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Role of Bone Marrow-Derived Mesenchymal Stem Cells in Healing of Femoral Bone Defect in Rat Model: *Behavioral Psychomotor Performance, Oxidative-inflammatory Markers, Osteoblast Markers and Mesenchymal Stem Cell Growth Factors*.

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Abstract

Background: **Various techniques have been used to restore large bone abnormalities, but they have limitations. Mesenchymal stem cells (MSCs) have the potential to differentiate into bone tissue and are a promising candidate for cell therapy.** ***Aim:*** **This study aimed to investigate the effectiveness of using bone marrow-derived mesenchymal stem cells (MSCs) to treat femoral bone defects in rats.** ***Material and Methods:*** **The study was carried out in the Faculty of Medicine, Suez Canal University. The study involved dividing 27 male rats into control, defect, and stem cell-treated groups. The rats' locomotor activity, oxidative stress, inflammatory markers [tumor necrosing factor (TNF-α) and interleukin (IL-6)], angiogenic markers, osteoblast markers [Osteopontin and transforming growth factor (TGF-β)], and bone healing were assessed through various tests and measurements (histologically and radiologically).** ***Results:*** **The stem cell-treated group exhibited significantly improved locomotor activity, levels of inflammatory markers, total antioxidant capacity, and osteoblast markers compared to the defect group. Histological and radiological assessments indicated improvements in the stem cell-treated group.** ***Conclusion:*** **bone marrow-derived MSCs can enhance experimental bone healing by modulating oxidative stress, and inflammatory and angiogenic markers leading to improved locomotor parameters.**

Keywords: bone healing, bone marrow-derived mesenchymal stem cells, total antioxidant, Osteopontin, transforming growth factor-beta.

Introduction

After experiencing comminuted fractures, bone infections, osteonecrosis, or tumor removal, individuals often face the challenge of large bone defects. To address this issue, various techniques have been utilized, such as autogenous bone grafting[(1)], and synthetic bone grafting using materials derived from calcium phosphate or hydroxyapatite[(2,3)]. One promising approach for cell therapy involves using mesenchymal stem cells (MSCs) obtained from bone marrow. BM-MSCs are easily acquired through bone marrow aspiration and can be expanded in large quantities before transplantation, avoiding ethical concerns. MSCs have shown great poten-

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tial in cell-based therapies for tissue regeneration[4]. **The** MSCs also release biologically active molecules and many known mediators of tissue repair, such as VEGF (vascular endothelial growth factor), PDGF (platelet derived growth factor), EGF (epithelial growth factor), and TGF (transforming growth factor) that influence cell migration, proliferation, and survival of the surrounding cells[5,6]. Many previous studies showed that MSCs have an important role in bone defect healing, MSCs could promote the regeneration of injured bone and improve morphometric and mechanical parameters of rat femur bone[7,8]. The study objectives were 1) to assess locomotor activity of rats after the bone marrow derived mesenchymal stem cells transplantation in femoral bone defect. 2) to compare between time of femoral bone defect healing in rats with bone marrow derived mesenchymal stem cells transplantation and defect group. 3) to assess oxidative stress, inflammatory markers, angiogenic markers, and osteoblast markers in rats with bone marrow derived mesenchymal stem cells transplantation and defect group. 4) to compare between of femoral bone defect healing histologically and radiologically in rats with bone marrow derived mesenchymal stem cells transplantation and defect group.

Material and Methods

This experimental study was conducted at the animal house and tissue culture unit of the Center of Excellence, Faculty of Medicine, Suez Canal University. The study involved 27 adult male albino rats with an average weight of 200 gm ± 50 gm. The rats were divided into three groups, each consisting of 9 rats: Group I: Control group - Rats normal and healthy that did not receive any intervention. Group II: Defect group - Rats with a 2mm bone defect, where phosphate buffered saline (PBS) was locally injected at the site of the defect during surgery. Group III: Stem cell treated group - Rats with a 2mm bone defect, where MSCs in PBS were locally injected at the site of the defect during surgery. The timeline of the study was three weeks.

1. Isolation of stem cells

1.1. Separation of BM-MSCs

The hind limbs of the rats were collected and cleaned by removing the skin, muscles, and connective tissue. The limbs were severed above the pelvis and below the ankle bone while ensuring the preservation of the bone ends to maintain the integrity of the bone marrow. Following the limb dissection, the knee joint was gently separated. The bones were then washed in sterile phosphate buffer saline (PBS) through six intervals. The ends of each bone were trimmed off using scissors under a laminar flow hood. Next, a syringe was used to force media through the bone shaft to extract all the red marrow into a 150 mm plate. This extraction procedure was repeated multiple times to ensure that all the marrow was collected. The cell mixture was pipetted several times to separate the cells. The resulting cell suspension was then centrifuged at 1500 rpm for 5 minutes, and the supernatant was carefully removed. The cell pellet obtained from the centrifugation was resuspended in 5.0 ml of pre-warmed complete conditioned media and centrifuged again at 1500 rpm for 5 minutes. Finally, the number of separated cells was determined using the Countess™ 3 Automated Cell Counter (Thermo Fisher Scientific Inc., Waltham, Massachusetts, US, Catalog no. AMQAX2000), and the viability of the cells was assessed using the trypan blue exclusion test[9].

