

Therapeutic approach for osteoporotic animal model by bone marrow mesenchymal stem cells

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ABSTRACT

Osteoporosis (OP) is a common debilitating metabolic bone disorder manifested by poor mineral density and deteriorated skeleton. Stem cell therapies are being considered as alternative therapeutic modalities. This study determined the therapeutic potential of mesenchymal stem cell (MSC) application in osteoporotic rats. To achieve this, an OP animal model was developed through ovariectomy and glucocorticoids in rats. MSCs were isolated from the same breed of rats and cultured MSCs were administered to osteoporotic rats via vein injections. After 4 and 8 weeks of MSCs administration, rats were sacrificed and all desired components, including serum and joints, were isolated. The present findings showed that the transplantation of MSCs into osteoporotic rats enhanced osteoblastic differentiation and function reverting OP. Calcium deposition, bone-specific alkaline phosphatase activity, and serum osteocalcin concentration were significantly elevated in MSCs-treated osteoporotic rats ($p < 0.05$). Notably, their levels were much higher in osteoporotic rats treated for 8 weeks than in those treated for 4 weeks ($p < 0.05$). Moreover, the histological image of the femur head showed uniform thinning of trabeculae (TB) in osteoporotic rats, resulting in the widening of intertrabecular spaces and disjointed bony ossicles interrupted by virtue of expanded fatty bone marrow spaces. Interestingly, after MSCs treatment, cancellous bone TB appeared normal with red bone marrow. Furthermore, a photomicrograph of osteogenic differentiation of MSCs of MSCs-treated osteoporotic rats showed significantly high scattered red calcified nodules ($p < 0.05$). In conclusion, this study illustrates that the transplantation of MSCs into osteoporotic rats provides a supplement for bone architecture and enhances osteoblastic differentiation and function, thus reverting OP. This work presents a unique method as a future application for the treatment of human OP.

INTRODUCTION

Osteoporosis (OP) is a systemic metabolic bone disease manifested by poor mineral density and deteriorated skeleton, which is common in every population all over the globe (Drake *et al.*, 2015). Numerous previously established theories contributed to its development but the most common was found to be a disturbance

in the balance of bone formation and resorption, which may lead to its onset due to the excessive loss of bone tissue (Thomsen *et al.*, 2012). Moreover, dysfunction of a number of hormones and interleukins was also found to have a link with the onset of OP (Chen *et al.*, 2016). Estrogen deficiency due to menopause was reported to activate osteoclasts, resulting in a reduction in bone formation (Miyamoto, 2015). At present, the drug treatment for primary OP targets symptoms and complications, without bone targeting of bone formation (Cosman *et al.*, 2014). The therapeutic mechanism for parathormone treatment includes the acceleration of bone formation but subsequently stimulates bone resorption (Makras *et al.*, 2015). Correcting abnormal bone revolution may

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improve bone weight and reduce the chance of fractures. However, pharmacotherapy can be prohibitive in cost, take a long time to be effective, and lead to adverse reactions. Bisphosphonate therapy may lead to extensive suppression of bone development and even necrosis of the mandibular bone (Rousselle and Heymann, 2002). The nonpharmacologic low-intensity pulsed ultrasound and physical exercise trials were done but yet it needs more justification according to the variability of sex, age, and bone status (Sun *et al.*, 2020). Additionally, some of these therapies employ virus-mediated gene or protein delivery, posing potential biological risks to the patient (Chen *et al.*, 2002). Therefore, it is essential to develop improved therapies for OP.

Recently, mesenchymal stem cell (MSC)-based therapies have been implemented for different diseased animal models and some of them are under consideration, but their success in clinical applications is yet to be established (Chen *et al.*, 2002). Studies have also shown that MSCs can produce a number of cellular components that are related to bone regeneration but their effect on the curve of bone formation and their applicability in therapeutics against the onset of OP has not yet been clearly defined. Moreover, live-cell industrialization and preservation have also not yet been available for clinical application. Furthermore, cell therapy alone also failed due to its short shelf life (Littman and Abo, 2015). Low bone formation was observed in osteoporotic conditions due to scanty recruitment and invasion of MSCs to the defect bone for bone healing (Almeida *et al.*, 2019; Sanghani-Kerai *et al.*, 2017). But still, stem cells possess many unique features such as self-renewal ability, potential of multiple cell lineages differentiation, and potential in regeneration of tissues (Fortier and Travis, 2011).

This study determined the therapeutic potential of MSCs application in osteoporotic rats. In light of the works discussed, the present study hypothesized that the transplantation of MSCs into osteoporotic rats may augment bone architecture and enhance osteoblastic differentiation and function, which might result in the reversion of OP conditions. To test this hypothesis, an OP animal model was developed by ovariectomy and glucocorticoids in rats. MSCs were isolated from the same breed of rats and cultured MSCs were administered to osteoporotic rats via vein injections. After 4 and 8 weeks of MSCs administration, rats were sacrificed, all desired components including serum and joint were isolated, and the levels of all top biomarkers involved in OP were determined. The present data proved that the transplantation of MSCs into osteoporotic rats provides a supplement for bone architecture and enhances osteoblastic differentiation or functions, thus reversing the key features of OP.

METHODS

Ethical approval

This work was conducted under the international ethical guidelines for animal research and was permitted by the Animal Research Ethical Committee of Qassim University (Approval No. 3739-cavm-2018-1-14-S).

Development of osteoporotic animal model

Female Wistar rats (50 days old, weighed 150–170 g) were used for induction of OP by ovariectomy and glucocorticoids. The rats were housed in equal groups, split into five rooms, fed standard

ration, and given water *ad libitum*. For ovariectomy, a bilateral operation was performed with a dorsal approach and all steps were conducted under ether anesthesia to minimize animal suffering (Wronski *et al.*, 1985). The incision was sealed with interrupted sutures using a nylon 4.0. Sham control rats were exposed to sham surgery in which ovaries were exteriorized. Two days after ovariectomy, administration of dexamethasone phosphate (1 mg/kg bwt) by intraperitoneal (i.p.) injections was performed daily for a period of 8 weeks, or with saline as untouched control. For ensuring OP, 10 animals were randomly selected; blood samples were collected for estradiol analysis (E2) using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, MI, USA). Measurements were performed every 2 weeks for 8 weeks after ovariectomy until serum E2 (>50%) was significantly detected compared with the sham control group.

