [34] Fawcett J, Buckley C, Holness CL, Bird IN, Spragg JH, Saunders J, Harris A, Simmons DL. Mapping the homotypic binding sites in CD31 and the role of CD31 adhesion in the formation of interendothelial cell contacts. *J Cell Biol* 1995;128(6):1229–41.
- [35] Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. *Semin Oncol* 2002;29(6 Suppl 16):10–4.
- [36] Griffin M, Iqbal SA, Bayat A. Exploring the application of mesenchymal stem cells in bone repair and regeneration. *J Bone Joint Surg Br* 2011;93(4):427–34.
- [37] Sivanathan KN, Gronthos S, Rojas-Canales D, Thierry B, Coates PT. Interferon-gamma modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. *Stem Cell Rev Rep* 2014;10(3):351–75.
- [38] Montes-Medina L, Hernández-Fernández A, Gutiérrez-Rivera A, Ripalda-Cemboráin P, Bitarte N, Pérez-López V, Granero-Moltó F, Prosper F, Izeta A. Effect of bone marrow stromal cells in combination with biomaterials in early phases of distraction osteogenesis: an experimental study in a rabbit femur model. *Injury* 2018;49(11):1979–86.
- [39] Jia Y, Qiu S, Xu J, Kang Q, Chai Y. Exosomes Secreted by Young Mesenchymal Stem Cells Promote New Bone Formation During Distraction Osteogenesis in Older Rats. *Calcif Tissue Int* 2020;106(5):509–17.
- [40] Ai-Aql ZS, Alagil AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *J Dent Res* 2008;87(2):107–18.
- [41] Deshpande SS, Gallagher KK, Donneys A, Nelson NS, Guys NP, Felice PA, Page EE, Sun H, Krebsbach PH, Buchman SR. Stem cells rejuvenate radiation-impaired vasculogenesis in murine distraction osteogenesis. *Plast Reconstr Surg* 2015;135(3):799–806.
- [42] Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes Cancer* 2011;2(12):1097–105.
- [43] Peng Y, Wu S, Li Y, Crane JL. Type II blood vessels in bone modeling and remodeling. *Theranostics* 2020;10(1):426–36.
- [44] Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature* 2014;507(7492):323–8.
- [45] Lin S, Lee WYW, Xu L, Wang Y, Chen Y, Ho KKW, Qin L, Jiang X, Cui L, Li G. Step-wise preconditioning enhances mesenchymal stem cell-based cartilage regeneration through epigenetic modification. *Osteoarthritis Cartilage* 2017;25(9):1541–50.
- [46] Deng J, Petersen BE, Steindler DA, Jorgensen ML, Laywell ED. Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. *Stem Cells* 2006;24(4):1054–64.
- [47] Lin W, Xu L, Lin S, Shi L, Wang B, Pan Q, Lee WYW, Li G. Characterisation of multipotent stem cells from human peripheral blood using an improved protocol. *J Orthop Translat* 2019;19:18–28.
- [48] Xu J, Wang B, Sun Y, Wu T, Liu Y, Zhang J, Lee WY, Pan X, Chai Y, Li G. Human fetal mesenchymal stem cell secretome enhances bone consolidation in distraction osteogenesis. *Stem Cell Res Ther* 2016;7(1):134.
- [49] Xu J, Wu T, Sun Y, Wang B, Zhang J, Lee WY, Chai Y, Li G. Staphylococcal enterotoxin C2 expedites bone consolidation in distraction osteogenesis. *J Orthop Res* 2017;35(6):1215–25.
- [50] Xu J, Sun Y, Wu T, Liu Y, Shi L, Zhang J, Kang Q, Chai Y, Li G. Enhancement of bone regeneration with the accordion technique via HIF-1 α /VEGF activation in a rat distraction osteogenesis model. *J Tissue Eng Regen Med* 2018;12(2):e1268–76.