POTENTIAL ROLE OF BONE MARROW DERIVED MESENCHYAL STEM CELLS WITH OR WITHOUT INJECTABLE CALCIUM PHOSPHATE COMPOSITE IN MANAGEMENT OF OSTEOPOROSIS IN RAT MODEL

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Received: 06 Jun 2013, Revised and Accepted: 28 Jun 2013

ABSTRACT

Objective: The purpose of the present study was to evaluate the possible therapeutic role of bone marrow mesenchymal stem cells (BM-MSCs) alone or in combination with injectable calcium phosphate composite in management of osteoporosis in ovariectomized rats.

Methods: The MSCs were harvested from femoral bone marrow of male rats, as sex mismatches, to track the MSCs fate and to ensure their homing to the injured femurs’ femurs. The isolated BM-MSCs proved their MSCs identity via their morphological appearance, multilineage potential and the positive expression for CD29, CD44 as well as CD106 and negative expression for CD14, CD34 and CD45. A total number of seventy adult female albino rats were used in the present study. The rats were classified as follows: group 1 was the gonad intact control, group 2 served as untreated ovariectomized (OVX) rats, while the groups from the third to seventh were OVX rats treated with, BM-MSCs, BM-MSCs with injectable bone substitute (IBS), IBS, calcitonin and calcitonin with IBS respectively. Core binding factor alpha-1 (Cbfα-1 or Runx-2) and nuclear factor kappa B (NFκB) gene expression levels in femur bones were detected using real time PCR. Serum osteoprotegerin (OPG) and monocyte chemoattractant protein-1 (MCP-1) were estimated using ELISA technique.

Results: The positive expression of Y-chromosome (sry) gene detected in the BM-MSCs treated groups indicated that the systemically delivered single dose of undifferentiated MSCs was able to home at the females’ femur bones. The expression level of Runx-2 showed down-regulation while that of NF-κB showed up-regulation in femur bones of OVX group. Additionally, serum OPG level was significantly reduced while serum level of MCP-1 was significantly elevated in OVX group as compared to gonad intact control group. The MSCs injection with or without the biphasic calcium phosphate hydroxy-propyl-methyl-cellulose (HPMC) composite produced significant up-regulation of Runx-2 gene expression associated with significant down-regulation of NF-κB gene expression levels in femur bones. Moreover, this type of treatments produced significant increase in serum OPG level associated with significant decrease in serum MCP-1 level when compared with the untreated OVX group.

Conclusion: These results demonstrate the usefulness of MSCs in management of osteoporosis. Additionally, the current study spots light on a novel approach of utilizing injectable biphasic calcium phosphate composite with undifferentiated BM-MSCs as a therapeutic application for osteoporosis.

Keywords: Osteoporosis, Ovariectomized rats, Bone marrow mesenchymal stem cells, Calcium phosphate composite.

INTRODUCTION

Bone tissue constitutes one of the most important units of the locomotor system. For many years it has been in the center of intense clinical and research activity within the musculoskeletal discipline. However, despite its property to heal without scar formation, its regenerative capacity remains limited. Consequently, the repair of bone defects and other bone diseases is a major socioeconomic issue. The World Health Organization has acknowledged this fact by declaring the years 2000–2010 “The Bone and Joint Decade”. Thus, the development of innovative bone-healing strategies is a prerequisite for the successful treatment of a variety of patients suffering from local bone defects [2].

Osteoporosis is a major health burden and the incidence of related fractures is predicted to have doubled by the year 2050. The risk of developing the disease is not only dependent on the rate of bone loss with ageing but also on the peak bone mass (PBM) attained by early adulthood [3]. Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [4]. Osteoporosis incidence is increasing in developed countries, as a result of the increase in the proportion of elderly people in the population, and increased fracture rates within the elderly population, which results from a more sedentary lifestyle [5]. Because bone loss occurs insidiously and is initially asymptomatic, osteoporosis is often only diagnosed after the first clinical fracture has occurred. Consequently, the aim of therapy is usually prevention of further fractures. Early assessment of an individual’s risk of osteoporosis is therefore important to prevent the first fracture [6].

