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Emerging role of stem cells and / or osteoinductive material in retrieving osteoporosis in rodents

Hanaa H. Ahmed[a*], El-Sayed M. El-Sayed Mahdy[b], Wafaa Gh. Shousha[b], Laila A. Rashed[c] and Sara M. Abdo[b]

[a]*Hormones Department, Medical Research Division, National Research Centre, Dokki, Giza, Egypt*
[b]*Cemistry Department, Faculty of Science, Helwan University, Cairo, Egypt*
[c]*Medical Biochemistry Department, Faculty of Medicine, Cairo University, Cairo, Egypt*

ABSTRACT

The objective of this study was to verify the therapeutic role of mesenchymal stem cells, osteoinductive material or both of them versus calcitonin therapy in betterment of osteoporosis in ovariectomized rat model. Mesenchymal stem cells (MSCs) were harvested from femoral bone marrow and excised from both the omentum and the inguinal fat pad of male rats. Ninety adult female rats were enrolled in the current study and assigned into nine groups: group (1) served as gonad intact control, group (2) served as untreated ovariectomized (OVX) rats, while the groups from the third to ninth were OVX groups treated with, bone marrow mesenchymal stem cells (BM-MSCs), adipose tissue derived mesenchymal stem cells (ASCs), BM-MSCs plus injectable bone substitute (IBS), ASCs plus IBS, IBS, calcitonin and calcitonin plus IBS respectively. BALP gene expression level was detected via real time PCR. In addition, levels of IL-7 in serum as well as level of DPD in urine were determined by ELISA. Moreover, BMD was measured using DEXA technique. The Results showed positive SRY gene expression in the femur bones of the MSCs treated groups, confirming that the intravenously injected MSCs could migrate to the site of injury. MSCs injection with or without IBS reversed the effect of ovariectomy on the studied biomarkers causing significant up-regulation in the expression level of BALP in femur bones and significant reduction in serum, IL-7 and urinary DPD levels. Moreover, the DEXA results revealed that MSCs therapy improved the ovariectomy induced decrease in BMD in some areas of rats' femur bones. In conclusion, the current results indicated the potent role of MSCs in retrieving of osteoporosis. Also, the synergism between MSCs and osteoinductive material could be beneficial for enhancement of the therapeutic effect.

Key words: Bone marrow mesenchymal stem cells, adipose tissue derived mesenchymal stem cells, osteoporosis, bone substitute, Rats.

INTRODUCTION

Osteoporosis is a skeletal disorder characterized by low bone mass, microstructure damage and more fragile bone leading to an increase of the bone fracture risk. The imbalance of bone remodeling seems to be the cause of osteoporosis, and the osteoblasts and osteoclasts come to be disproportionate. However, the detailed pathologic mechanism, especially the molecular cause of this disorder, keeps more mystery [1].

The average survival of osteoblast is the critical factor affecting the outset of osteoporosis in postmenopausal women. In case of estrogen insufficiency, the remodeling units increase and an unbalanced coupling of bone resorption and formation occur. Consequently, the rate of initiation of remodeling cycles and bone resorption increases, leading to decrease in bone density [2]. Moreover, it has been reported that estrogen insufficiency causes remodeling imbalance along with substantial increase in bone turnover that leads to loss of trabecular bone as a result of increased osteoclastogenesis [3].

Stem cells are undifferentiated cells characterized by self-renewal and multipotential differentiation [4]. These cells have the ability to differentiate to more specialized cell types that perform specific functions within tissues [5]. Stem cell based therapies potential could be allied to their ability to differentiate into cell lineages *in vitro* and/or reconstitute tissue *in vivo* [6]. With this concept, the multipotency and ready availability of mesenchymal stem cells (MSCs) make them promising source for regenerative therapies [7].

The *in situ* differentiation of MSCs to become normal components of the recipient cytoarchitecture, supporting stroma after recruitment to the injury site and their action *via* a paracrine mechanisms are the two possible mechanisms responsible for the beneficial effects of MSCs [8]. Additionally, these cells could modulate the inflammatory and immune reactions, protect from cell death and stimulate endogenous progenitor cells [9, 10].

Calcium phosphate cements (CPCs) showed a promise for repairing defects as they are similar to the mineral phase of bone. As well the high reactivity of these cements is considered the principal cause for their usage in bone enhancement, as the reaction products result in crystallization into an apatite-like phase, like the inorganic constituent of bones [11, 12]. Also, calcium phosphate ceramics [hydroxyapatite (HA) and beta-tricalcium phosphate] are the main two substances used for bone substitutes [13], as they possess osteoinductive ability similar to that of macroporous ceramics [11]. Treatment of osteoporotic bone defects requires often the insertion of bone substitution materials to improve bone healing and stabilization. The choice of the most suitable material for osteoporotic bone is controversially discussed [14]. However, injectable bone cements (IBCs) are biocompatible materials used for defect improvement of the weakened osteoporotic bone [15].

This work goaled to investigate the promising role of mesenchymal stem cells, injectable bone substitute or both of them contra calcitonin therapy in alleviating osteoporosis in ovariectomized rats.

MATERIALS AND METHODS

BM-MSCs and ASCs isolation

Bone marrow was harvested from femur bones of 6-week-old male Wistar rats by flushing these bones with DMEM (GIBCO/BRL, USA) supplemented with 10% FBS (GIBCO/BRL). Nucleated cells were isolated *via* a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in a culture medium supplemented with 1% penicillin–streptomycin (GIBCO/BRL). Then, cells were incubated at 37 °C in 5% humidified CO_2 incubator until formation of large colonies [16].