1.2. Culture of MSCs

The MSCs were cultured in 25 cm2 flasks at a concentration of 1x106 mononuclear cells/cm2. The cells were initially cultivated

with complete media, which consisted of DMEM supplemented with 1% glutamine, 1% antibiotic/antimycotic, and 20% fetal bovine serum. The flasks containing the cells were maintained in a humid environment at 37°C with 5% CO2. After 24 hours of incubation, non-adherent cells were removed from the culture. The culture media was then replaced every four days. The cellular development was monitored daily using an inverted microscope to observe the growth and morphology of the cells. Once the cells reached 50-60% confluence, they were harvested. This was achieved by treating the cells with a solution of 0.025% trypsin and ethylenediaminetetraacetic acid (EDTA). The trypsin/EDTA treatment facilitated the detachment of the cells from the flask surface, allowing for their extraction and further analysis or use in experimental procedures(10).

1.3. Trypan Blue Test of Cell Viability

The dye exclusion test was employed to assess the number of viable cells within the cell suspension. This test relies on the principle that viable cells, with intact cell membranes, exclude certain dyes, whereas nonviable cells, with compromised membranes, take up the dye[11]. In the dye exclusion test, a dye such as trypan blue is added to the cell suspension. The dye stains nonviable cells, giving them a distinctive color, while viable cells remain unstained. The stained and unstained cells are then counted to determine the number of viable cells present in the suspension. By performing the dye exclusion test, researchers were able to quantify the proportion of viable cells and gain insights into the overall viability and health of the cell population under investigation[9].

1.4. Labeling of BM-MSCs:

To prepare for cell transplantation, the cultured BM-MSCs underwent a labeling process. For this purpose, the cells were treated with an injectable solution of ferumoxides (feridex) at a concentration of 25 micrograms of iron per milliliter. This labeling solution, provided by Bayer Health Care Pharmaceuticals, contained ferumoxides which are magnetic iron oxide nanoparticles. The labeling process involved incubating the BM-MSCs with the feridex solution for a duration of 24 hours. After incubation, the labeled MSCs were washed with PBS to remove any excess feridex particles. Subsequently, the cells were trypsinized, which involved detaching them from the culture flask surface using an enzyme called trypsin, followed by additional washing steps. Finally, the labeled cells were resuspended in a solution of 0.01 M PBS at a concentration of 1×10,000,000 cells per milliliter. The effectiveness of the labeling process was assessed histologically using a staining method called Prussian blue. Prussian blue staining allows for the visualization of iron particles, enabling researchers to confirm the successful labeling of the BM-MSCs with feridex[12].

2. Surgical procedure

The rats were administered anesthesia using a combination of ketamine hydrochloride at a dose of 60 mg/kg and xylazine at a dose of 10 mg/kg. A 2 cm long incision was made in the middle third of the right femur. This allowed for exposure of the femur bone. Using a 2 mm-sized drill, a transcortical defect was created within the distal epiphysis of the femur bone. In the stem cells group, immediately after creating the bone defect, a total of 2 × 106 cells in a volume of 0.2 ml of phosphate-buffered saline (PBS) were injected. This injection was performed using a 21-G stainless steel needle connected to a micropipette. The needle was inserted tangentially to the femur bone, with the tip of the needle placed near the center of the bone defect

and the bevel of the needle facing downward[7]. In the defect group, a volume of 0.5 ml of PBS was locally injected at the site of the bone defect immediately after its formation. Subsequently, the tissue was sutured using a 4.0 silk thread to close the incision. Post-operative care involved the application of local antibiotics, specifically gentamycin ointment, and the application of betadine to the surgical wound until it healed properly. This ensured proper wound management and reduced the risk of infection.

3. The open-field test for evaluation of locomotor behavior

To assess locomotor behavior, we utilized a wooden open-field arena with dimensions of 100 × 100 cm and walls measuring 40 cm in height. The floor of the arena was divided into 25 squares, each measuring 20× 20 cm, with white lines marking the divisions. Individually, the rats were introduced into the open-field arena, and their behavior was recorded using video cameras for a duration of 5 min. The recordings were conducted under high light conditions to ensure clear visibility of the rats' movements. During the observation period, the rats' ambulation was measured by counting the number of squares they crossed using both forelimbs. Additionally, the number of rearings, which refers to the instances where the rats stood on their hind legs with their forelimbs lifted off the ground, was also recorded. To ensure impartial data collection, an experienced observer who was unaware of the treatment groups assessed these open-field behaviors by watching the recorded videos. This evaluation was performed for all groups after the completion of the 2nd week. The locomotor parameters recorded in this test provided valuable insights into the rats' activity levels and exploratory behavior[13].

4. Biochemical analysis

Two milliliters of blood were collected from the retro-orbital sinus of the rats using heparinized microhematocrit capillary tubes. The collected blood samples were then centrifuged to separate the serum, which was subsequently frozen at a temperature of -20°C until further chemical analysis.

4.1. Measurement of inflammatory markers

To measure the levels of inflammatory markers, specifically tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), in the blood, sandwich-based enzyme-linked immunosorbent assay (ELISA) kits were utilized. The ELISA kits used were specifically designed for TNF-α (Catalog No. CSB-E11987r) and IL-6 (Catalog No. CSB-E04640r) and were obtained from Cusabio. The measurements for these markers were performed for all groups at the end of the 1st, 2nd, and 3rd week of the study.