The strength and integrity of the human skeleton depends on a delicate equilibrium between bone resorption by osteoclasts and bone formation by osteoblasts. Accelerated bone resorption by osteoclasts has been established as a principal mechanism in osteoporosis [7]. However, recently, in the pathogenesis of osteoporosis, impairment of both mesenchymal stem cells (MSCs) functionality and microenvironment add to the known detrimental effect of increased osteoclast activity, resulting in decreased bone formation [8].

Given their role in maintaining and replenishing tissues, stem cell therapy undoubtedly holds much promise for the treatment of many diseases. Theoretically, these cells may exert beneficial functional effects by replacing lost or damaged cells, or by protecting threatened host cells via immunomodulatory effects [9]. Mesenchymal stem cells are among the candidates for the regenerative participants in the regenerative of injured adult tissues, as they can potentially be mobilized into the circulation in response to injury signals [10]. Mesenchymal stem cells are multipotent cells that are able to differentiate to osteoblasts, chondrocytes, and adipocytes and can be isolated from bone marrow, adipose tissue, and other mesodermal tissues. This multipotency, especially the ability to differentiate to osteoblasts and chondrocytes, is attractive for tissue engineering of bone and cartilage in various disorders, including osteoporosis [11,12]. A promising alternative to the existing strategies employed in the treatment of musculoskeletal injuries is to reinforce the inherent reparative capacity of the body by delivering MSC harvested from the patient’s own tissues to the site of injury [10].

The main goal of the present study was to evaluate the therapeutic effect of bone marrow derived mesenchymal stem cells (BM-MSCs) with or without injectable calcium phosphate composite in management of osteoporosis in ovariectomized rats as a good model of postmenopausal osteoporosis in women.


**MATERIALS AND METHODS**

**Isolation and preparation of BM-MSCs**

Bone marrow was harvested by flushing the femurs of 6-week-old male albino rats with Dulbecco’s modified Eagle’s medium (DMEM, Gibco/BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco/BRL). Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin–streptomycin (Gibco/BRL). Cells were incubated at 37 °C in 5% humidified CO₂ for 12–14 days as primary culture or upon formation of large colonies.

When large colonies of BM-MSCs developed (80–90% confluence), cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% trypsin in 1mM EDTA (Gibco/BRL) for 5 min at 37 °C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50 cm² culture flask (Falcon). The resulting cultures were referred to as first-passage cultures [13]. To ensure the MSCs identity, the cells were characterized morphologically by inverted microscope examination. Additionally, multilineage potential was confirmed via in vitro differentiation to adipocytes, chondrocytes and osteocytes. Furthermore, PCR detection of CD29, CD44, CD106, CD14, CD34 and CD45 genes expression were done also.

**In vitro differentiation of BM-MSCs**

Bone marrow derived cells were grown until confluence and the growth medium was replaced with the inductive medium consisting of Iscove’s modified Dulbecco’s medium (Invitrogen, USA) 20% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.05 mM β-mercaptoethanol supplemented with specific differentiation reagents as follows:

**a) Adipogenesis assay**

Cells were incubated for 3 weeks with 5 μg/mL insulin (Sigma, USA) and 10⁻⁵ M dexamethasone. Adipogenic differentiation was visualized in phase-contrast microscopy by the presence of highly refractive intracellular lipid vacuoles [14]. Oil Red O (Sigma, USA) staining was used to assay the accumulation of lipid droplets in these vacuoles.

**b) Chondrogenesis assay**

The stem cells were harvested and 6 x 10⁵ cells were centrifuged to form a pellet on the bottom of a 15 mL polystyrene tube (Falcon). The micro mass was cultured in 500 μL of chondrogenic medium that consisted of 50 μg/mL ascorbic acid 2-phosphate and 1 ng/mL TGF-β1 (Sigma, USA) [15]. After 3 weeks of culture, cell clumps were harvested, embedded in paraffin, cut into 3μm sections, and stained for glycosaminoglycans using 0.1% Alcian blue (Sigma, USA).

**c) Osteogenesis assay**

Stem cell cultures were fed twice a week for 3 weeks with 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid 2-phosphate and 10⁻⁶ M dexamethasone [16]. Then cells were fixed with 10% formalin for 20 min at room temperature and mineralization (presence of calcium-rich hydroxyapatite) of the extracellular matrix was assessed by staining for 20 min with 2% wt/vol Alizarin Red S, adjusted to pH 4.1 with ammonium hydroxide [15], all reagents were obtained from Sigma, USA.

