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Effects of Bone Marrow Stromal Cell Transplantation on Repair of Bone Defect in Rats

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Abstract

Background: Mesenchymal stem cells are one type of adult stem cells, which are able to give rise to mesodermal origin tissues. The application of mesenchymal stem cells for tissue and organ regeneration offer advantages because of the relative ease of collection and their potential to differentiate to 3 cell lineages.
Objectives: This research was designed to study and evaluate the effect of mesenchymal stem cells on the repair of bone defects in Wistar rat models and to compare autologous and non-autologous cell transplantation in repairing bone defects.
Methods: The mesenchymal stem cells were cultured and expanded in MEM medium supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin by incubation at 37°C in 5% CO_2. Immunocytochemistry analysis was performed using CD44 and fibronectin markers to detect the mesenchymal stem cells. A transcortical defect was created within the distal epiphyses of the femur bone. After 3 days of injury, Brdu-labeled cells were injected at the site of injury. The animals were sacrificed after 4 weeks of transplantation and the femurs with the recipient area were removed and the length, weight, area, density, and biomechanical parameters were evaluated.
Results: The obtained results showed that although neither non-autologous nor autologous cell transplantation significantly improved the effect on length and area of the defective femur bone, the mass and bone density of the cell-injected transplanted groups had a significant increase in comparison with the control and sham groups.
Conclusion: It seems that cell transplantation could improve these parameters, improving the mean mass, length, area, and density of parameters during the the regeneration process.

Keywords: BMSCs, Cell Transplantation, Bone Repair, Rat on the Bone Regeneration

1. Background

Bone regeneration is a process that has common features with embryonic development of the skeleton and involves molecular pathways mediating chondrogenesis and osteogenesis during fetal development (1, 2).

Different strategies have been defined to promote bone healing, which rely on cells that can participate in bone regeneration process through cell contacts, growth factors, cytokines, and extracellular matrix proteins, creating micro domains or niches and regulating self-renewal and differentiation (3, 4). Bone marrow-derived mesenchymal stem cells (MSCs) represent a considerable candidate for cell therapy due to being easily obtained from a bone marrow aspiration and expanded to large number before transplantation, without ethical problems. In general, cell-based therapies have shown promising results for tissue regeneration using MSCs (5). In addition, the use of MSCs for tissue and organ regeneration offers advantages because of the relative ease of collection using a simple bone marrow aspiration and by showing their potential to differentiate to 3 lineages of cells (6-10). Additionally, they can be easily cultured outside the body for several passages without losing their renewal (11).

Mesenchymal stem cells are able to give rise to tissues of mesodermal origin, such as dentine, bone or periodontal ligament (12), and bone regenerative potential of MSCs have been evaluated in bone defects in animals (13, 14). The international society of cellular therapy defined mesenchymal stem cells as pluripotent cells (15) that are usually present in damaged tissue, and through cell-to-cell interactions, or by secreting proteins can repair the affected

area andthe influence of MSCs on damaged areas (16, 17). Some studies also suggested that MSCs lack certain receptors on their surface, which allows them to escape the T-cell component of the immunity system (18, 19).

The MSCs also release biologically active molecules and many known mediators of tissue repair, such as VEGF, PDGF, bFGF, EGF, KGF, and TGF-β that influence cell migration, proliferation, and survival of the surrounding cells (20). Systemic transplantation of MSCs showed that the cells have the ability to migrate through the circulatory system and participate in regeneration of damaged tissue (21-23). In addition, injection of the cells to specific target tissues has shown that these cells contribute to the tissue cell phenotype (24-28).

Autologous and none-autologous MSCs transplantation are considered as ideal procedures and may play a role in the clinical application of stem cells, particularly for bone defects, since they can be easily obtained without moral constraint and their presence and proliferation can be traced (29).

2. Objectives

This study aimed at evaluating the effect of bone marrow mesenchymal stem cells on the repair of bone defects in femur bone of rats and comparing autologous and non-autologous cell transplantation for repairing bone defects.