4.2. Total antioxidant capacity (TAC) in blood

The total antioxidant capacity (TAC) in the blood samples was assessed using an OxiSelect™ TAC assay kit (Catalog No. sta-360) obtained from Cell Biolabs. This assay allowed for the quantification of the overall antioxidant capacity of the blood samples[14].

4.3. Serum VEGF

The levels of serum vascular endothelial growth factor (VEGF) were estimated using the enzyme-linked immunosorbent assay (ELISA) technique. Specific ELISA kits designed for rat adhesion molecules were used for this purpose (Catalog No. ab 100786). These chemical analyses provided valuable information regarding the inflammatory status, antioxidant capacity, and VEGF levels in the blood samples, allowing

for a comprehensive assessment of the systemic response in the study groups. After behavioral and radiological assessments, rats were decapitated under anesthesia and the femur bone removed.

5. Histological examinations

Histological examinations using hematoxylin and eosin (H/E) staining was performed and grading system was done for assessment of bone healing according to amount of fibrous tissue, cartilage, immature bone and mature bone based on the scale recommended by Huo et al[15] as following: Grade 1: Fibrous tissue. Grade 2; Mostly fibrous tissue, little cartilage. Grade 3; Equal amounts of fibrous and cartilage tissue. Grade 4: Mostly cartilage, little fibrous tissue. Grade 5: Cartilage tissue. Grade 6; Mostly cartilage, little immature bone. Grade 7; Equal amounts of cartilage and immature bone tissue. Grade 8; Mostly immature bone, little cartilage tissue. Grade 9; Bone healing with immature bone Grade 10; bone healing with mature bone.

6. Immunohistochemistry:

This was done in all groups after 1st,2nd and 3rd week after the surgery.

6.1. Osteopontin as osteoblast marker

This was done using 2 sites specific osteopontin antibodies:10A16 monoclonal antibody and O-17 polyclonal antibody)

6.2. TGF-β

This was used as MSCs growth factor (using Avidin-Biotin Complex ABC method) that stimulate osteoblast replication.

7. Radiographic evaluation

X-ray images high-energy electromagnetic radiation of the right femur were taken by VALUETIK VET 100 MA/670 MAS MOBILE X-ray system every week until three weeks after the surgery and scoring system was done to assess bone healing showed varying grades, with absent callus and visible fracture line (grade 1) or callus and visible fracture line (grade 2), callus and visible fracture line were observed, with bridging callus (grade 3)and remodeled callus and an invisible fracture line grade 4[16].

Statistical Analysis

All quantitative data were analyzed with SPSS statistical software version 20 and were reported as mean ± standard deviation (SD), cut point of the p-value ($P < 0.05$). One way analysis of variance (ANOVA) was used for comparing means of a variable in the three groups. In case a statistically significant difference was detected by ANOVA, Post hoc tests followed by Tukey were used to further analyze the results. Fisher exact was used to calculate difference between qualitative variables as indicated.

Ethical consideration

This study considered the ethical considerations involved in the experimental animal studies as mandatory. The study protocol was carried out under approval of the Ethics Committee, Faculty of Medicine; Suez Canal University, Egypt with ethical approval number (4233).

Ethical consideration for the animal experiment

The rats in this experimental study were used for valid scientific aims, and there were no other replacement alternatives at this stage of knowledge to their use. The experiment had a good probability of meeting the stated objectives, which have a reasonable chance of contributing to human welfare.

Results

1. The open-field test for evaluation of locomotor behavior

This test was done to all groups after 2nd week. General locomotor activity was evaluated in the open-field test (Table 1). One-way ANOVA indicated significant differences among the study groups in both ambulation distance [F (3,20)= 49.55, P<0.001] and rearing frequency [F(3,20)= 20.85, P<0.001]. Both parameters were significantly reduced in the defect group in 2nd week compared to the control group (34 ± 7.29 vs. 90 ± 9.48) and significantly greater in stem cell treated group (69 ± 3.6).

Table 1: Performance of behavioral motor activity (horizontal and vertical ambulation) of rats at the second week in open field test.

Groups	Horizontal ambulation (number of squares that crossed/5min)	Vertical ambulation (rearing number/ 5min)
Control group	90 ± 9.48	17 ± 4.7
Defect group	34 ±7.29[a]	2 ± 1.2[a]
Stem cell treated group	69 ± 3.6[ab]	12 ± 2.8[ab]

Data are expressed as mean ± SD and analyzed using One- way ANOVA indicated significant differences among the study groups in both ambulation distance [F(3,20) = 49.55, P < 0.001] and rearing frequency [F(3,20)= 20.85, P < 0.001]. Superscripts [a, b] represents a statistically significant difference when compared to control and defect groups respectively.

2. Biochemical analysis

2.1. Inflammatory markers

Serum IL-6 and TNF-α increased significantly in defect group rats ($p<0.05$) and decreased in stem cell treated rats (Table 2).

2.2.TAC assessment:

TAC showed an average (±SD) of 0.68 ±0.05, 0.32±0.06, and 0.57±0.03 mM/L, where the TAC decreased significantly in defect group rats ($p<0.05$) and increased in stem cell treated rats ($p<0.05$) as revealed by one-way ANOVA and Duncan's Multiple range test, ANOVA revealed a highly significant difference (p<0.001) between treated and defect groups (Table 3).