**PCR detection of CD14, CD29, CD34, CD44, CD45 and CD106 genes expression**

**RNA extraction from MSCs**

In order to confirm that the isolated cells from bone marrow are mesenchymal stem cells, total RNA was extracted from cultured cells using RNeasy mini kit for purification of total RNA from animal cells (Qiagen, Germany) according to the manufacturer’s instructions.

**Reverse Transcription**

The reverse transcription was carried out on the extracted RNA (1 μg) using the high capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer’s instructions.

**Conventional PCR detection of CD29, CD34 and CD45 genes expression**

The conventional PCR reaction mix for CD29, CD34 and CD45 was 12.5 μL of master mix (Qiagen, Germany), 1 μL of the corresponding forward primer (10 pmol/μL), 1 μL of the corresponding reverse primer (10 pmol/μL) (Invitrogen, USA) Table 1, 5 μL cDNA and 5.5 μL nuclease free water.

**Real time PCR detection of GAPDH, CD14, CD44 and CD106 genes expression**

**Drugs**

Macalc® ampoules, 1ml contains 100 IU of synthetic salmon calcitonin (Novartis Pharma Stein, Switzerland) were used in the present study representing the conventional therapeutic agent for osteoporosis. Calcitonin administered in a dose of 15 IU kg⁻¹ dissolved in distilled water [22].
Biomaterials
Injectable bone substitute (IBS) is a composite biomaterial obtained by associating a polymer and biphasic calcium phosphate (BCP) granules (40% w/v). BCP granules contained 60% hydroxy apatite (HA) (Sigma-Aldrich Co, USA), 40% tricalcium phosphate (β-TCP) (Merck KGaA Co, Germany). The polymer is a cellulose derivative (hydroxy-propyl-methyl cellulose – HMPC) (Winlab Co., UK). An aqueous solution of 3% HPMC was prepared by dissolving raw, dry HPMA powder in bi-distilled water under stirring for 48 h. BCP granules were added under stirring and the biomaterial was prepared in ready-to-use glass flasks sterilized by steam at 121 °C for 20 min [23]. Then the IBS was transferred into 1ml syringe mounted with 18G needle and injected sub mucosal adjacent to the femur surface. Two injections, one for each femur, 64μl each was performed for each rat [24].

Experimental animals
Adult female albino rats weighing 130-150 g were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt, and acclimated for one week in a specific area where temperature (25±1°C) and humidity (35%). Rats were cared for according to the guidelines for Animal Experiments which were approved by the Ethical Committee of Medical Research at National Research Centre, Cairo, Egypt.

Experimental osteoporosis was induced in the adult female rats surgically by ovarioectomy. Under general anaesthesia using diethyl ether, the rats were bilaterally ovarioctomized by dorsal approach [25]. The surgical ovarioectomy operation was performed at Hormones Department, Medical Research Division, National Research Centre. Ovarioctomized (OVX) rat is a suitable experimental model for postmenopausal osteoporosis that faithfully reproduces the changes observed in human subjects and has an additional benefit that the effects are detectable only a few months after intervention [26].

This study included 70 adult female rats, which were divided into seven experimental groups (10 rats/group) as follows after three months from the surgical ovarioectomy: group I (Controls intact group): healthy female rats as negative control group, group II (OVX group): untreated ovarioctomized rats, group III (BM-MSCs group): ovarioctomized rats infused with a single dose of undifferentiated BM derived MSCs (3 x 10^6 cells/rat) intravenously [27,28], group IV (BM-MSCs and IBS group): ovarioctomized rats infused with a single dose of undifferentiated BM derived MSCs (3 x 10^6 cells/rat) intravenously and IBS submucosally, group V (IBS group): ovarioctomized rats injected with IBS submucosally, group VI (Calcitonin group): ovarioctomized rats administered with calcitonin subcutaneously weekly for three months [22] and group VII (Calcitonin and IBS group): ovarioctomized rats injected with IBS submucosally and administered with calcitonin subcutaneously weekly for three months.