Isolation of MSCs from bone marrow

Five female Wistar albino rats 50 days old were used for isolation of MSCs from bone marrow as described previously with some modification (Abo-Aziza *et al.*, 2019a). Briefly, sodium pentobarbital anesthesia was used for euthanization of rats and bone marrow was harvested from hind limbs. The limbs were cut over the hip and under the ankle, keeping the bone ends to ensure bone marrow sterility. Bones were washed three times in a dish using phosphate buffer saline (PBS). The ends of each bone were snipped off to extract more bone marrow in sterile PBS. A 10 cc syringe containing prewarmed complete conditioned Dulbecco's modified Eagle's media [1.0 g/l glucose + fetal bovine serum (FBS) 10%, Sigma-Aldrich, USA] was used and the media were pushed several times by the needle through the bone shaft to extract all the red marrow into a 100 mm Petri dish containing 1 ml heparin (2,000 IU/0.2 ml). The cell mixture was pipetted up and down several times using a syringe. The suspension was washed twice by centrifugation using PBS for tissue remnants removal. Alpha minimum essential medium (α -MEM), (Lonza, Germany) was then added, and the bone marrow was used on the equal volume of buffer solution of sodium carbonate buffer solution (0.1%) for lyses of erythrocytes, as described previously (Lee *et al.*, 2015). The mononuclear cells (MNC) were separated and were grown as described previously (Abo-Aziza *et al.*, 2019b; Lee *et al.*, 2015).

Immunophenotype analysis for isolated MSCs

The immunophenotype analysis for bone marrow MSCs was performed through the detection of cell surface markers using the flow cytometry as described previously (Ssadh *et al.*, 2019). All major cell surface markers for MSCs such as CD34, CD45, CD14, CD105, CD166, CD90, CD73, CD146, and CD106 were assessed using FACS Calibur flow cytometer (BD Bioscience, San Jose, CA).

Application of stem cells to osteoporotic rats

Stem cell therapy was applied to develop osteoporotic rats. MSCs (0.5 million/rat) were delivered via tail vein injection with two different time points of harvest, 4 and 8 weeks after stem cell injection. Rats were divided into four groups with *n* of 20 per each group as follows: Group 1: untouched control; Group 2: sham control; Group 3: α -MEM (OP +ve control); Group 4: OP + MSCs. Over half of the animals of the last two groups were sacrificed after 4 weeks via overdose *i.p.* injection of 3% sodium pentobarbitone

(50 mg/kg), followed by the collection of blood and bone samples, while the other half were sacrificed 8 weeks after injection.

Biomarker evaluation: serum osteocalcin (OCN), serum bone-specific alkaline phosphatase (B-ALP), calcium levels, and phosphorus levels

The experimental rats were sacrificed by the use of sodium pentobarbitone, following which desired components, such as blood, joint tissues, bone tissue, and bone marrow, were collected for the biomarkers analysis to detect the stem cell application on osteoporotic rats. Serum samples obtained from the experimental rats were evaluated for OCN levels by eBioscience ELISA kits (San Diego, CA). B-ALP was quantified by Metra Biosystems ELISA kits (Quidel Corp., CA, USA). Meanwhile, the levels of calcium (Ca) and phosphorus (P) were measured by colorimetric kits (Sigma-Aldrich, USA).

Stem cell sampling

Bones were also collected from the experimental rat's groups and the acquired bone marrow was harvested to isolate and culture MSCs for osteoblastic differentiation. MSCs (passage 3) were tested for viability by trypan blue stain and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (Sigma-Aldrich, USA) assays before culturing in osteogenic media as described previously (Alabdulmonem *et al.*, 2018; Rasheed *et al.*, 2010). The cell suspension was done at an appropriate concentration of 1×10^5 /ml in α -MEM as described previously (Quan *et al.*, 2016). The proliferation capability of the stem cells was measured in both primary cultures and subcultures. Cells were incubated with 0.2 mg MTT/ml α -MEM media for 1 hour at 37°C in 6-well plates to form formazan. The media were then collected with the addition of 1 ml isopropanol containing 0.04 N HCl. Formazan quantity was assayed calorimetrically at a wavelength of 570 nm after 5 minutes of shaking (Zaki *et al.*, 2020).

Osteoblastic differentiation

Osteoblastic differentiation was used as described previously (Abo-Aziza *et al.*, 2019a). Briefly, the culture medium of passage 3 confluent cultures was replaced by an osteogenic medium containing α -MEM with 10% FBS, penicillin-streptomycin, and GlutaMAX complemented with 100 μ M L-ascorbic acid 2-phosphate, 10 nM dexamethasone, 2 mM L-glutamine, and 2 mM β -glycerophosphate (Lonza, Germany). The differentiation of cultures continued for 3 weeks. The media were changed every 2 days. Calcified tissues and mineralization potentiality inside differentiated cells were evaluated using Alizarin red stain, as described previously (Huang *et al.*, 2009).

B-ALP activity for osteogenic differentiation

B-ALP activity was assayed to indicate osteogenic differentiation as described previously (Choi *et al.*, 2005) and the levels of its activity were measured using the B-ALP-specific assay kit (Abcam, Cambridge, UK).

Calcium deposition assay for osteogenic differentiation

The differentiation of MSCs to bone tissue was indicated by the amount of calcium deposited (Salaszyk *et al.*, 2004). A fixed number of colonies from 80% confluent plates were washed twice

with PBS. Calcium was extracted from cells by agitation for five hours at 4°C and centrifugation at low speed using hydrochloric acid extracting solution (0.5 N). Ca was assessed in the supernatant by colorimetric assay kit (Abcam, Cambridge, UK).

Bone histomorphometry

The femurs of all rats were separated from their attached muscles and fixed immediately for 2 days in neutral buffered formaldehyde (10%, Sigma-Aldrich, USA). After that, the distal femurs of the trabecular bone were collected from the midline longitudinal sections and mediolateral plane and decalcified for 8 days with the decalcifying solution, which consisted of formic acid 24.4% and sodium hydroxide 0.5 N (Sigma-Aldrich, USA). Paraffin blocks were possessed, and 5 mm thick sections were serially performed and stained with hematoxylin and eosin stain (H&E) and examined, as previously described by Eça *et al.* (2009).