3. Histological examination

Examination of H & E-stained femur sections of the control group showed the normal general histological structure with cortical lamellar bone tissue, scattered osteocytes within lacunae, scattered osteoblasts, Intact bone surface, regularly arranged lamellae and no evidence of immature bone, cartilage or fibrous tissue (Figure 1A).

Table 2: Serum IL-6 and TNF-α in different studied groups in three weeks.

Groups	IL-6 pg/mg	TNF-α pg/mg
Control group	30.43 ± 3.45	20.10 ± 5.26
Defect group at 1st week	132.97 ± 4.86[a]	100.16 ± 7.51[a]
Defect group at 2nd week	100.87 ± 3.76[a]	88.54 ± 4.86[a]
Defect group at 3rd week	96.44 ± 5.2[a]	70.71 ± 4.86[a]
Stem cell treated group at 1st week	75 ± 7.24[ab]	55.90 ± 5.58[ab]
Stem cell treated group at 2nd week	62.30 ± 7.12[ab]	40.04 ± 3.54[ab]
Stem cell treated group at 3rd week	45.04 ± 6.54[ab]	31.040 ± 3.64[ab]

Results are expressed as mean ± SD and compared among groups by one-way ANOVA and Tukey's post-hoc test. A p-value < 0.05 is considered statistically significant for all tests. Superscripts [a, b] indicates statistically significant differences compared to control, and defect group in the same week respectively.

Table 3: Serum TAC and VEGF in different studied groups in three weeks		
Groups	TAC mM/L	VEGF (pg/ml)
Control group	0.68±0.05	56.9±17.83
Defect group at 1st week	0.32±0.06[a]	210.6±63[a]
Defect group at 2nd week	0.37± 0.06[a]	198± 60.4[a]
Defect group at 3rd week	0.41± 0.04[a]	123± 35[a]
Stem cell treated group at 1st week	0.54± 0.02[ab]	289.3± 25.1[ab]
Stem cell treated group at 2nd week	0.57±0.03 [ab]	278± 32.4 [ab]
Stem cell treated group at 3rd week	0.62± 0.03[ab]	214± 28.4[ab]

Results are expressed as mean ± SD and compared among groups by one-way ANOVA and Tukey's post-hoc test. A p-value < 0.05 is considered statistically significant for all tests. Superscripts [a, b] indicates statistically significant differences compared to control, and defect group in the same week respectively.

Defect group at the 1st week showed a defect formed predominantly by fibrous tissue with many proliferating spindled fibroblasts & scattered osteoclast type giant cells. Proliferating capillaries scattered inflammatory cells and foci showing islands of cartilaginous tissue.At the 2nd week, the defect formed predominantly by irregular immature woven bone trabeculae. Numerous osteoblasts, giant cells and rimming bone trabeculae were seen in the defect. At the 3rd week, many areas showing healing mainly by large bony trabeculae with osteoblasts & minimal areas showing complete healing by islands of cartilage & irregular woven bone (Figure 1B). Stem cell treated group at the 1st week showed a defect gap obliterated mainly by irregular woven immature bone trabeculae with maturation surrounded by active osteoblasts. At the 3rd week, the defect gap almost completely obliterated mainly by mature lamellar bone with very few foci showing irregular woven bone and very few osteoblasts (Figure 1C).

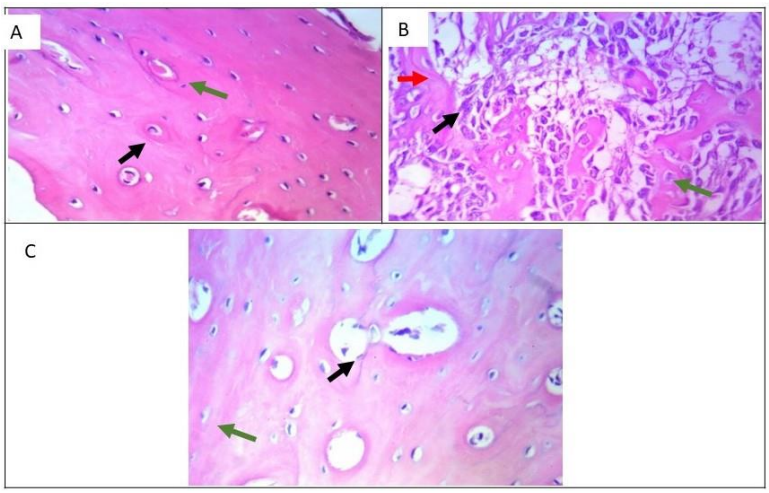

Figure 1: Photomicrographs of femur stained with H&E X 400 from different groups. **(A)** control group shows cortical lamellar bone tissue (green), scattered osteocytes within lacunae. (black), intact bone surface, regularly arranged lamellae and no evidence of immature bone, cartilage or fibrous tissue. **(B)** defect group at the 3rd week shows a defect in which many areas showing healing mainly by large bony trabeculae (red) with osteoblasts (black)& minimal areas showing complete healing by islands of cartilage & irregular woven bone (green). **(C)** Stem cell treated group shows a defect gap almost completely obliterated mainly by mature lamellar bone (green) with very few foci showing irregular woven bone and very few osteoblasts (black).