At the end of the experimental period, after three months of MSCs injection and calcitonin administration, all animals were fasted for 12 h and the blood samples were withdrawn from retro-orbital venous plexus under diethyl ether anesthesia [29]. The blood samples were left to clot and the sera were separated by cooling centrifugation (4°C) at 1800 xg for 10 min and then stored immediately at -80°C till analysis. While the rats’ femur bones were immediately dissected, cleaned and carefully frozen in liquid nitrogen, then stored at -80°C for the molecular genetics analyses.

PCR detection of male-derived MSCs in femur bones of treated females
The genomic DNA was prepared from femur bones of female rats treated with BM-MSCs using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA). The presence or absence of the sex determination region on the Y chromosome male (sry) gene in the femur bones of recipient female rats was assessed by quantitative PCR. Primer sequences for sry gene (forward 5′-CAT GGA AGG GTT AAA GTC CCA-3′, reverse 5′-ATA GTG TGT AGG TTG TTC-3′) were obtained from the previous published sequences [30] and amplified to a product of 104 bp. The PCR conditions were as follows: incubation at 94 °C for 4 min; 35 cycles of incubation at 94 °C, 50 s/60 °C for 30 s, and 72 °C for 1 min; with a final incubation at 72 °C for 10 min. PCR products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide.

Molecular genetics analyses
Detection of core binding factor alpha-1 (Runx-2) and nuclear factor-κB (NF-κB) gene expression levels using real time PCR
RNA extraction
Total RNA was isolated from femur bones of female rats using SV Total RNA Isolation system (Promega, Madison, WI, USA) according to manufacturer’s instruction. The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically, then the concentration of the RNA was assayed using the OD 260/280 ratio, and only samples with ratios above 1.5 were used in the experiments.

cDNA synthesis
First-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, Netherlands) by mixing 2 μg total RNA with 0.5 μg of oligo (dT) 12-18 primer in a total volume of 12μL. After heating the mixture at 70 °C for 10 min, a solution containing 50 mmol/L Tris HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 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 42 °C for 1 h.

Real-time quantitative polymerase chain reaction (PCR)
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, Foster City, CA, USA) and 200 ng of each primer, (shown in Table 2). PCR reactions consisting of 95 °C for 10 min (1 cycle), 94 °C for 15 s, and 60 °C 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 version 1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the beta actin genes [31].

Table 2: Primers sequences of the studied genes used for real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>Cbfa-1 or (Runx-2)</td>
<td>Forward: 5′-GGCACGCCTCACTGAGATATTTA-3′ Reverse: 5′-TGACACGGGTGTCATCAGTCT-3′, according to the published sequences [32].</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Forward: 5′-AAGATGCTTTGCCCTCCATC-3′ Reverse: 5′-TGCTCTCTTCGCCAGAGGA-3′, according to the published sequences [33].</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5′-TCTGGACCCACACTTCTACATG-3′ Reverse: 5′-ACGACAGCTGGAATAGAACG-3′, according to the published sequences [34].</td>
</tr>
</tbody>
</table>

Biochemical analyses
Serum osteoprotegerin (OPG) and monocyte chemoattractant protein-1 (MCP-1) levels were assayed by ELISA technique using kits purchased from Glory Science Co., USA, according to the manufacturer’s instructions.

Statistical analyses
In the present study, all results 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
RESULTS

Stem cells morphology

The photomicrographs in Fig. 1 show the shape of the BM-MSCs at the first day of isolation and culture (Fig. 1a) and the spindle shape of cells which is the typical morphological aspects of mesenchymal stem cells derived from bone marrow through culture flask at day 10 (Fig. 1b) and day 14 (Fig. 1c).

In vitro differentiation of BM-MSCs

Adipogenic differentiation

Bone marrow stem cells are differentiated into adipocytes through 1 week of culture (Fig. 2b) and continued to increase in cells number through 2 weeks of culture (Fig. 2c). Adipocytes are stained with special Oil Red O stain (Fig. 2d).

Chondrogenic differentiation

Bone marrow derived stem cells are differentiated through 1 week into chondrocytes (Fig. 3b), as indicated by their round configuration and the development of an extracellular matrix that stained with special Alchian blue stain (Figs. 3c and 3d).