Statistical analysis

The data found through the results were expressed as mean \pm standard error (SE). Statistical Package for the Social Sciences version 20.0 software was used. Analysis of variance was done for all groups in the study to find out if the results were significant. Ca deposition, phosphorous, B-ALP activity, and OCN concentrations in serum as well as Ca and B-ALP concentrations in lysed differentiated MSCs were compared through the Student *t*-test, and *p*-values <0.05 and <0.01 were considered as statistically significant. The viability % was calculated by the following formula: $100 \times \text{number of viable cells} / \text{number of harvested cells}$.

RESULTS

MSCs morphology

MNCs were isolated from rat bone marrow and seeded to adhere to the culture dish bottom. One to two days later, cells were monitored using an inverted microscope. It was found that the cells started to develop extensions and some cells appeared spindled as fibroblasts. The proliferation and propagation of cells continued from 50% confluence (Fig. 1A) until the targeted 80% confluence was achieved, where the dish bottom appeared covered with cells after 3 weeks (Fig. 1B). Throughout the expansion cultures in P2 and P3, the cell number continued to propagate. The subcultures from the 80% confluence cells have a tendency to grow faster, so the cultures take a shorter time to reach confluence as compared to primary cultures.

Phenotypic analysis

Immunophenotypic analysis for characterization of MSCs surface markers was performed using the FACS Calibur machine (Fig. 1). The positive stained population indicated the expression of MSCs surface markers. The results showed a low +ve stained population of MSCs for CD34 (10%), CD45 (4.9%), and CD14 (26%) that indicated negative expression. However, a high +ve stained population was recorded for CD105 (73.9%), CD166 (23%), CD90 (46.6%), CD73 (50.1%), CD146 (58.6%), and CD106 (62.9%) indicating positive expression. The -ve sample with the untreated isotope was used as the control. It expressed a very low positive population (0.6%).

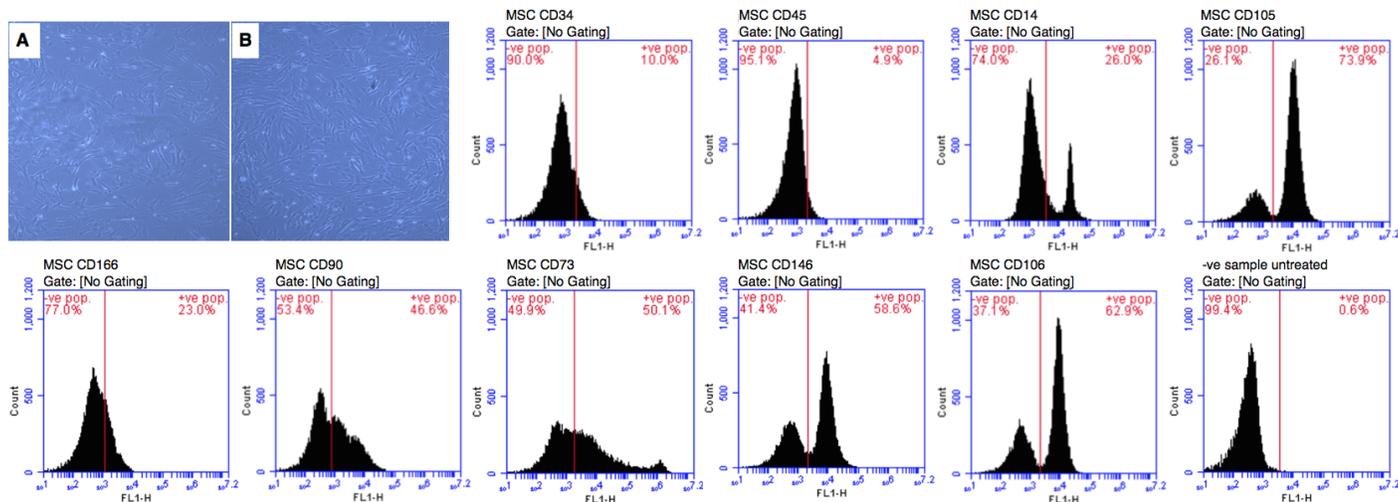


Figure 1. MSCs morphology and immunophenotype analysis for MSCs surface markers using FACS Calibur machine. Spindle-shape appearance of MSCs by inverted microscope showing 50% (A) and 80% confluence (B); $\times 20$. Percentages of immunophenotype analysis indicated the amount of negative and positive stained populations. The positive population indicated the expression of MSCs surface markers. MSCs showed negative expression of CD34 (10%), CD45 (4.9%), and CD14 (26%) and positive expression of CD105 (73.9%), CD166 (23%), CD90 (46.6%), CD73 (50.1%), CD146 (58.6%), and CD106 (62.9%). Isotype was used as –ve sample untreated control (0.6%). MSCs: mesenchymal stem cells, Pop: population.