Based on the histological grading system, there was a statistically significant difference between the defect group and the stem cell treated group at the 1st and 2nd week, indicating that the treatment with stem cells had a positive impact on the histological healing process (Table 4). The histological findings demonstrated that the stem cell treatment contributed to the formation of more mature and organized bone tissue within the defect, leading to enhanced healing compared to the defect group alone.

4.1. Osteopontin

There was statistically significant difference between the three studied groups regarding area of distribution of osteopontin immunostaining in which the defect group showed the higher levels of immune reaction in the newly formed bone trabeculae than stem cell treated group all over the three weeks (Figure 2).

4. Immunohistochemistry

4.2. TGF-β

There was statistically significant difference between the three studied groups regarding area of distribution of TGF-β immunostaining in which the defect group showed the higher levels of immune reaction in the newly formed bone trabeculae than stem cell treated group all over the three weeks (Figure 3).

Table 4: Histological bone healing grades of femoral bone defect among studied groups in the three weeks

Histological variable	1st week			P value
	Control group	Defect group	Stem cell treated group	
Grade 2; Mostly fibrous tissue, little cartilage	0	2	0	0.007*
Grade 3; Equal amounts of fibrous and cartilage tissue	0	1	0	
Grade 6; Mostly cartilage, little immature bone	0	0	1	
Grade 7; Equal amounts of cartilage and immature bone tissue	0	0	2	
Grade 10; bone healing with mature bone	3	0	0	
	2nd week			
Grade 8; Mostly immature bone, little cartilage tissue	0	2	0	0.014
Grade 9; Bone healing with immature bone	0	1	3	
Grade 10; bone healing with mature bone	3	0	0	
	3rd week			
Grade 8; Mostly immature bone, little cartilage tissue	0	1	0	0.250
Grade 9; Bone healing with immature bone	0	1	0	
Grade 10; bone healing with mature bone	3	1	3	

*Results were expressed as the number of rats per group using Fisher exact test; *p is significant at <0.05.*

5. labeling of BM-MSCs Homing:

Numerous Prussian blue-stained iron particles were detected in the cytoplasm of MSCs in bone defect of stem cell treated group indicating homing of stem cell in the defect as shown in figure 4B.

6. Radiological examination

After 1st week, in defect group, one rat showed grade 1 as there was absent callus and visible fracture line and two rats showed callus and visible fracture line so graded 2. In stem cell treated group, two rats showed callus and visible fracture line while one rat graded 3 and showed bridging callus and visible fracture line (Figure 5). After 3rd week, in defect group, one rat showed grade 3 with bridging callus and visible fracture line and 2 rats showed grade 4. In stem cell treated group, all 3

rats showed remodeled callus and invisible fracture line similar to control group with no significant difference between the three studied groups as shown in table 5.

Discussion

Fractures are commonly caused by high-energy trauma such as accidents or sports injuries[17]. The healing of fractures involves a complex series of growth factors and cytokines that regulate the activation, proliferation, and differentiation of local mesenchymal stem or progenitor cells[18]. Recently, it has been demonstrated that MSCs transplantation is successful in adopting regenerative powers in fracture repair[19]. In our study, general locomotor activity was evaluated using the open-field test for all groups after 2nd week and both ambulation distance and rearing frequency were significantly reduced in the defect group compared to the control group (34 ± 7.29 vs. 90 ± 9.48).

Table 5: Radiological bone healing scoring of femoral bone defect among studied groups in the three weeks

Experimental groups	1st week				P value
	1 (absent callus + visible fracture line)	2 (present callus+visible fracture line)	3 (bridging +visible fracture line)	4 (remodeled + invisible fracture line)	
Control group	0	0	0	3	0.036*
Defect group	1	2	0	0	
Stem cell treated group	0	1	2	0	
2nd week					
Control group	0	0	0	3	0.079
Defect group	0	2	1	0	
Stem cell treated group	0	0	2	1	
3rd week					
Control group	0	0	0	3	>0.999
Defect group	0	0	1	2	
Stem cell treated group	0	0	0	3	

*Results were expressed as the number of rats per group using Fisher exact test; *p is significant at <0.05.*

However, the stem cell treated group showed significant improvement in these parameters (69 ± 3.6) indicating the potential of MSCs to enhance locomotor behavior. Recent studies have shown that transplantation of MSCs can enhance bone regeneration and facilitate fracture repair. MSCs can exert their effects through three possible mechanisms: direct differentiation into osteoblasts and chondroblasts, modulation of inflammation and immune responses, and paracrine signaling through the secretion of factors such as TGF-β1, VEGF, and BMP-1. These factors play crucial roles in osteogenesis, angiogenesis, cell migration, proliferation, and osteoblast differentiation[20]. In this study, Inflammation markers IL-6 and TNF-α were significantly increased in the defect group but decreased in the stem cell treated group. This is consistent with previous findings by Zhang et al[18] that MSCs can modulate inflammation and immune responses by secreting growth factors and anti-inflammatory cytokines, promoting a more favorable environment for bone healing. Angiogenic factors, such as VEGF, are crucial for fracture healing as they sup