Adipose tissue was excised from both abdominal and inguinal fat pad of male Wistar rats according to the method described by Tomiyama et al. [17]. The isolated adipose tissue was resected and placed into a sterile tube that contains 15 mL of phosphate buffered solution (PBS, Gibco/Invitrogen, USA). Enzymatic digestion was done using 0.075% collagenase II (Serva Electrophoresis GmbH, Mannheim) in Hank's Balanced Salt Solution for 60 min at 37°C with shaking. Digested tissue was filtered and centrifuged, then erythrocytes were removed. The cells were transferred to tissue culture flasks containing DMEM supplemented with 10% FBS for 24 hours then, the non-adherent cells were removed by PBS wash. Attached cells were cultured in DMEM media supplemented with 10% FBS, 1% penicillin-streptomycin, and 1.25 mg/L amphotericin B (Gibco/BRL), then incubated at 37 °C in 5% humidified CO_2 incubator until formation of large colonies.

When BM-MSCs and ASCs reached 80–90% confluence, cultures were washed two times with PBS and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL) for 5 min at 37 °C. Thereafter, cells were resuspended with serum-supplemented medium and incubated in 50 cm^2 culture flask after centrifugation [18]. To assure the identity of the isolated MSCs, the cells were recognized morphologically by inverted microscope examination, *in vitro* differentiation into adipocytes, chondrocytes and osteocytes and detection of CD14, CD44 and CD106 genes expression by real time PCR, as in our previous work [16, 19].

Drug

Calcitonin (Miacalcic®) was provided from Novartis Pharma Stein (Switzerland) as 1 ml ampoules containing 100 IU of synthetic salmon calcitonin.

Biomaterial

Hydroxy-propyl-methyl-cellulose (HPMC, Winlab Co., U.K) was prepared (3%) by dissolving raw, dry HPMC powder in bidistilled water under stirring for 48 h. Then, biphasic calcium phosphate (BCP, Sigma-Aldrich Co., USA) granules were added during stirring. The biomaterial was then transferred in ready-to-use glass flasks sterilized by steam at 121^0C for 20 min [20].

Animals

Ninety female Wistar rats whose weights at the time of randomization ranged from 130 to 150 g were assigned into nine groups (n=10) and then allowed one week to acclimatize to animal room conditions before ovariectomy. All procedures concerning animal experiments were done with proper approval of Ethics Committee of Medical Research at National Research Centre, Giza, Egypt.

Surgical overiectomy was done as described by Mattila [21] to induce primary osteoporosis. Ovariectomized (OVX) rat is an ideal model mimic postmenopausal bone loss in human subjects [22].

Experimental setting

The animals were divided after three months from the surgical ovariectomy into 9 experimental groups as follows: **group 1** (Gonad intact group): healthy female rats, **group 2** (OVX group): untreated ovariectomized rats, **group 3** (BM-MSCs group): ovariectomized rats injected intravenously with a single dose of undifferentiated BM derived MSCs (3 x 10^6 cells/rat) [23], **group 4** (ASCs group): ovariectomized rats injected intravenously with a single dose of undifferentiated adipose derived MSCs (3 x 10^6 cells/rat) [24], **group 5** (BM-MSCs and IBS group): ovariectomized rats treated with single dose of BM-MSCs intravenously and two injections (each of 640 µl for each rat) of IBS submuscularly adjacent to the femur surface [25], **group 6** (ASCs and IBS group): ovariectomized rats treated with single dose of ASCs intravenously and two injections of IBS submuscularly, **group 7** (IBS group): ovariectomized rats injected with IBS submuscularly, **group 8** (Calcitonin group): ovariectomized rats treated with calcitonin subcutaneously in a dose of 15 IU kg^{-1} dissolved in distilled water once a week for three months [26] and **group 9** (Calcitonin and IBS group): ovariectomized rats injected with IBS submuscularly and administered with calcitonin subcutaneously once a week for three months.

After the completion of this round (3 months after MSCs injection), 24 hours urine samples were collected and all animals were fasted for 12 h. Thereafter, the blood samples were collected from retro-orbital venous plexus under anaesthestic condition. After clotting, the sera were separated by centrifugation (4ºC) at 3000 r.p.m for 10 min and then stored immediately at -80ºC till the analysis.

After blood collection, the animals were rapidly sacrificed and the rats' right femur bones were immediately frozen in liquid nitrogen and stored at -80ºC for the molecular study. Meanwhile, the rats' left femur bones were cleaned carefully for measuring bone mineral density (BMD) using the dual energy X-ray absorptiometry technique (DEXA).

PCR detection of SRY gene

The DNA was isolated from femurs of ovariectomized rats which were treated with MSCs using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA). SRY gene was detected in femur bone of the treated female rats by PCR. SRY gene primer sequence (F: 5′-CATCGAAGGGTTAAAGTGCCA-3′, R: 5′-ATAGTGTGTAGGTTGTTGTCC-3′) were according to the previous published sequences with product size of (104 bp) [27]. PCR cycling conditions were performed as mentioned in our previous work [16].

Biochemical analyses

Serum interleukin-7 (IL-7) and urinary deoxypyridinoline DPD levels were determined by ELISA using kit purchased from Glory Science Co. (USA), according to the manufacturer's instructions.