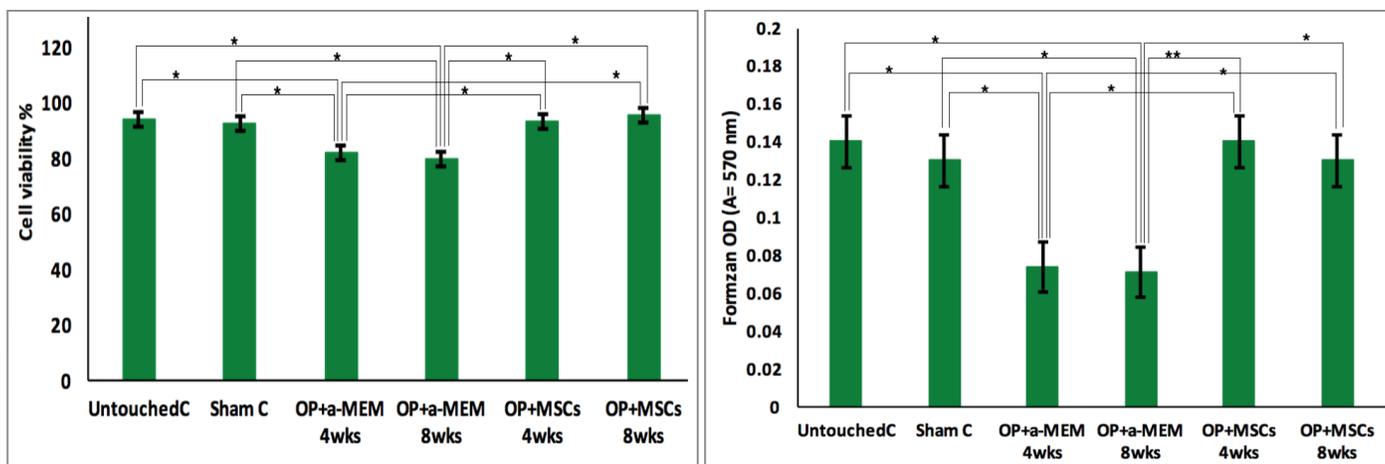


Figure 2. Cell viability (%) and the proliferation assay as expressed by formazan OD of cultured MSCs derived from the different groups of rats. Bars represent mean \pm SE of groups statistically analyzed and marked by * and ** as significantly different at $p < 0.05$ and $p < 0.01$, respectively. C: control, OP: osteoporosis, α -MEM: alpha-modified Eagle medium, and MSCs: mesenchymal stem cells.

Cell viability and MTT assay

Cell viability percentage and the proliferation of cultured MSCs derived from the different groups of rats were measured (Fig. 2). The cell viability percentage of sham-operated rats was 92.52%, which was not significantly different from the untouched control group (94.13%). However, the cell viability percentage of MSCs derived from OP at 4 (82.27%) and 8 (79.83%) weeks experienced a significant decline compared to the untouched control and sham control groups ($p < 0.05$). It was noticed that cell viability of MSCs derived from OP + MSCs after 4 (93.52%) and 8 (95.36%) weeks after treatment showed significant elevation compared to OP at 4 and 8 weeks ($p < 0.05$). The quantity of formazan was measured using an MTT assay to evaluate the proliferation of MSCs (Fig. 2). The results showed that the formazan optical densities (ODs)

of MSCs of sham-operated and untouched control rats were 0.14 ± 0.04 and 0.13 ± 0.02 , respectively, and did not differ significantly. It was observed that formazan ODs of MSCs derived from OP at either 4 weeks (0.074 ± 0.003) and 8 weeks (0.071 ± 0.004) were significantly declined compared to the untouched control and sham control groups ($p < 0.05$). However, formazan ODs of MSCs derived from OP + MSCs at 4 weeks (0.14 ± 0.01) and 8 weeks (0.13 ± 0.006) after treatment were significantly elevated compared to OP at the fourth and eighth weeks ($p < 0.05$ and $p < 0.01$, respectively).

Serum biomarkers levels of MSCs-treated and MSCs-untreated osteoporotic rats

Serum biomarkers OCN, calcium, phosphorus, and B-ALP levels were determined after OP induction as well as after

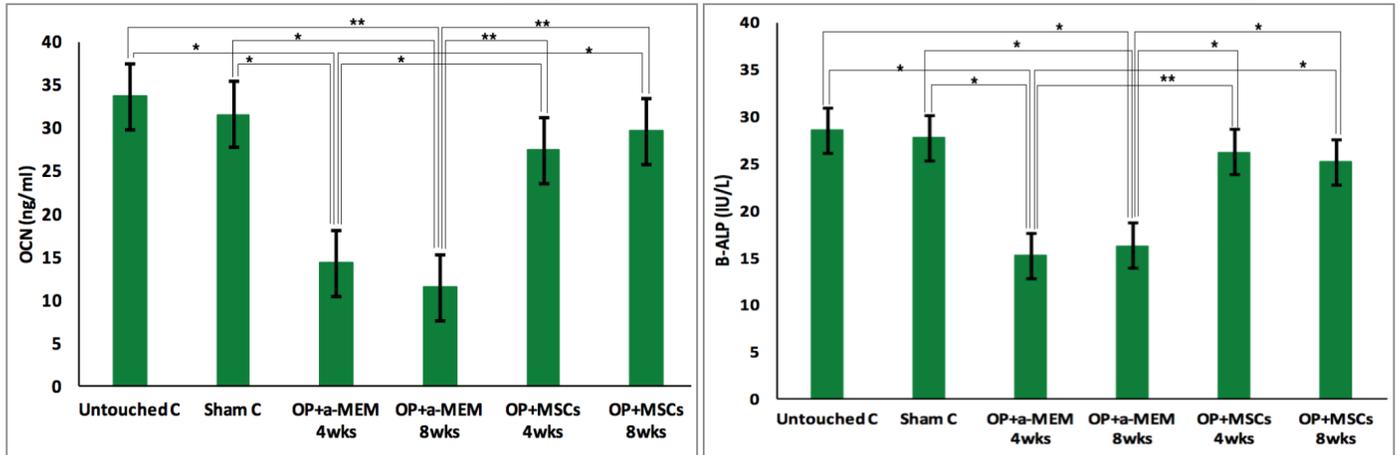


Figure 3. OCN concentration and B-ALP activity in the serum of the different groups of rats. Bars represent mean \pm SE of groups and marked by * and ** as significantly different at $p < 0.05$ and $p < 0.01$, respectively. C: control, OP: osteoporosis, α -MEM: alpha-modified Eagle medium, and MSCs: mesenchymal stem cells.

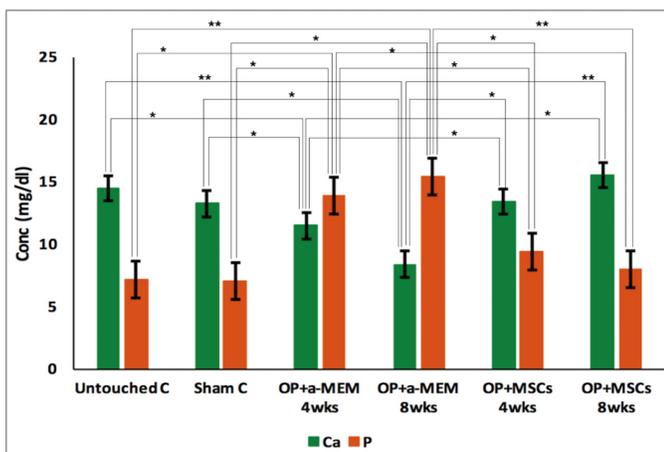


Figure 4. Calcium and phosphorus concentrations in the serum of the different groups of rats. Bars represent mean \pm SE of groups and marked by * and ** as significantly different at $p < 0.05$ and $p < 0.01$, respectively. C: control, OP: osteoporosis, α -MEM: alpha-modified Eagle medium, and MSCs: mesenchymal stem cells.