port the growth of new blood vessels at the site of injury[21,22]. Treatment with MSCs in led to a significant increase in the release and expression of VEGF, indicating enhanced vascularization and bone formation, it is also a promising tool to treat osteonecrosis. Our study aligns with previ ous studies as treatment with stem cells significantly increased the release and expression of VEGF $P < 0.05$. This also aligns with studies that have combined MSC injection with bisphosphonate therapy to promote vascularization in bone healing processes[18]. Studies have also shown that MSCs can improve osteoporosis and induce specific differentiation of naive MSCs, promoting vascularization in the fracture healing process [3,23]. Additionally, according to Zhang et al[18], transplantation of MSC markedly enhanced angiogenesis and bone healing processes in a rat model of femoral fracture. In vitro, other than enhancing osteogenic differentiation, MSC increased the expression of VEGF and hypoxia inducible factor-1a (HIF-1a) which agree with our results. Additionally, MSCs have been found to possess antioxidant properties, which can help reduce oxidative damage associated with various diseases[24]. MSCs can instantly ameliorate oxidative stress-related damage. Because oxidative stress is associated with cellular damage, inflammation, and dysregulated metabolism, it is a critical pathophysiological factor in many illnesses. Chemical components inherent in all living cells that perform analogous functions create oxidative stress and redox imbalance[24].

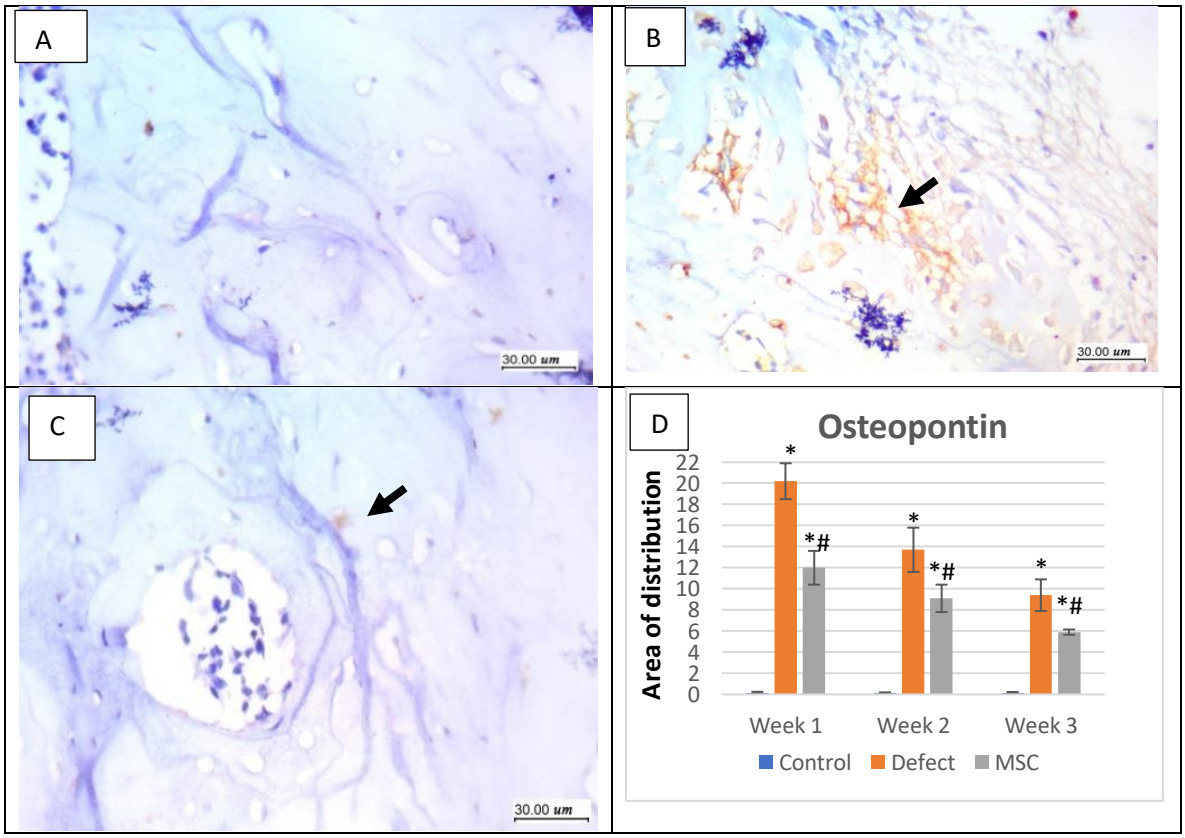

Figure 2: Photomicrographs of the bone defect stained with anti osteopontin immunostain from different groups at the 3rd week **(A)** (Control group) shows almost negative immunoreaction in bone matrix **(B)** Defect group shows positive osteopontin expression in osteoblast and osteocyte cells (black arrow) **(C)** Stem cell treated group shows a low level of osteopontin immune reaction in the newly formed bone trabeculae (D) Quantification of anti osteopontin immunostaining using computer software image J. Values were expressed as mean ± S.E. All data were analyzed using one way ANOVA followed by the Tukey post hoc test. *****significant *p-value* in comparison to control group, #significant *p-value* in comparison to defect group.