Detection of bone alkaline phosphatase (BALP) gene expression

Total RNA was isolated from femur bones using SV Total RNA Isolation system (Promega, USA) according to manufacturer's instruction. Then, first-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Netherlands) by mixing 2 µg of total RNA with 0.5 µg of oligo (dT) 12-18 primer in a total volume of 12 µL. After the mixture was heated at 70° C for 10 min, a solution containing 50 mmol/L Tris HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 0.5 µL RNase inhibitor, and 200 U superscript reverse transcriptase was added, resulting in a total volume of 20.5 µL. This mixture was incubated at 42ºC for 1 h.

For real-time quantitative PCR, 5 µL of first-strand cDNA was used in a total volume of 25 µL, containing 12.5 µL 2x SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 ng of each primer, which shown in **Table (1)**. PCR reactions consisting of 95ºC for 10 min (1 cycle), 94ºC for 15 s, and 60ºC for 1 min (40 cycles), were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems). Data were analyzed with the ABI Prism 7500 sequence detection system software and quantified using the v1·7 sequence detection software from PE Biosystems (Foster City, CA). Relative expression of BALP gene was calculated using the comparative threshold cycle method. All values were normalized to the beta actin gene [30].

Table (1): Primers sequence of the tested genes used for real-time PCR

Genes	Primers sequence
BALP	F: 5′- CATGTTCCTGGGAGATGGTA-3′ R: 5′- GTGTTGTACGTCTTGGAGAGA-3′, according to Qi et al. [28] published sequence.
β-actin	F: 5′-TCTGGCACCACACCTTCTACAATG-3′ R R: 5′- AGCACAGCCTGGATAGCAACG -3′, A according to Porichi et al.[29] published sequence.

Measurements of bone mineral density (BMD) of rat femur bones

The left femur bone of each animal was cleaned and stored in formalin buffer (10%). Then, the length and weight of each femur were recorded. The BMD of each femur bone was measured in proximal, mid, distal and total areas [31] by DEXA technique using Norland XR46, version 3.9.6/2.3.1 instrument, which could be equipped with dedicated software for small animal measurements.

Statistical analyses

In the current work, all data were expressed as Mean ± standard error (S.E) of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 17 followed by least significant difference (LSD) to compare significance between groups [32]. Difference was considered significant when P value was < 0.05.

RESULTS

I- Homing of the injected mesenchymal stem cells

The agarose gel electrophoresis **(Fig. 1)** represented that the femurs of OVX rats treated with MSCs either derived from bone marrow or adipose tissue showed positive expression of SRY gene. While, the femurs from gonad intact as well as ovariectomized rats showed negative SRY gene expression. These findings indicated the accommodation of the male donor stem cells to the site of injury in femurs of the female recipients.

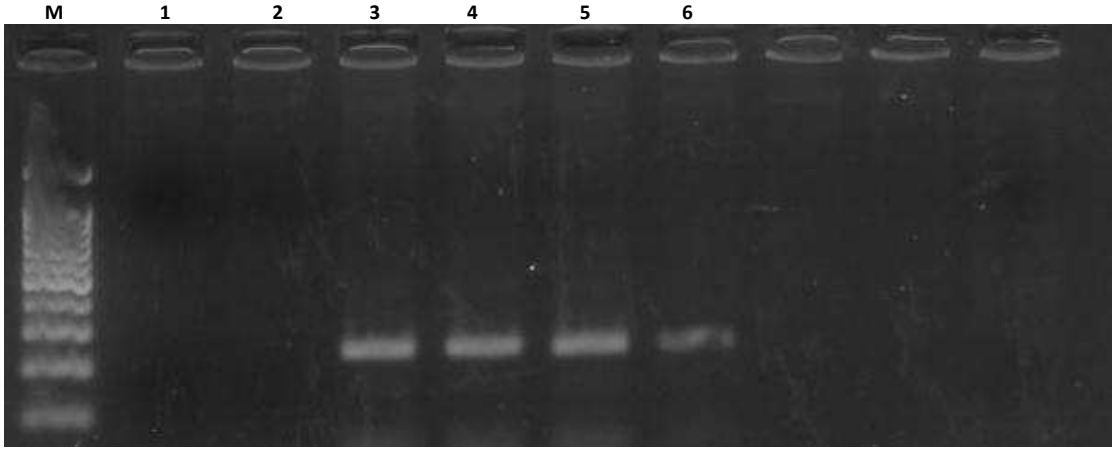

Fig. (1): Agarose gel electrophoresis representing the PCR products of *SRY* gene expression in the studied groups (M): DNA ladder; Lane (1): gonad intact group; Lane (2): OVX group; Lane (3): BM-MSCs group; Lane (4): ASCs group; Lane (5): BM-MSCs + IBS group and Lane (6): ASCs +IBS group

II- **Biochemical analyses**

The results in **Table (2)** represented the influence of treatment with MSCs, IBS and calcitonin on serum IL-7 and urinary DPD levels in osteoporotic rats. Serum IL-7 level increased significantly ($P < 0.05$) in osteoporotic group versus gonad intact group. Conversely, all studied groups recorded significant ($P < 0.05$) decrease in serum IL-7 level as compared to osteoporotic group with percent of change ranged from 11.7 % to 27.5 %. On the other hand, the infusion of BM-MSCs or BM-MSCs plus IBS revealed significant ($P < 0.05$) decrease in serum IL-7 level as compared with that in calcitonin treated group. The change in serum IL-7 level in IBS, calcitonin and calcitonin plus IBS groups revealed significant ($P < 0.05$) increase when compared to that of BM-MSCs. Furthermore, the entire groups showed significant ($P < 0.05$) increase in serum IL-7 level with respect to BM-MSCs plus IBS treated group, except the BM-MSCs and ASCs plus IBS groups that recorded insignificant change ($P > 0.05$) in serum IL-7 level as compared to BM-MSCs plus IBS treated group.