3. Methods

3.1. Animals

Fifty-six male adult Wistar rats, weighting 200 $\pm$ 20 g, were maintained under standard laboratory conditions. Animals were housed in an environment of 21 $\pm$ 0.5°C with a relative humidity of 50 $\pm$ 10% and a 12-hour light-dark cycle. Food and water were always available. Rats were randomly divided to 4 equal groups (14 rats in each group) as follows: 1- Rats with bone defect and no treatment (control group), 2- Rats with bone defect and culture medium treatment (sham group), 3- Rats with bone defect and non-autologous MSCs transplantation (Experimental group 1), and 4- Rats with bone defect and autologous MSCs transplantation (Experimental group 2).

3.2. Preparation of Mesenchymal Stem Cells culture

Under sterile conditions, MSCs were obtained from bone marrow and harvested during anesthesia by aspiration to a syringe containing 1 mL of DMEM medium from the adult male rat femurs femoral of adult male Wistar rats. Approximately 1 mL of bone marrow was obtained and MSCs were isolated by adherence to the plastic surface of the culture plates. The MSCs were further cultured and expanded in alpha-minimum essential medium (MEM; Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco), and 2% penicillin/streptomycin mixture (Pen/Strep, 10 000 IU/mL; Gibco), by incubation at 37°C in a 5-% CO_2 atmosphere. On In the second day, the non-adherent cells were removed. The medium was replaced every 2 days with fresh medium and the cells were maintained in culture with cell passaging.

3.3. Immunocytochemistry

Immunocytochemistry analysis was performed using CD44 and fibronectin markers to detect the MSCs. Medium was removed from plates, cells were washed in PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. For investigations of protein expression, goat anti-rat CD44 and fibronectin antibody was used and incubated at 37°C for 12 hours with 5 mg/mL of the following monoclonal antibodies (Santa Cruz). The staining protocol was continued with secondary antibody binding (60 minutes), substrate addition, and hematoxylin counterstaining of the nuclei. The nuclei were counterstained with hematoxylin.

The quantification assessment was performed using the Motic software with at least 5 randomly selected fields at 400X magnification and counting positive cells.

3.4. Surgical Procedure

Rats were anesthetized with 60 mg/kg ketamine hydrochloride and 10 mg/kg xylazine and a 2-cm longitudinal incision was caused in the middle third of the femur. The rat femur bone was exposed and a transcortical defect was created within the distal epiphyses of the femur bone using a drill sized 1 mm. After 3 days of injury, 2×10^5 cells/mL of Brdu labeled cells were suspended in DMEM medium and injected at the site of injury using an insulin syringe. All animals received 2,400,000 U of penicillin, Intramuscularly (IM) up to 6 days.

3.5. Detection of Brdu-labeled Mesenchymal Stem Cells

Four weeks after cell transplantation, tissue sections prepared from the bone defect of rat femur were treated with polyclonal antibodies specific for BrdU. The implants were fixed overnight in 10% formaldehyde and decalcified for 7 days in 10% formic acid. The resulting specimens were paraffin embedded and sectioned at a thickness of 5 micrometers. Sections were de-paraffinized and rehydrated and washed with Phosphate Buffered Saline (PBS), and were then incubated with rat monoclonal anti-Brdu (1:100 dilution; Abcam) for 12 hours at 4°C, followed by

Biotin and Avidin with HRP-conjugated anti-rat IgG for 1-hour. Specimens were colored by diaminobenzidine (DAB) as a substrate at room temperature. Sections were counterstained with Hematoxylin. The new bone formation occupying space within the bone defects was measured using an image analysis system coupled with a light microscope, and then the Brdu-labeled cells were counted at different microscopic fields.

3.6. Biomechanical Testing

The effect of MSCs transplantation on the mechanical properties of healing bone was evaluated after 4 weeks of transplantation. Briefly, the animals were sacrificed and the femurs were harvested, wrapped in saline soaked gauze, and stored at -20°C until the time of testing. Prior to testing, the femurs were thawed at room temperature and kept moist in a saline solution. The proximal and distal ends of each femur were then placed in the grips of a hydraulically material testing machine (Zwick, Germany). The bones were tested at 3 points, bending until failure, at a constant angular displacement rate of 2 degrees per second. The stiffness and the breaking strength (Fmax) of the femur bones were tested for all groups.