Thus, MSCs' capacity to control these processes may explain the range of disease models curable by MSCs, this is consistent with the increase in total antioxidant capacity observed in the stem cell treated group compared to the defect group in our study. Histological assessment revealed that stem cell-treated groups showed superior results compared to groups without stem cells[25] which is similar to our results at 1st week that revealed the stem cell-treated groups exhibited filling of the fracture gaps with hard callus and the presence of mature lamellar bone, indicating enhanced bone formation. However, at the 2nd and 3rd weeks there were no significant difference between both groups that may be related to small sample size per group. The expression of osteopontin (OPN), an osteogenic marker, was found to be enhanced in the stem cell-treated group. OPN is involved in bone development and acts as a chemotactic molecule for inflammatory cells, promoting their migration to the wound site. It also plays a role in modulating the immune response and enhancing the expression of certain cytokines and matrix-degrading enzymes[20]. TGF-β1 plays a pivotal role in the process of fracture healing as it enhances the proliferation and differentiation of (MSCs), increases the production of extracellular matrix and is chemotactic on bone cells.

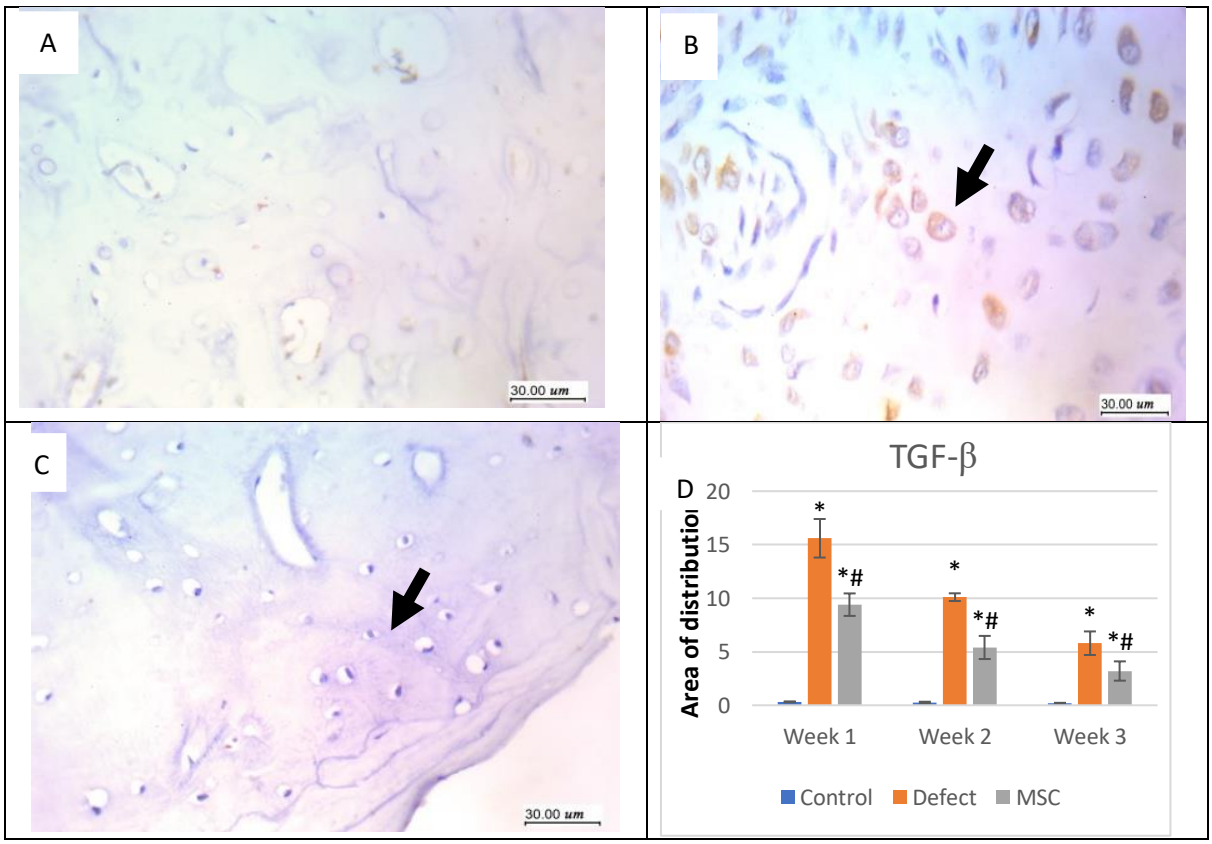

Figure 3: Photomicrographs of the bone defect stained with anti TGF-β immunostain from different groups at the 3rd week **(A)** (Control group) shows almost negative immunoreaction in bone matrix **(B)** (Defect group) shows positive brownish immunoreaction in bone matrix **(C)** (Stem cell treated group) shows almost negative immunoreaction with few positive immunoreaction (D) Quantification of anti TGF-β immunostaining using computer software image J. Values were expressed as mean ± S.E. All data were analyzed using one way ANOVA followed by the *Tukey post hoc* test. *significant *p-value* in comparison to control group, #significant *p-value* in comparison to defect group.