MSCs treatment after 4 and 8 weeks and are shown in Figures 3 and 4. OCN concentrations were measured in the different groups by ELISA kits. A significant decline was observed in the serum concentrations of OCN of OP at 4 weeks (14.29 ± 1.53 ng/ml) and 8 weeks (11.51 ± 1.09 ng/ml) as opposed to the untouched control (33.62 ± 4.23 ng/ml) and sham control (31.48 ± 2.88 ng/ml) groups ($p < 0.01$). After treatment with MSCs, the results revealed a significant elevation in OCN concentrations in the sera of treated rats in the OP + MSCs group at 4 weeks (27.39 ± 2.87 ng/ml) and 8 weeks (29.61 ± 3.11 ng/ml) compared to OP at 4 weeks and 8 weeks ($p < 0.05$ and $p < 0.01$, respectively).

B-ALP activity was assayed in the serum of different groups of rats (Fig. 3). A significantly decreased serum B-ALP activity was noticed in OP at 4 weeks and 8 weeks (15.17 ± 1.13 and 16.23 ± 2.65 IU/l, respectively) compared to the untouched control (28.49 ± 3.11 IU/l) and sham control (27.68 ± 3.08 IU/l) groups ($p < 0.01$ and $p < 0.05$, respectively). However, a significant elevation was observed

in B-ALP activity in the serum of the OP + MSCs group after 4 weeks (25.11 ± 3.15 IU/l) and 8 weeks (26.22 ± 3.87 IU/l) compared to OP ($p < 0.01$). Sham-operated rats did not show any significant differences in all serum measured parameters compared to untouched control.

Calcium and phosphorus concentrations were measured in the serum of rats using the calorimetric method (Fig. 4). Serum Ca concentration was significantly lower in OP at 4 weeks (11.45 ± 1.07 mg/dl) and 8 weeks (8.36 ± 1.66 mg/dl) after induction compared to the untouched control (14.42 ± 1.11 mg/dl) and sham control (13.23 ± 1.23 mg/dl) groups ($p < 0.05$). It was observed that serum Ca concentrations were significantly elevated after MSCs treatment as shown in the OP + MSCs group after both 4 weeks (13.39 ± 1.34 mg/dl) and 8 weeks (15.50 ± 1.67 mg/dl) compared to OP ($p < 0.05$). Serum phosphorus concentration was significantly elevated ($p < 0.05$) in OP at 4 and 8 weeks (13.89 ± 1.43 and 15.36 ± 1.98 mg/dl, respectively) compared to the untouched control (7.11 ± 0.63 mg/dl) and sham control (6.99 ± 0.98) groups. However, OP + MSCs showed a significant decrease in the concentration of serum P after 4 and 8 weeks of MSCs treatment (9.41 ± 1.01 and 7.96 ± 0.97 mg/dl, respectively) compared to OP ($p < 0.01$).

Calcium level and B-ALP activity in lysed osteogenic differentiated MSCs from MSCs-treated and MSCs-nontreated osteoporotic rats

Calcium concentrations and B-ALP activity were measured in the lysed differentiated MSCs to indicate osteogenic differentiation of MSCs derived from different groups (Fig. 5). Ca concentration in the lysed differentiated cells of untouched control was 20.44 ± 2.33 mg/well and of the sham control group was 18.55 ± 1.56 mg/well. It was clear that Ca concentration in the lysed differentiated cells of sham control did not show any significant difference compared to the untouched control group. However, Ca concentrations in the lysed differentiated cells of OP at 4 weeks (11.12 ± 1.17 mg/well) and 8 weeks (8.54 ± 1.09 mg/well) were significantly decreased compared to the untouched control and sham control groups ($p < 0.05$).

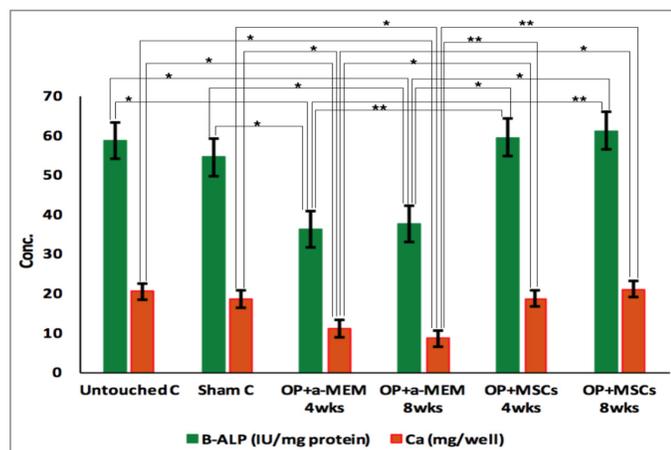


Figure 5. Calcium concentration and B-ALP activity in the lysed differentiated MSCs from the different groups of rats. Bars represent mean \pm SE of groups and marked by * and ** as significantly different at $p < 0.05$ and $p < 0.01$, respectively. C: control, OP: osteoporosis, α -MEM: alpha-modified Eagle medium, and MSCs: mesenchymal stem cells.

The results revealed that MSCs treatment led to a significant elevation of Ca concentrations in the lysed differentiated cells as shown in the OP + MSCs group after either 4 weeks (18.65 ± 2.81 mg/well) or 8 weeks (21.02 ± 3.21 mg/well) compared to OP at 4 and 8 weeks ($p < 0.01$).