3.7. Assessment of Morphological Parameters

Animals were sacrificed after 4 weeks of MSCs transplantation and the femurs with the recipient area were removed and the length, weight, area, and density parameters were examined as below:

Length: the length of the femur was measured in all groups. The ruler caliper was used, and length (mm) was recorded for each sample and the average sizes were calculated separately for the 4 groups. Weight: The weight of all samples was measured using a mass balance and were recorded and compared between groups. Area: Finally, the area was measured using caliper diameters anterior-posterior and internal-external of the shaft for each sample using the mean diameter obtained and surface area per square millimeter for each sample using the formula below:

$A = \pi r2$ between groups were compared.

Density (g/mm^3): Density was obtained according to the following formula and was compared between groups.

$V = L \times A$

$P = M / V$

3.8. Statistical Analysis

The obtained data were calculated and expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparing the data in all groups. A significance level of 0.05 was predetermined for all statistical analyses.

4. Results

4.1. Isolation of BMSCs

The MSCs were isolated from bone marrow and characterized as the expression of fibronectin and CD44 markers. Results from immunohistochemistry analysis showed that more than 93% and 95% of MSCs expressed these markers, respectively (Figure 1).

4.2. Detection of Brdu-Labeled Cells

Immunohistochemical study of MSCs in the bone callus showed that in the cell therapy groups, which were previously labelled, MSCs were established and recognized in new bone and detected as brown cells. Hematoxylin staining was used as counter staining and the percentage of labeled cells and non-labeled cells were counted under a microscopic field (Figure 2).

4.3. Biomechanical Results

Four weeks after transplantation of MSCs, biomechanical testing of the femur bones of rats showed that the average maximum bone strength (Fmax) versus bending force was 1.287 ± 0.0613 in the control group, 1.299 ± 0.0368 in the sham group, 1.95 ± 0.02 in the non-autologous and 1.56 ± 0.113 in the autologous group. No significant differences were shown in the mean Fmax between sham and control groups. The mean Fmax significantly increased in the autologous group compared with the control group ($P < 0.05$). However, the mean Fmax increased in non-autologous group compared with the control group yet this difference was not statistically significant ($P = 0.07$).

4.4. Morphological Results

Length: After 4 weeks, mean length of femur in the control group (34.6 ± 0.34) and sham group (36.7 ± 0.68) was not significantly different. In addition, mean length of femur in non-autologous (35.7 ± 0.47) and autologous (36.4 ± 0.43) groups showed no significant difference when compared with the control group (Figure 3).

Weight: The results of the samples showed the average mass of the control (0.68 ± 0.022) and sham groups (0.68 ± 0.045) were not significantly different but non-autologous (0.88 ± 0.027) and autologous (0.87 ± 0.06) groups showed significant increase compared with the control group ($P < 0.05$) (Figure 4).

Area: The mean area of transverse section of tibia in control (3.5 ± 0.12), sham (3.6 ± 0.12), non-autologous (3.6 ± 0.07) and autologous (3.5 ± 0.10) groups showed no significant differences among the groups in this regard (Figure 5).