The data in **Table (2)** also illustrated that urinary DPD levels increased significantly ($P < 0.05$) in osteoporotic

group with respect to gonad intact group. In contrary, urinary DPD level decreased significantly ($P < 0.05$) in all treated groups as relative to osteoporotic group. The percent of change from osteoporotic group was 23.9 %, 17.8 %, 29.7 %, 21.2 %, 16.2 %, 17.3% and 15.6% for BM-MSCs, ASCs, BM-MSCs plus IBS, ASCs plus IBS, IBS, calcitonin and calcitonin plus IBS groups respectively. The osteoporotic group treated with BM-MSCs and BM-MSCs plus IBS revealed significant ($P < 0.05$) decrease in urinary DPD level versus calcitonin treated group. On the other hand, the groups of rats treated with IBS, calcitonin and calcitonin plus IBS revealed significant ($P < 0.05$) increase in urinary when compared to that of BM-MSCs group. Moreover, all treated groups showed significant ($P < 0.05$) increase in urinary DPD level in comparison with BM-MSCs plus IBS treated group, except the BM-MSCs group which showed insignificant ($P > 0.05$) change in urinary DPD level.

Table (2): Influence of MSCs, IBS and Calcitonin therapy on serum IL-7 and urinary DPD levels in osteoporotic rats

Groups	IL-7 (pg/ml)	DPD (nmol/mmol Creatinine)
Gonad intact	733.11 ± 16.69	24.81 ± 0.53
OVX	1083.82 ± 66.45^{a}	39.91 ± 0.86^{a}
OVX + BM-MSCs	827.34 ± 22.42^{bc}	30.36 ± 1.31^{bc}
OVX + ASCs	889.16 ± 26.16^{be}	32.82 ± 0.98^{be}
OVX + BM-MSCs + IBS	786.08 ± 17.63^{bc}	28.06 ± 0.81^{bc}
OVX + ASCs + IBS	853.3 ± 21.68^{b}	31.45 ± 1.09^{be}
OVX + IBS	937.74 ± 27.85^{bde}	33.43 ± 1.03^{bde}
OVX + Calcitonin	913.9 ± 20.81^{bde}	33.01 ± 0.44^{bde}
OVX + Calcitonin + IBS	957.24 ± 17.12^{bde}	33.69 ± 0.69^{bde}

*Data are represented as Mean ±S.E of 10 rats /group. **a:** Significant change at P< 0.05 in comparison with gonad intact group. **b:** Significant change at P< 0.05 in comparison with the osteoporotic group. **c:** Significant change at P< 0.05 in comparison with the calcitonin- treated group. **d:** Significant change at P< 0.05 in comparison with the BM-MSCs- treated group. **e**: Significant change at P< 0.05 in comparison with the BM-MSCs + IBS- treated group.*

III- Molecular analyses

Regarding the alterations in BALP gene expression following MSCs, IBS and the calcitonin therapy, the results given in **Table (3)** revealed significant ($P < 0.05$) decrease in BALP gene expression in osteoporotic group as compared to gonad intact group. On the other side, BM-MSCs, ASCs plus IBS and BM-MSCs plus IBS groups showed significant ($P < 0.05$) increase in BALP gene expression relative to osteoporotic group with percent of change 54.9 %, 39.4 % and 83.1 % respectively from osteoporotic group. The BM-MSCs and BM-MSCs plus IBS groups produced significant ($P < 0.05$) increase in BALP gene expression versus calcitonin group. On the other hand, BM-MSCs plus IBS produced significant ($P < 0.05$) increase in BALP gene expression with respect to BM-MSCs group (1.3 versus 1.1), while the groups treated with ASCs, IBS, calcitonin and calcitonin plus IBS displayed significant ($P < 0.05$) decrease in BALP gene expression with respect to BM-MSCs group. In addition, all the treated groups revealed significant ($P < 0.05$) decrease in BALP gene expression with respect to BM-MSCs plus IBS group.

Table (3): Effect of MSCs, IBS and Calcitonin therapy on BALP gene expression in osteoporotic rats

Groups	Relative BALP gene expression
Gonad intact	1.72 ± 0.11
OVX	0.71 ± 0.03^{a}
OVX + BM-MSCs	1.1 ± 0.05^{bce}
OVX + ASCs	0.83 ± 0.04^{de}
OVX + BM-MSCs + IBS	1.3 ± 0.08^{bcd}
OVX + ASCs + IBS	0.99 ± 0.07^{be}
OVX + IBS	0.81 ± 0.04^{de}
OVX + Calcitonin	0.82 ± 0.04^{de}
OVX + Calcitonin + IBS	0.79 ± 0.03^{de}