It has a key role on the promotion of cartilage formation and increases the formation of callus and bone strength[20]. In the current study, Osteopontin and TGF-β levels were higher in the defect group but lower in the stem cell-treated group, suggesting a late-stage bone matrix formation in the stem cell group. In our study regarding the downregulation of OPN expressions levels in stem cell treated group at post-implantation 2 and 3weeks, this might be correlated to the normal reduction in the synthesis of bone matrix proteins in the late stages of bone healing as reported by Gao, et al[26] who investigated the osteoblasts and osteocytes potential for bone matrix proteins production during bone formation and reported the significant increase in bone matrix proteins production toward the 2nd week then decreased. Radiological examinations showed a faster rate of bone defect repair in the stem cell-treated group, with complete filling of the defects with new bone observed at the 3rd week. These findings are consistent with previous studies showing that stem cell-treated groups exhibit larger callus volumes and faster healing compared to control groups. To add to that, similar study by Zhang et al[18] evaluated the bone formation in femoral fracture healing. A hard callus with bridging of the fracture gap was observed in X-ray images, and the fracture gap was obvious on post-operative day 14 i n all three groups. The border between the newly formed hard callus and the existing cortical bone had disappeared with the ob served remodeling processes. However, the stem cells group had significant larger callus volumes than the other groups.

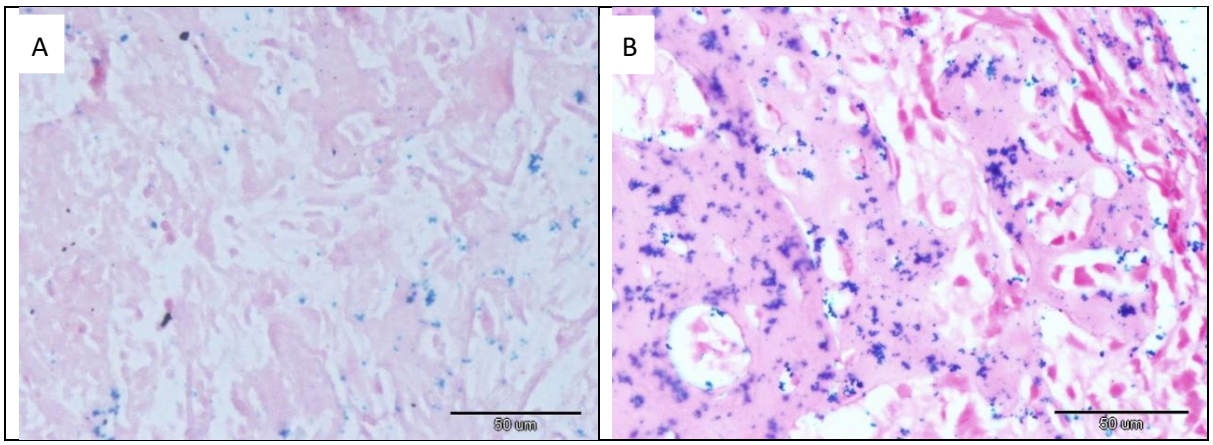

Figure 4: Prussian blue stain in defect (A) & stem cells treated groups (B) which shows positive staining of stem cells in stem cell treated group.

Conclusion

In summary, our study showed that bone marrow-derived mesenchymal stem cells (BM-MSCs) can accelerate the healing of experimental bone defect through multiple mechanisms. The BM-MSCs exhibited antioxidant capacity, which can help reduce oxidative damage associated with the healing process. Additionally, the paracrine mechanisms of BM-MSCs were observed, as they secreted anti-inflammatory factors, VEGF, and TGF-β.

Conflict of Interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or

financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

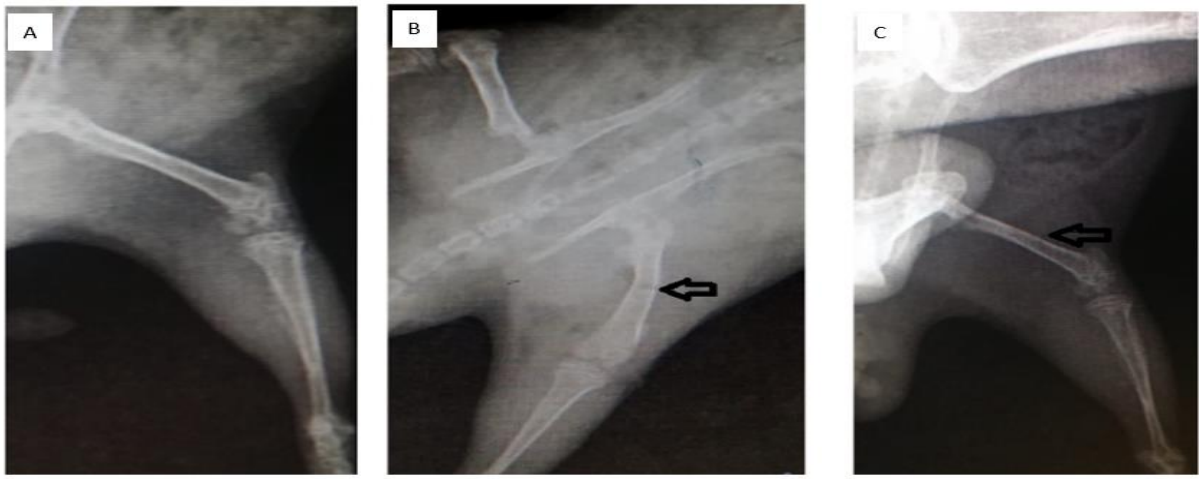

Figure 5: Radiological Assessment among the three studied groups after 1st Week shows the defect point in defect and stem cell treated groups. (A) Control group, (B) Defect group, (C) stem cell treated group. Arrow indicates defect point.

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