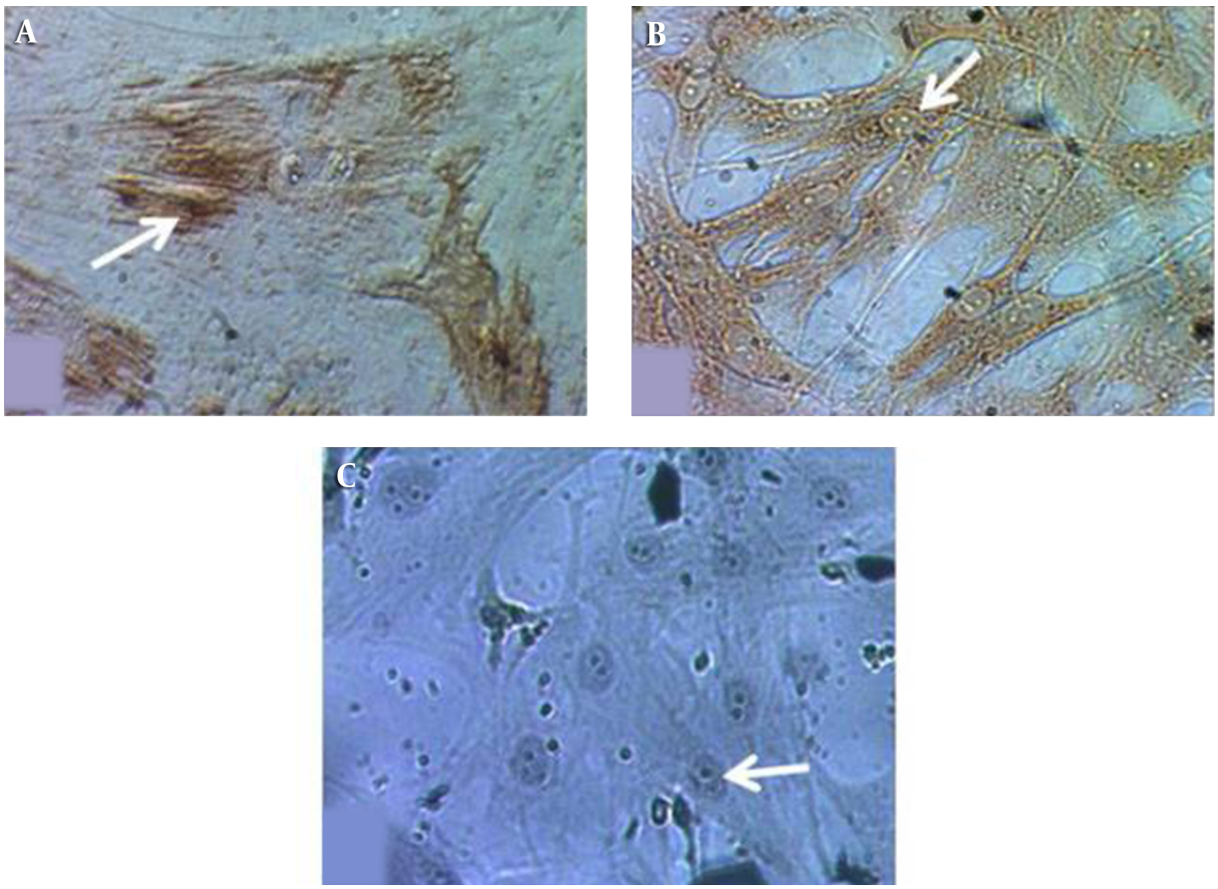

Figure 1. Photomicrograph of Mesenchymal Cells After Immunocytochemistry. A - C, are Related to Fibronectin Antibody, CD44 and Negative Control Tests, Respectively. Fibronectin and CD44 Positive Cells in A and B Were Detected Due to DAB Stained Brown Color (Original Magnification, 400 ×).