*Data are represented as Mean ±S.E of 10 rats /group. **a:** Significant change at P< 0.05 in comparison with gonad intact group. **b:** Significant change at P< 0.05 in comparison with the osteoporotic group. **c:** Significant change at P< 0.05 in comparison with the calcitonin- treated group. **d:** Significant change at P< 0.05 in comparison with the BM-MSCs- treated group. **e**: Significant change at P< 0.05 in comparison with the BM-MSCs + IBS- treated group.*

IV-Dual-energy X-ray absorptiometry

The data shown in **Table (4)** represented the alterations in bone mineral density of proximal area of the osteoporotic rats left femur bones following MSCs, IBS and the calcitonin therapy. Ovariectomy produced significant ($P < 0.05$) decrease in BMD of this area when compared with gonad intact group (102.7 mg/cm^2 versus 111.8 mg/cm^2, with 8.1 %). Meanwhile, BM-MSCs, ASCs, BM-MSCs plus IBS and ASCs plus IBS treated groups revealed significant ($P < 0.05$) increase in BMD of proximal area when compared with osteoporotic group with percent of change ranged from 3.3 % to 8.2 %. With respect to the calcitonin group, the BM-MSCs, BM-MSCs plus IBS and ASCs with IBS treated groups showed significant ($P < 0.05$) increase in BMD of proximal area. In addition, the results indicated

significant ($P < 0.05$) decrease in BMD of proximal area in ASCs, IBS, calcitonin and calcitonin with IBS groups as compared to BM-MSCs group. Moreover, all treated groups showed significant ($P < 0.05$) decrease in BMD of proximal area as compared to BM-MSCs plus IBS group except the BM-MSCs treated group which showed insignificant ($P < 0.05$) change in BMD of this area.

The left femur bones of the osteoporotic rats showed significant ($P < 0.05$) decrease in BMD of distal area when compared with gonad intact group. On the other side, BM-MSCs, BM-MSCs plus IBS, ASCs and ASCs plus IBS treated groups displayed significant ($P < 0.05$) increase in BMD of distal area with respect to osteoporotic group with percent of change 10.8%, 12.8%, 5.1% and 6.9% respectively. Within the treated groups, rats injected with BM-MSCs, BM-MSCs plus IBS and ASCs plus IBS showed significant ($P < 0.05$) increase in BMD of distal area when compared with calcitonin group. The results also clarified that treatment with ASCs, ASCs plus IBS, IBS, calcitonin and calcitonin plus IBS recorded significant ($P < 0.05$) decrease in BMD of distal area as compared to BM-MSCs group. Moreover, all treated groups showed significant decrease in BMD of distal area as compared to BM-MSCs plus IBS group except the BM-MSCs treated group which showed insignificant change ($P > 0.05$) in BMD of this area **(Table 4)**.

Regarding the BMD of mid area in the osteoporotic rats left femur bones following MSCs, IBS and the calcitonin therapy, the results given in **Table (4)** revealed significant ($P < 0.05$) decrease in BMD of this area in osteoporotic group as compared to gonad intact group (103.6 mg/cm^2 versus 115.2 mg/cm^2, with 10.1 %). However, treatment with BM-MSCs, BM-MSCs plus IBS, ASCs and ASCs plus IBS caused significant ($P < 0.05$) increase in BMD of mid area with percent of change 6.1%, 7.9%, 3.2% and 3.9% respectively as compared to osteoporotic group. Only, the BM-MSCs and BM-MSCs plus IBS treated groups showed significant ($P < 0.05$) increase in BMD of mid area as compared to calcitonin group. On the other hand, IBS, calcitonin and calcitonin plus IBS groups showed significant decrease in BMD of mid area with respect to the BM-MSCs treated group. Furthermore, all treated groups recorded significant ($P < 0.05$) decrease in BMD of mid area when compared with BM-MSCs plus IBS group except for the BM-MSCs treated group which showed insignificant ($P > 0.05$) change in BMD of this area.

The alterations in the BMD of total area of osteoporotic rats left femur bones illustrated that ovariectomy resulted in significant ($P < 0.05$) decrease in BMD of total area as compared with gonad intact group (108.8 mg/cm^2 versus 117.2 mg/cm^2, with 7.2%). In contrast, BMD of total area increased significantly ($P < 0.05$) in BM-MSCs and BM-MSCs plus IBS treated groups as compared to osteoporotic group with percent of change 4.9 % and 5.4 %. In addition, the results indicated that only BM-MSCs and BM-MSCs plus IBS treated groups displayed significant ($P < 0.05$) increase in BMD of total area as compared with calcitonin group. On the other hand, the groups injected with ASCs, IBS, calcitonin and calcitonin plus IBS revealed significant ($P < 0.05$) decrease in BMD of total area when compared with BM-MSCs treated group. Besides, all treated groups showed significant ($P < 0.05$) decrease in BMD of total area in comparison with BM-MSCs plus IBS group except the BM-MSCs treated group which displayed insignificant ($P > 0.05$) change in BMD of this area **(Table 4)**.