Osteogenic differentiation

Bone marrow derived stem cells are differentiated into osteocytes through 1 week of culture (Fig. 4b). The osteocytes are stained with special Alizarin Red S stain (Fig. 4c and 4d).

Cell surface markers for BM-MSCs

The agarose gel electrophoresis shows that BM-MSCs used in the present study are positive for CD29, a cell surface marker for MSCs as the positive band appears at 261 bp (Fig. 5), and negative for CD45 as well as CD34 which are cell surface markers associated with hematopoietic precursor cells and endothelial cells (Figs. 5 and 6 respectively).

Fig. 1: Morphological aspects of BM-MSCs in culture flask.

(a)  
(b)  
(c)  

Fig. 2: Differentiation of BM-MSCs into adipocytes. (a): Undifferentiated BM-MSCs at 2 weeks in culture; (b): BM-MSCs at 1 week of differentiation into adipocytes; (c): BM-MSCs at 2 weeks of differentiation into adipocytes; (d): Differentiated BM-MSCs into adipocytes stained with special Oil Red O stain.

(a)  
(b)  
(c)  

Fig. 3: Differentiation of BM-MSCs into chondrocytes. (a): Undifferentiated BM-MSCs at 2 weeks in culture; (b): BM-MSCs at 1 week of differentiation into chondrocytes; (c and d): Differentiated BM-MSCs into chondrocytes stained with special Alchian blue stain.

(a)  
(b)  
(c)  

Fig. 4: Differentiation of BM-MSCs into osteocytes. (a): Undifferentiated BM-MSCs at 2 weeks in culture; (b): BM-MSCs at 1 week of differentiation into osteocytes; (c and d): Differentiated BM-MSCs into osteocytes stained with special Alizarin Red S stain.
Fig. 5: Agarose gel electrophoresis for CD29 and CD45 genes expression for BM-MSCs sample. Lane (1) represented DNA ladder, lane (2) represented CD29 gene expression and lane (3) represented CD45 gene expression.

Fig. 6: Agarose gel electrophoresis for CD34 gene expression for BM-MSCs sample. Lane (1) represented DNA ladder, lane (2) represented CD34 gene expression.

Moreover, the amplification plots of the real time PCR for CD14, CD44 and CD106 show that the used BM-MSCs are negative for CD14 gene expression, cell-surface marker associated with hematopoietic precursor cells and positive for CD44 as well as CD106 genes expression, cell-surface markers for MSCs (Figs. 7 and 8 respectively). It is obvious that in Fig. (8), the expression of CD44 exceeds the threshold which is equal 1.733. While, the expression of CD106 exceeds the threshold which is equal 2.313.

Fig. 7: The real time PCR amplification plot for CD14 gene for the BM-MSCs sample.
Mesenchymal stem cells homing (PCR detection of male-derived MSCs)

The bones of femur from untreated OVX rats show negative expression of sry gene as shown in the agarose gel electrophoresis from DNA fragments. While, those represent the bones of femur of OVX rats treated with BM-MSCs showed positive expression of sry gene (Fig. 9). These results confirm the homing of the male donor stem cells to the injured femur bones of the female recipients.

Molecular genetics analyses

The alteration in Runx-2 and NF-κβ gene expression levels in femur bones of osteoporotic rats following MSCs injection with or without IBS as well as calcitonin administration are shown in Table (3). The data illustrate that the Runx-2 expression level decreased significantly in osteoporotic group in comparison with gonad intact control group. In contrast, Runx-2 expression increased significantly in all treated groups as compared to the osteoporotic group.