B-ALP activity in the lysed differentiated MSCs of sham control (54.4 ± 3.18 IU/mg protein) did not show any significant difference compared to the untouched control group (58.6 ± 4.11 IU/mg protein). The activities of B-ALP in the lysed differentiated MSCs of OP at 4 weeks (36.11 ± 3.75 IU/mg protein) and 8 weeks (37.55 ± 2.65 IU/mg protein) show a significant decrease, as compared to the untouched control and sham control groups ($p < 0.05$). However, a significant elevation of B-ALP activity was noticed after MSCs treatment as shown in the lysed differentiated MSCs of the OP + MSCs group at 4 and 8 weeks (59.45 ± 4.54 and 61.14 ± 5.85 IU/mg protein, respectively) as compared to their corresponding OP groups ($p < 0.01$).

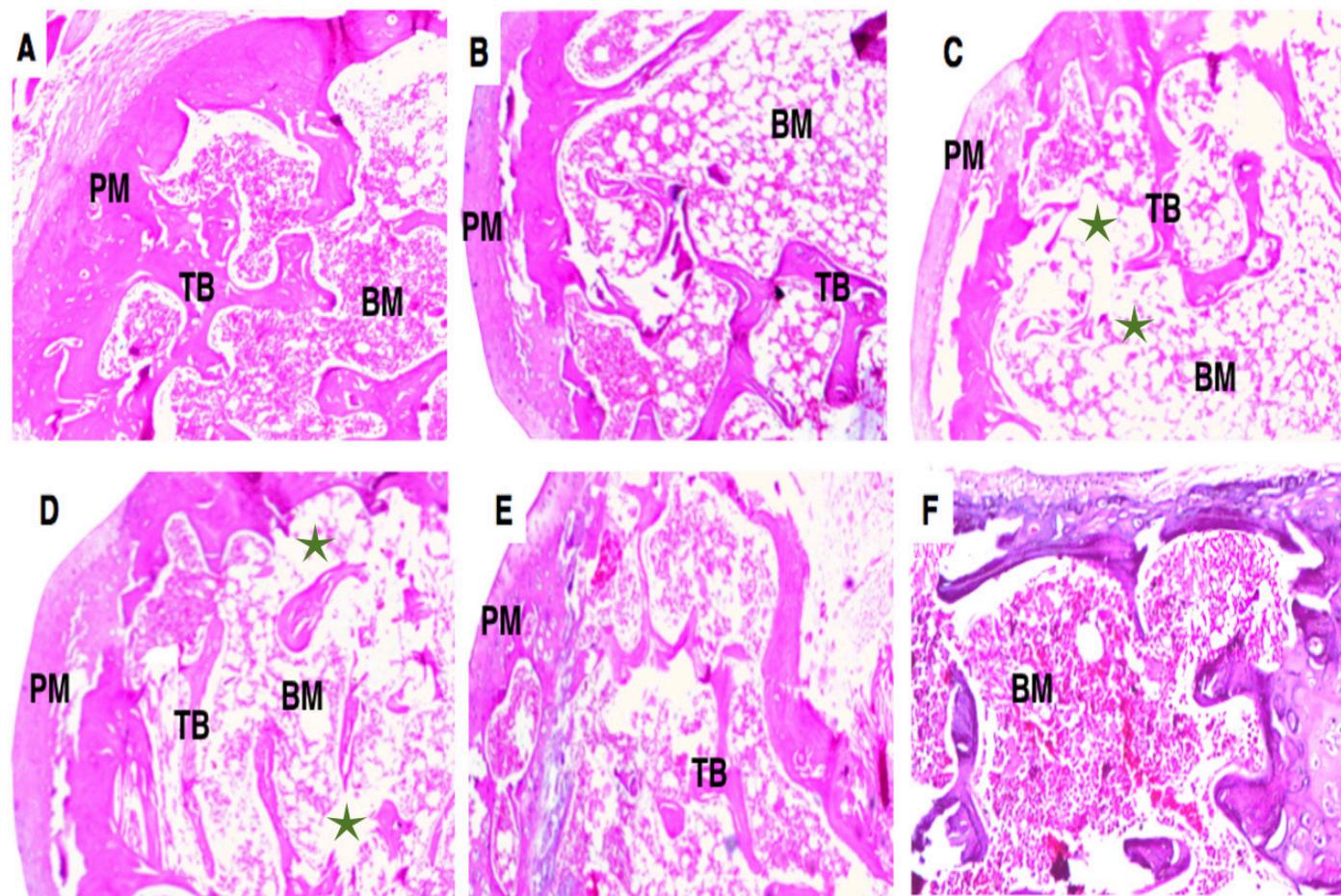


Figure 6. Histological architecture of soft regions of the left femur of different groups stained by hematoxylin-eosin. Untouched control (A) and sham control (B) showed uniform TB within abundant bone marrow. The PM is seen in the outermost layer of bone. Soft bone of OP group after 4 weeks (C) and 8 weeks (D) showed thin TB with wide intertrabecular spaces and disjointed bony ossicles interrupted by expanded fatty bone marrow spaces (green stars). On the other hand, cancellous bone of OP groups treated with MSCs showed normal TB with red bone marrow after 4 weeks (E) and 8 weeks (F). All micrographs were visualized by $\times 40$ magnification. OP: osteoporosis and MSCs: mesenchymal stem cells.

Bone histomorphometry from MSCs-treated and MSCs-nontreated osteoporotic rats

The criteria for the histological sections of the left femoral diaphysis for the untouched and control groups stained by H&E presented normal red bone marrow and trabecular bone and periosteum (PM) with narrow intertrabecular spaces. The histopathological examination of the cancellous bone revealed continual thinning of the trabeculae (TB) in the OP after 4 or 8 weeks leading to widening of intertrabecular spaces in the head of the femur and intermittent bony ossicles interrupted with extended fatty bone marrow spaces. After MSCs treatment, cancellous bone TB appeared normal with red bone marrow (Fig. 6).

In vitro osteoblastic differentiation from MSCs-treated and MSCs-nontreated osteoporotic rats

In vitro osteoblastic differentiation of MSCs in response to culturing with osteogenic media was analyzed by Alizarin red staining (Fig. 7). The differentiation of MSCs showed highly red-stained dispersed calcified nodules of the untouched control (Fig. 7A) and sham control (Fig. 7B) groups in response to red

Alizarin. Photomicrograph of *in vitro* osteogenic differentiation of MSCs derived from OP group after 4 (Fig. 7C) and 8 (Fig. 7D) weeks showed very low scattered red calcified nodules in response to Alizarin red staining while treatment with MSCs in OP + MSC group expressed moderate to high scattered red calcified nodules after 4 (Fig. 7E) and 8 (Fig. 7F) weeks, respectively, after Alizarin red staining.