Table (4): Effect of MSCs, IBS and Calcitonin therapy on BMD in osteoporotic rats

Groups	BMD of proximal area (mg/ cm^2)	BMD of distal area (mg/ cm^2)	BMD of mid area (mg/ cm^2)	BMD of total area (mg/ cm^2)
Gonad intact	111.8 ± 0.98	122.7 ± 2.2	115.2 ± 1.34	117.2 ± 0.87
OVX	102.7 ± 0.83^{a}	106.4 ± 1.78^{a}	103.6 ± 1.06^{a}	108.8 ± 0.93^{a}
OVX + BM-MSCs	109.9 ± 0.5^{bc}	117.9 ± 0.41^{bc}	109. 9 ± 0.91^{bc}	114.1 ± 0.96^{bc}
OVX + ASCs	106.1 ± 0.97^{bde}	111.8 ± 1.48^{bde}	106.9 ± 0.71^{be}	110.7 ± 1.02^{de}
OVX + BM-MSCs + IBS	111.1 ± 0.52^{bc}	120 ± 1.51^{bc}	111.8 ± 1.06^{bc}	114.7 ± 0.78^{bc}
OVX + ASCs + IBS	108.2 ± 0.71^{bce}	113.7 ± 1.61^{bcde}	107.6 ± 1.26^{be}	111.4 ± 0.51^{e}
OVX + IBS	103.6 ± 1.32^{de}	107.1 ± 0.34^{de}	105.3 ± 1.13^{de}	109.5 ± 1.19^{de}
OVX + Calcitonin	104 ± 1.2^{de}	109.1 ± 0.66^{de}	106.4 ± 1.15^{de}	110.2 ± 1.32^{de}
OVX + Calcitonin + IBS	103 ± 1.09^{de}	106.9 ± 1.2^{de}	104.7 ± 1.6^{de}	109.3 ± 1.12^{de}

Data are represented as Mean ±S.E of 10 rats /group. ***a:*** *Significant change at P< 0.05 in comparison with gonad intact group.* ***b:*** *Significant change at P< 0.05 in comparison with the osteoporotic group.* ***c:*** *Significant change at P< 0.05 in comparison with the calcitonin- treated group.* ***d:*** *Significant change at P< 0.05 in comparison with the BM-MSCs - treated group.* ***e****: Significant change at P< 0.05 in comparison with the BM-MSCs + IBS - treated group.*

DISCUSSION

Mesenchymal stem cells have the ability for self-renewal, the potential to differentiate into multilineage cells, and seem to be the one of greatest therapeutic resources for gene therapy, cell therapy and tissue engineering [7]. Several reports indicated that the intrinsic properties of MSCs were altered in osteoporosis and should be relevant for therapeutic use of MSCs [33]. With this concept, the current work goaled to elucidate the potency of MSCs, IBS or both of them versus calcitonin in the betterment of experimental osteoporosis.

Treatment of ovariectomized rats with MSCs evoked positive expression of SRY gene in their femurs. This documented the homing of the intravenously injected MSCs into the site of injury [16]. This character could be allied to the mediators released due to injury and gave migratory cues for intravenously injected stem cells. It has been suggested that these cues promote the amplification of selectins and stimulation of integrins on the surfaces of stem cells, inducing cells to interact with the endothelium. Then, the stem cells adhere and transmigrate through the layer of endothelium into the tissues [34].

Interleukin-7 is a causal molecule of bone loss produced by estrogen deficiency [3]. Our results indicated that ovariectomy increased the level of serum IL-7. This result is in great agreement with that of Weitzmann [35] who demonstrated that ovariectomy induces the formation of IL-7 and the antibody neutralizing IL-7 prevents ovariectomy-induced loss of bone. IL-7 neutralization could enhance bone formation and this suggests that IL-7 is an osteoblast inhibitor and in turn it is considered as bone formation suppressor *in vivo*. In the same context, Tani *et al.* [36] reported that ovariectomy increases the release of IL-7 and the circulating IL-7 level was reduced by estrogen treatment, as estrogen is able to suppress the generation of proinflammatory modulators like, IL-1, IL-6, IL-7 and TNF-α [37]. The amplification of IL-7, as a result of estrogen deficiency, plays a key role in OVX-induced bone loss by uncoupling bone formation from bone resorption. IL-7 achieves this by enhancement of osteoclastogenesis, while at the same time, it limits the magnitude of the compensatory increase in bone formation necessary to compensate the elevated bone resorption and to restore bone homeostasis [3].

Urinary DPD serves in monitoring bone resorption, as the bone remodeling is estimated by biochemical markers for bone turnover. The present results indicated that OVX produces significant increase in urinary DPD levels versus the gonad intact group. Our results are in accordance with the previous observation of Cheng *et al.* [38], who indicated that OVX results in a significant increase in urinary DPD/creatinine ratio. Also, Khosla *et al*. [39] stated that the withdrawal of estrogen due to ovariectomy or menopause leads to marked increase in bone resorption indices.

Bone-type alkaline phosphatase (BALP) is a well-established metabolic marker of osteoblastic bone formation [40,41]. Our data revealed significant decrease in BALP expression in the OVX rats with respect to gonad intact group. These data are in consistence with those of Chang *et al.* [42] who stated that in the OVX group compared with the control group, the expression of the osteogenic marker, BALP, in the femoral tissue is markedly decreased. This supports the concept that following estrogen deficiency, bone resorption outstrips bone formation leading to net bone loss. This is because of estrogen directly affects all types of bone cells leading to suppression of bone remodeling, reduction of bone resorption, and maintenance of bone formation, respectively [39].