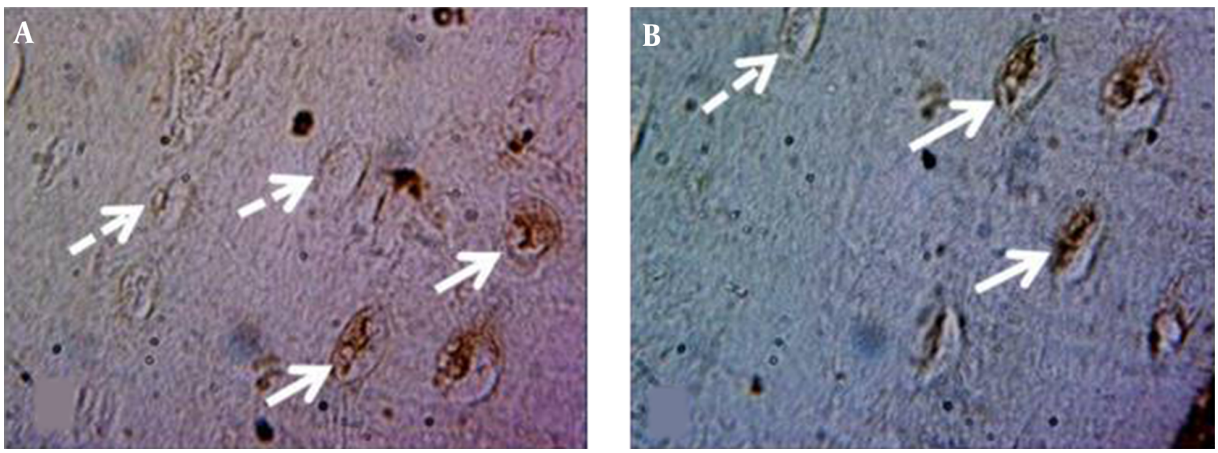

Figure 2. Photomicrograph of Labeled Cells in the Callus Tissue Sections of Non-Autologous (A) and Autologous (B) Stem Cell Transplantation (Magnification, 1000 ×). Continuous Arrows Show BrdU-Labeled Cells and Disrupted Arrows Show the Cells That Are Not Labeled for BrdU.

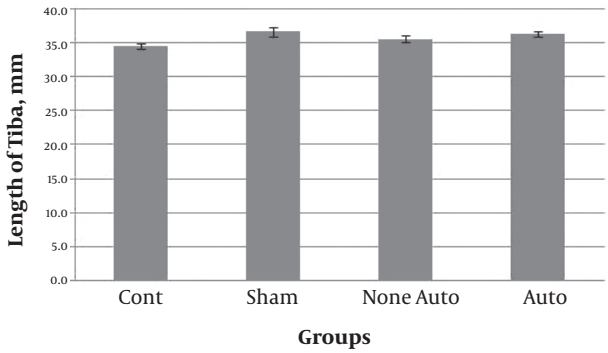

Figure 3. The Mean of the Length in Four Experimental Groups at Four Weeks After Transplantation Data is Showing Mean ± SD.

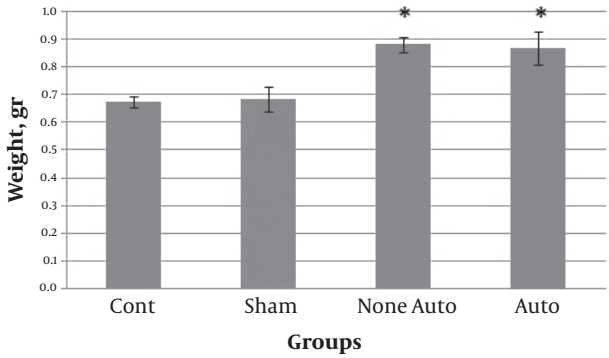

Figure 4. The Mean of Femoral Bone Mass at Four Weeks After Transplantation. Data Is Showing Mean ± SD.

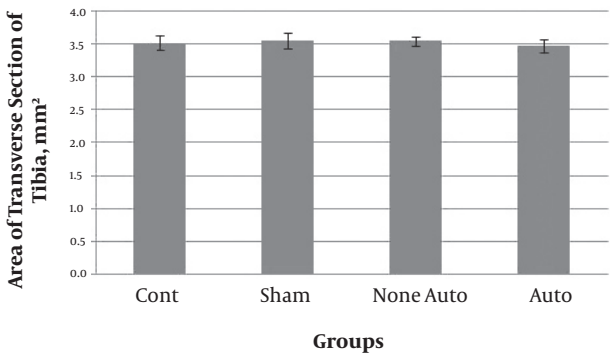

Figure 5. The Average Size of Tibia at Four Weeks After Transplantation. Data Is Showing Mean ± SD.

Density: evaluating the mean of bone density indicated no significant difference between the control (0.005 ± 0.0001) and sham (0.005 ± 0.0001) groups, yet the mean density in the non-autologous group (0.007 ± 0.0002) and autologous group (0.006 ± 0.0004) was significantly increased compared with the control group (P = 0.001 and P = 0.013) (Figure 6).

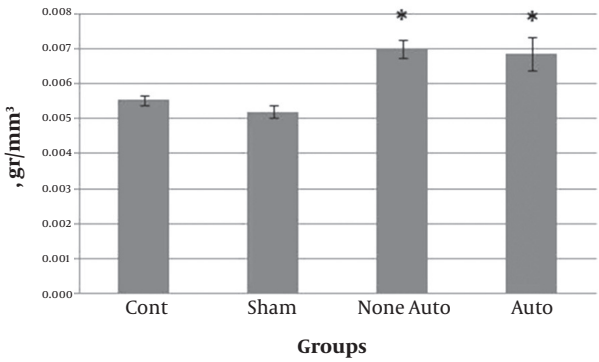

Figure 6. The Average Size of Experimental Groups at Four Weeks After Transplantation. Data Is Showing As Mean ± SD.