DISCUSSION

This approach determined the therapeutic potential of MSCs application in induced osteoporotic rats. The data obtained show that the transplantation of stem cells into osteoporotic rats provides a supplement for bone architecture and enhances osteoblastic differentiation, suggesting reverting OP. Bone formation is a biological process by which the old bone is replaced by a new one. The old bone was resorbed by osteoclasts while the new formation was caused by osteoblasts (Bellido, 2014). To date, it is not yet clear how osteogenesis or adipogenesis can be optimized and differentiated. MSCs are nonhematopoietic cells with the potentiality of self-renewal and multilineage differentiation and have been extensively used in the cellular therapeutic field

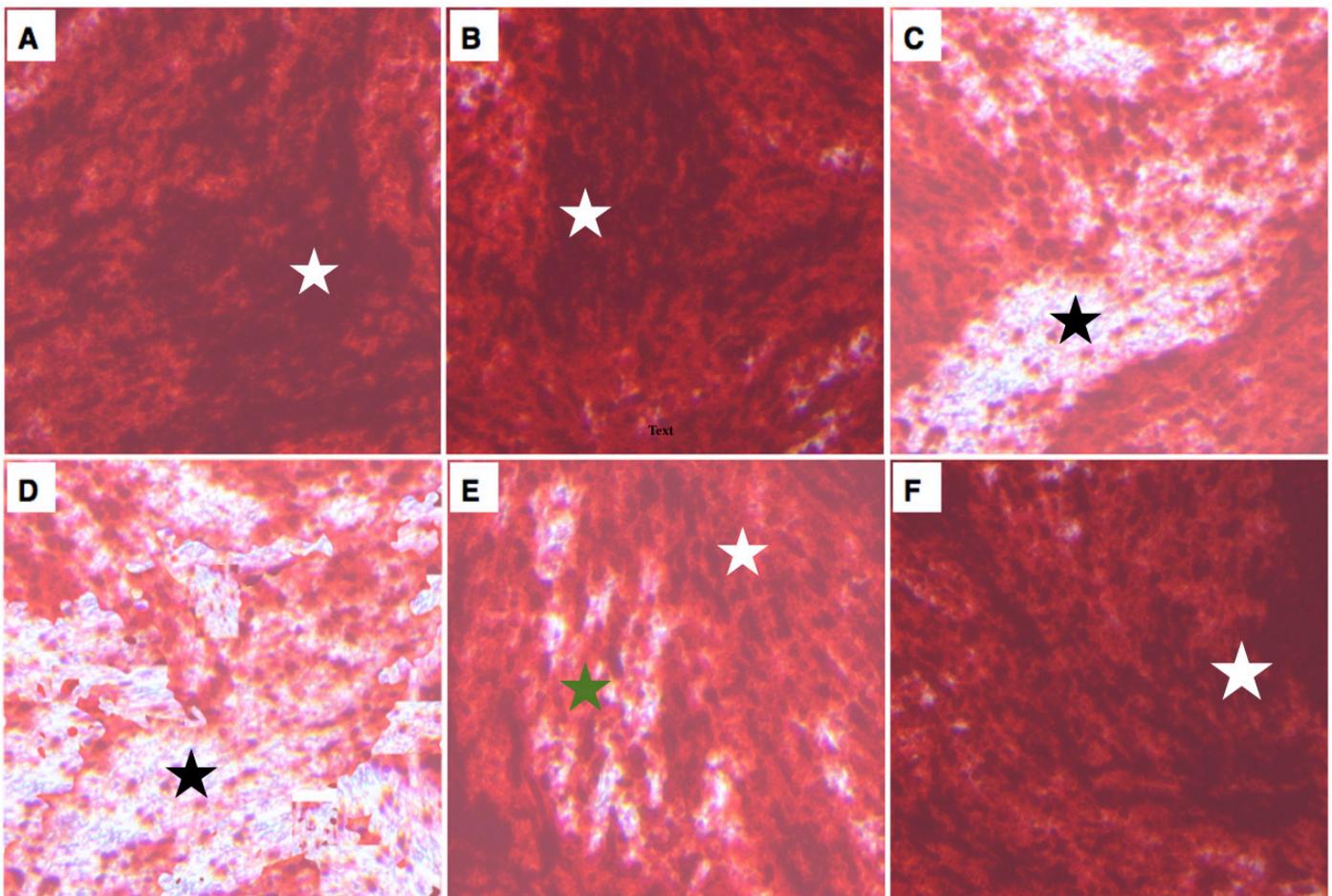


Figure 7. Photomicrograph of *in vitro* osteoblastic differentiation of MSCs derived from different groups of rats after Alizarin red staining. Untouched control (A) and sham control (B) showed highly scattered red calcified nodules (white star). Osteogenic differentiated cells of OP groups showed very low scattered red calcified nodules (black star) after 4 weeks (C) and 8 weeks (D) of induction. Osteogenic differentiated cells of OP + MSCs group expressed moderate (green star) to high (white star) scattered red calcified nodules after 4 weeks (E) and 8 weeks (F). All photomicrographs were visualized by $\times 40$ magnification. OP: osteoporosis and MSCs: mesenchymal stem cells.