The dual energy X-ray absorptiometry data of the present study revealed that ovariectomy leads to a reduction in BMD of rats' femur bones (proximal, distal, mid and total areas). These results agreed with those of Zhang *et al.* [43] who stated that ovariectomy results in the reduction of BMD of rats. Also, Chang *et al.* [42] supported these results as they recorded that DEXA results of the left femur (BMD) in the OVX group are significantly lower than that of the gonad intact control group. Consistent with this finding, Donmez *et al.* [44] reported a reduced bone mass in OVX rats, and they indicated that the increased fracture risk and the alteration in mechanical properties of bone are generally associated with the reduced BMD found in osteoporotic bone. Moreover, ovariectomy stimulates bone remodeling and bone loss in rats which manifests itself with reduced BMD. Therefore, a combined effect of increased bone-remodeling units and a negative remodeling balance is the main base for the quick depletion in bone mass following ovariectomy [45]. Furthermore, Cheng *et al.* [38] indicated that with an ovariectomy, the marked decrease in BMD is due to an increased bone turnover in OVX rats.

The cell therapy results denoted significant betterment in osteoporosis following MSCs infusion. These results are in respect with Jones and Yang [46] reasonable proposal that the implanted MSCs can act as ‘‘seeds’’ or ‘‘signalling centres’’, orchestrating and organizing the recepient response to the injury. This is beside their direct role as a source of new osteoblasts and osteoprogenitors. Also, our data came in line with Ocarino *et al.* [47] who reported that transplantation of differentiated MSCs in osteoporotic rats led to the improvement in the osteoporotic fracture of the treated group versus osteoporotic ones.

The immunosuppressive effect of MSCs could be attributed to the secretion of immunosuppressive soluble mediators which contribute to the inhibition of osteoclastogenesis [48]. These lines of evidences might also contribute to the significant decrease in serum IL-7 and urinary DPD levels showed in the osteoporotic rats following the MSCs infusion. Cytokines have a direct role in the promotion of osteoclastogenesis and may implicate to bone loss accompanied by certain pathological conditions, including inflammation [35]. The anti-inflammatory action of MSCs might explain the benefits of implantation of MSCs in the treatment of osteoporosis as

a growing body of evidences have suggested that inflammation exhibits strong impact on bone turnover in osteoporosis [37, 49].

ALP is a marker of the osteoblast differentiation stage of bone formation [42]. The present study manifested a significant upregulation in BALP gene expression following the MSCs treatment in OVX rats. These results agree with Wan [50] and Aggarwal *et al.* [52] who indicated that in osteoporosis, the differentiated osteoblasts from MSCs is compromised, paralleled by a rise in marrow adipogenesis, resulting in bone loss. These authors added that the infusion of stem cells induces significant recovery of osteoporosis, via elevation of bone formation presumably by osteoblasts paralleled by a reduction in the number of adipocytes and osteoclasts. This hypothesis may explain the upregulation of BALP expression after the infusion of MSCs, as its expression is responsible for the osteoblast phenotype among a wide variety of genes including those encoding osteocalcin, osteopontin, TGF-β, type I collagen and fibronectin [52].

The MSCs succeeded to increase BMD of rats' femur bones (with variable degrees) in the different areas. These findings agree with Ocarino *et al*. [47] who reported that MSCs increased bone mass and trabecular bone percentage. In addition, Jones and Yang [46] indicated that, the beneficial role of implanted MSCs may not be solely restricted to their direct trasformation into osteoblasts. Based on a great number of evidences, it appears that implanted MSCs possess other actions, such as paracrine effects, including anti-apoptotic effects, immunomodulatory function, and induction of host cell migration. Moreover, Tortelli *et al.* [53] and Scotti *et al.* [54] suggest that these ‘‘indirect’’ functions of transplanted MSCs, particularly in attracting host vasculature, play an important role in bone tissue regeneration as their direct role to form new bone.

Our study exemplifies a neoteric strategy utilizing biphasic calcium phosphate composite consisting from HMPC and BCP granules, plus MSCs from different origin for treatment of experimental osteoporosis. The improvements in the osteoporotic rats were associated with a significant decrease in serum IL-7 and urinary DPD levels in concomitant with significant up regulation of BALP gene expression after the infusion of MSCs with IBS. These results agree with Brennan *et al.* [55] who investigated the clinical scenario which mimics how large bone defects shall be regenerated. After eight weeks from implantation of MSCs, mineralized bone containing mature bone marrow territories was formed in ectopic sites. This work mentioned the safety and efficacy of MSCs/BCP combinations for the implementation of MSC therapy for bone regeneration. Unfortunately they reported significant loss of cell viability, as MSCs were mixed with BCP biomaterial prior to subcutaneous implantation and only 1.5 % of the original number of transplanted cells remained after 37 days. However, this limitation completely overcame in our experimental protocol. Also, our results are in accordance with Lobo *et al.* [56] who demonstrated that the genes involved in osteogenesis were over expressed upon seeding of MSCs on bioceramics which suggests that BCPs may have osteoinductive potency. In addition, depending on the physical features and chemical composition of BCPs, they can modulate stem cell behavior.

Extensive studies supported the notion that seeding a threshold amount of stem cells into CaP-based biomaterial is a key determinant for CaP-driven ectopic bone formation [25, 57]. Gauthier *et al.* [58] previously reported that the injectable bone substitute generates some good healings of bone defects in animal model. However, the combination of calcium phosphate with polymers serves to improve the mechanical properties and enhances the osteoconductive properties of the composite [11]. Soon after injection, the polymer phase of the injectable bone substitute is biodegraded and the rest of BCP granules is considered as a scaffold for osteogenesis leading to their incorporation in newly formed bone trabeculae [59].