5. Discussion

Stem-cell-based therapies have been a promising alternative for bone regeneration. Selection of appropriate donor cell types plays an important role in successful cell transplantation. Mesenchymal stem cells are of great interest, because they are a suitable source of osteogenic precursors, which may be used in both cell therapy and tissue engineering applications. Many studies expanding MSCs in vitro could enhance bone formation by increasing the quantity of the cells (30, 31). Osteo-progenitor cells derived from MSCs have been expanded in tissue culture-formed corticocancellous bone when transplanted to animal models (32-38). In utero transplantation of the cells, contribution of the cells in bone formation of the recipient animals has been indicated (39-41).

This study was designed to perform a morphological evaluation of MSC transplantation in femur bone healing and to compare non-autologous and autologous cell transplantation effects on bone regeneration and improvement of mean mass, length, area, and density parameters. The current results showed that autologous and non-autologous cell transplantation had no significant effect on length and area of the repaired femur bone, yet the mass and density of the injected groups had a significant difference in comparison with the control and sham groups. The results of the current study were compatible

with previous findings. Li et al. transplanted bone marrow-derived MSCs to treatment mouse with osteoporosis imperfecta (OI), which had a significant effect on bone regeneration, and transplanted cells differentiated into osteoblasts in vivo and contributed to new bone formation (42).

In agreement with the results of Li and Wang, in the current study it was shown that MSCs contribute to bone cell phenotype and bone formation in osteogenesis imperfecta bones (OI). The MSCs were marked with GFP and it was shown that transplanted cells differentiated to osteoblasts and contributed to bone formation in vivo (38).

In addition, Horwitz et al. transplanted non-autologous stromal stem cells to children with osteogenesis imperfecta; 3 months after treatment, they found high bone density, and reduced fracture rate (40). Also, Kon et al. also revealed the ability of marrow-derived osteoprogenitor cells to promote repair of critical-size tibial gaps upon autologous transplantation in a sheep model.

The current biomechanical results at 4 weeks after autologous and non-autologous MSCs transplantation showed the average maximum mechanical strength of bone against force to break increased in both cell transplanted groups, yet the increase was significant in comparison with non-transplanted groups only in theautologous group, and the non-autologous group did not show a statistically significant difference (P = 0.07). These results showed that MSC transplantation could induce regeneration in damaged femur bone and could improve some histomorphometric and biomechanical properties of regenerating bone. Although results were comparable between non-autologous and autologous groups, autologous cell transplantation showed a greater improvement effect, especially in regards to mechanical strength of the bone.

Shin et al. also compared the autologous group of stromal cells in the distal femur of demineralized dogs without a cell treatment with allograft transplantation, and it was shown that bone matrix demineralized dog (PDBM) without cells and untreated group showed a significant increase; these results are in agreement with the findings of the current study. Increase in bone strength in groups that were under cell therapy in the above study and the current research was because of increased accelerationinduced in bone formation anda more complete healing (43).

In contrast of the current results, other studies on goats and sheep showed that resistance of bone 8 and 32 weeks after cell therapy was significantly increased compared to controls (44).

It seems that the function of stem cells in bone regeneration is due to their differentiation in osteoblasts, which can produce the extracellular matrix, and secret bone-specific proteins and cytokines to enhance new bone formation. On the other hand, transplanted cells stimulated endogenous cells for repairing through interaction with the cellular matrix. In addition, they produced growth factors and other factors, such TGF-β. However, these cells could secrete a large amount of cytokines, which play an essential role in repair and activate osteogenesis at sites of damage (45).

The current results showed that autologous cell transplantation was more effective in comparison with non-autologous transplantation, which could explain why autologous cell transplantation had a lower risk of rejection and transplanted cells appeared easily in damaged tissue (REF) (46).

In conclusion, the current results showed that mesenchymal stem cells could promote the regeneration of injured bone and improve morphometric and mechanical parameters of rat femur bone.

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