(Fortier and Travis, 2011). MSCs are the most interesting cells to date, because of their assorted functions. In addition, they have fascinating characteristics in tissues and organs regeneration, which suggest that MSCs are capable of directing toward injured tissue, to undergo differentiation and immunomodulation (Aguirre *et al.*, 2010). In the current investigation, bone marrow MSCs were used for the treatment of osteoporotic rats, and before that, their morphology and phenotypic characteristics were examined and confirmed. Previous studies have shown that the OP was induced by ovariectomy (Kikuta *et al.*, 2013), glucocorticoid supplementation (Aggarwal *et al.*, 2012), or immune-deficient osteoporotic murine model (Aggarwal *et al.*, 2014). In the present study, a model was chosen to induce OP in rats by ovariectomy and glucocorticoids injection for eight weeks. It was known that glucocorticoids have a dual effect, increase bone resorption decrease, and decrease bone formation (Khalifa *et al.*, 2020). Ovariectomy removes the osteogenic effect of estrogen (Saito *et al.*, 2018). Then stem cells were isolated for determination of its criteria and ability to relieve OP. In this investigation, data revealed that the MSCs viability % and formazan OD derived from OP at 4 and 8 weeks were significantly decreased in comparison with the untouched and sham control groups. The calcium concentration and B-ALP activity were measured in the lysed differentiated MSCs to indicate osteogenic differentiation of MSCs derived from different groups. It was clear that calcium concentration and B-ALP activity in the lysed differentiated cells of sham control did not show any significant difference when compared to the untouched control group. However, calcium concentration and B-ALP activity in the lysed differentiated cells of OP at 4 and 8 weeks were significantly decreased as compared to the untouched and sham control groups. These results corroborate the findings on the OP rat model outlined by Khalifa *et al.* (2020).

In this investigation, the concentrations of OCN, Ca, P and B-ALP in serum of OP rats were obtained. A significant decline was noticed in the serum concentrations of OCN, Ca, and B-ALP activity of OP at 4 and 8 weeks compared to the untouched control and sham control groups. However, serum P concentration was significantly higher in OP at 4 and 8 weeks after induction compared to the untouched control and sham control groups. These results agree with the research conducted previously by (Khalifa *et al.*, 2020). The histopathological image of the femur head displayed a constant reduction of the trabecular bone in the OP group after 4 or 8 weeks leading to broadening of spaces between TB and disjointed bony ossicles interrupted by expanded fatty bone marrow spaces. Photomicrograph of *in vitro* osteogenic differentiation of isolated and proliferated MSCs of untouched and sham control groups showing highly dispersed red to brown calcified nodules by Alizarin red staining. Photomicrograph of MSCs isolated from OP model after 4 and 8 weeks of induction and undergoing *in vitro* osteogenic differentiation showed very low dispersed red to brown calcified nodules by Alizarin red staining. The loss of stem cells from bone marrow and their osteoplastic progeny is assumed to be a prominent factor for reduction of bone mass and now stem cell therapies for OP were under consideration, but their success in clinical applications is yet to be established. Due to the plenty of MSCs from bone marrow, it can be considered as an ultimate cell-based therapy to treat bone disease like OP. Due to ethics

concerning embryonic stem cell usage, laboratory attention has been focused on adult stem cells. However, MSCs combined two basic characteristics of self-renewal and multilineage differentiation. Bei *et al.* (2018) showed that MSCs have the ability to alleviate OP locally and systemic, through direct and indirect mechanisms. Systematic manipulation was preferred as in the present study to avoid the traumatic issue by local injection. The mechanism by which MSCs alleviate OP might be the engraftment to local osteoporotic lesion or direct anabolism of osteogenic differentiation or secretion of trophic factors for osteoblast (Sui *et al.*, 2016).

It was noticed in the present study that cell viability % and formazan OD of MSCs derived from OP + MSCs after 4 and 8 weeks after treatment were significantly higher than that of OP at 4 and 8 weeks. The results revealed also that Ca concentration and B-ALP activity in the lysed differentiated cells of OP + MSCs group at 4 weeks after treatment were significantly elevated compared to OP at 4 and 8 weeks. After treatment with MSCs, the results revealed that OCN, Ca, and OCN concentration and B-ALP activity in the serum of OP + MSCs rats at 4 weeks after treatment were elevated compared to OP at 4 and 8 weeks. On the other hand, OP + MSCs showed a significant decrease in serum P concentration at either 4 or 8 weeks after MSCs treatment compared to OP at 4 and 8 weeks. The histological image showed that, after MSCs treatment, cancellous bone TB appeared normal with red bone marrow. This data indicated a significant elevation of bone formation rate with the restoration of estrogen deficiency-induced trabecular and cortical bone loss due to ovariectomy as previously reported that bone histomorphometry analysis revealed a reduction in the number and width of trabecular bone of the tibia in OP rats (Yao *et al.*, 2013).

MSCs have the ability to alleviate OP through local and systemic or direct and indirect mechanisms (Sui *et al.*, 2017). In the present study, due to traumatic issues by local injection, the mechanism by which MSCs alleviate OP might be the engraftment to local osteoporotic lesion or direct anabolism of osteogenic differentiation or secretion of trophic factors for osteoblast (Sui *et al.*, 2016). Photomicrograph of *in vitro* osteogenic differentiation of MSCs of OP after 4 and 8 weeks showed very low dispersed red to brown nodules by Alizarin red staining while treatment with MSCs of OP expressed moderate to high scattered red calcified nodules after 4 and 8 weeks, respectively, in response to Alizarin red staining. This is consistent with the previously reported *in vitro* findings (Shen *et al.*, 2018; Sui *et al.*, 2018) which attributed the relief to immunosuppression/anti-inflammation and resident stem cell recovery.

CONCLUSION

Based on the present findings, it is concluded that the transplantation of MSCs into osteoporotic rats augments bone architecture and enhances osteoblastic differentiation and function, resulting in reverting OP because these cells provide the required complementary effect to the bone. This present study provides evidence for the nonimmunogenic therapy of MSCs for the treatment of osteoporotic murine models. This work presents a unique method as a future application for the treatment of human OP. Pharmacotherapy can be prohibitive in cost, taking a long time to be effective, and lead to adverse reactions. Bisphosphonate

therapy may lead to extensive suppression of bone development. Therefore, the combination of MSCs with bisphosphonate is put in perspective for control of OP, which needs to address many of the challenges that exist in this application in the future study.

AUTHORS' CONTRIBUTION

ASMA, AAZ, and FAMA contributed to the conception and design of the study. ASMA, WA, ZR, and FAA contributed to the acquisition of data. ASMA, WA, ZR, FAA, and FAMA contributed to the analysis and interpretation of data. ASMA, AAZ, and FAMA prepared the final manuscript for submission. All authors read, revised, and approved the final version of the article for submission.

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CONFLICT OF INTEREST

The authors have no conflicting financial interest.

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