It was proposed that biomolecules could adsorb growth factors from the body fluids, which in turn would allow the recruitment and accommodation of pluripotent stem cells to form new bone [60]. On the other hand, it was suggested that osteoinductive agents, such as bone morphogenetic protein 2 (BMP-2), are adsorbed on the surface of CaP after implantation, and involved in bone formation and this reflects the intrinsic osteoinductivity of CaP [61]. Moreover, Chang *et al.* [62] cited that CaP and BCP induced osteogenesis because of the presence of Ca ions in these materials. *In vitro*, both CaP and BCP could stimulate osteogenic differentiation of hMSCs, indicating that chemical composition was the essential condition of osteogenesis. Also, Song *et al.* [63] reported that BCP granules with HA/β-TCP in a ratio of 5/1, were successed in adsorbing growth factors from the body fluids, which was represented by imparting an osteoinductivity to the BCP to as certain ectopic bone formation.

The injectable calcium phosphate cements (i-CPCs) are nontoxic, biocompatible and most importantly being integrated into the tissue by the same processes active in remodeling healthy bone [12]. However, the participation of the injectable biphasic calcium phosphate composite alone in osteoporosis improvement was not observed within the IBS group. This could be due to the injection method used in the present work contrasts the direct or local implantation model used in the previous reports [20]. However, our results agreed with other studies reported that

HA–TCP implanted subcutaneously in mice without loading MSC cells resulted in the absence of osteoid formation [64].
No *et al.* [15] reported that although calcium phosphate cement, has some advantages, it also possesses some drawbacks that prevents it from gaining universal acceptance and some researches have focused modulating different injectable materials using nanomaterials to render them suitable for bone tissue regeneration. Also, several studies indicated that the first generation IBS composites, which consists of a water soluble 3% cellulosic polymer (like HPMC), needed some improvements in their properties by grafting silane to HPMC. This Si-HPMC hydrogel have been prepared to make it easy to manipulate *in vitro* and to inject *in vivo* [25].

Although bone marrow-derived MSCs are among the most frequently used types in bone regeneration research studies, several investigator have suggested the use of other sources of MSCs, including the adipose tissue-derived MSCs. However, there are no clear guidelines indicating which sources are the most suitable for bone regeneration [65]. Regarding the source of MSCs, our results addressed variation between BM-derived MSCs and adipose-derived MSCs with or without IBS in management of osteoporosis. These results are in consistence with the comparative study on the differentiation capability between BM-MSCs and adipose tissue-derived MSCs. Hayashi *et al.* [66] clearly demonstrated super osteogenic differentiation capacity of BM-MSCs in comparison with ASCs-derived from the same donor rat. In addition for, the *in vivo* assay, composites of these cells and hydroxyapatite ceramics were subcutaneously implanted into syngeneic rats. Micro-computed tomographic analysis and histological investigation indicated that new bone formation is demonstrated in the composites using BM-MSCs and it was hard to demonstrate in ASCs composites. Also, in the rabbit model, Ye *et al.* [67] suggested that autologous osteogenic-induced ASCs might be useful to alleviate osteoporosis temporally.

Salmon calcitonin (sCT), is used as a therapeutic option for metabolic bone diseases characterized by excessive bone turnover, such as osteoporosis [68]. The results of calcitonin therapy indicated insignificant change in BALP and DEXA versus the osteoporotic group, outlining its inability to reverse the OVX-induced effects on these parameters. Calcitonin is a potent antiresorptive agent, inhibits or reduces bone resorption by modulating both the number and activity of osteoclasts. Besides, calcitonin is able for inducing the rearrangement of osteoclasts causing the supression their motility. Those antiresorptive effects are mediated by its interaction with calcitonin receptors found primarily upon bone-resorbing osteoclasts, with a rapid but short lived action on osteoclast function [68, 69]. Calcitonin performed its osteoclastic activity inhibition without decreasing osteoblastic collagen synthesis [70]. In addition to its ability for preventing osteoblasts and osteocytic cells apoptosis [71]. Also, calcitonin enhances the bone biomechanical properties and improves the process of fracture healing in fractured osteoporotic bone [72].

Previous research studies in postmenopausal women with osteoporosis revealed small increases in BMD with calcitonin [73]. However, our results clarified the potential effects of MSCs over those of calcitonin in management of osteoporosis. This might be due to the short duration of treatment with calcitonin or the therapeutic utility of sCT to treat osteoporosis is severely limited by the short half-life of sCT as a result of rapid systemic clearance and enzymatic degradation. Collectively, those contribute to sCT poor and variable bioavailability [68].

In summary, our results provided clear evidence for the ability of MSCs to reverse ovariectomy-induced alterations in serum IL-7 and urinary DPD levels, BALP gene expression in femur bones as well as BMD. In addition, stem cell therapy was significantly better than calcitonin in management of osteoporosis. Noteworthy, the BM-MSCs with IBS recorded the best results over all the treated groups.

CONCLUSION

In conclusion, the present study highlighted the potent role of MSCs in management of osteoporosis. The favorable effect of MSCs therapy was likely attributable to their direct ability to generate osteoprogenitors and osteoblasts, their paracrine effects and their impact on osteoclastogenesis. Also, the synergism between MSCs and osteoinductive material could be beneficial for considerable anti-osteoporotic effect.

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