Table 3: Effect of BM-MSCs with or without IBS and calcitonin therapy on Runx-2 and NF-κβ expression levels in femur bones of OVX rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Runx-2 Relative expression</th>
<th>NF-κβ Relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad intact control</td>
<td>1.19 ± 0.06</td>
<td>1.54 ± 0.07</td>
</tr>
<tr>
<td>OVX</td>
<td>0.56 ± 0.02 ( ^a )</td>
<td>3.24 ± 0.13 ( ^a )</td>
</tr>
<tr>
<td>OVX + BM-MSCs</td>
<td>0.81 ± 0.03 ( ^b,^c )</td>
<td>1.99 ± 0.11 ( ^b,^c )</td>
</tr>
<tr>
<td>OVX + BM-MSCs + IBS</td>
<td>0.94 ± 0.02 ( ^b,^d )</td>
<td>1.89 ± 0.11 ( ^b,^c )</td>
</tr>
<tr>
<td>OVX + IBS</td>
<td>0.69 ± 0.03 ( ^b,^d )</td>
<td>2.7 ± 0.08 ( ^b,^d )</td>
</tr>
<tr>
<td>OVX + Calcitonin</td>
<td>0.71 ± 0.02 ( ^b,^d )</td>
<td>2.54 ± 0.10 ( ^b,^d )</td>
</tr>
<tr>
<td>OVX + Calcitonin + IBS</td>
<td>0.66 ± 0.01 ( ^b,^d )</td>
<td>2.82 ± 0.08 ( ^b,^d )</td>
</tr>
</tbody>
</table>

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.
Biochemical analyses

The data in Table (4) represent the effect of BM-MSCs with or without IBS on serum OPG level as compared to calcitonin-treated group (Table 4).

Regarding the serum level of MCP-1, the osteoporotic group showed significant increase in serum MCP-1 level when compared to gonad intact control group. However, the all treated groups produced significant decrease in serum MCP-1 level when compared to the osteoporotic group (Table 4). In rats received BM-MSCs and BM-MSCs with IBS, the decreased serum MCP-1 level was significant compared to that of calcitonin-treated group (Table 4).

Table 4: Effect of BM-MSCs with or without IBS and calcitonin therapy on serum OPG and MCP-1 levels in OVX rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>OPG (pg/ml)</th>
<th>MCP-1 (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>Gonad intact control</td>
<td>13.89 ± 0.31</td>
<td>69.84 ± 1.56</td>
</tr>
<tr>
<td>OVX</td>
<td>6.9 ± 0.26</td>
<td>110.52 ± 2.74</td>
</tr>
<tr>
<td>OVX + BM-MSCs</td>
<td>10.05 ± 0.48</td>
<td>83.69 ± 1.49</td>
</tr>
<tr>
<td>OVX + BM-MSCs + IBS</td>
<td>12.64 ± 0.57</td>
<td>79.49 ± 2.02</td>
</tr>
<tr>
<td>OVX + IBS</td>
<td>7.55 ± 0.32</td>
<td>93.63 ± 0.82</td>
</tr>
<tr>
<td>OVX + Calciton</td>
<td>8.17 ± 0.32</td>
<td>92.77 ± 0.83</td>
</tr>
<tr>
<td>OVX + Calciton + IBS</td>
<td>7.32 ± 0.24</td>
<td>94.17 ± 1.24</td>
</tr>
</tbody>
</table>

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.

DISCUSSION

Mesenchymal stem cells have been shown to possess the capability of multilineage differentiation in vitro [11,12]. Yet, in vivo, how they exercise auto specific function is not fully understood [36]. It has been believed that injury trigger the mobilization of MSC into the circulation and these cells contribute to healing by homing to the damaged tissues. Such a response is thought to be an inherent one that can potentially be augmented by enhancing the endogenous MSC pool with exogenously administered MSC [10]. Based on these findings, the present study used the sex mismatch model and postulated that the males MSCs may be able to migrate and homed to the females’ femurs and manage ovariectomy induced osteoporosis in these adult female rats.

The confirmatory results for BM-MSCs properties indicated their adhesiveness and fusiform shape in culture flask which is in agreement with that of Rochefort et al. [37]. Also, isolated BM-MSCs showed the ability to differentiate into adipocytes, chondrocytes and osteocytes as defined by Pittenger et al. [38]. Moreover, the PCR results demonstrated that the isolated BM-MSCs were positive for CD29, CD44 as well as CD106 and negative for CD14, CD34 and CD45 and these findings are in accordance with those of Pittenger et al. [38] and Kumar et al. [39]. Meanwhile, the positive expression of sry gene in the femurs of the ovariectomized female rats treated with BM-MSCs confirmed the migration capacity of the intravenously infused BM-MSCs to the site of injury. This property could be attributed to the chemokines, cytokines, and growth factors released upon injury and provided migratory cues for systemically or locally administered stem cells. The cues induce up-regulation of selectins and activation of integrins on the stem cell surface, enabling cells to interact with the endothelium. Stem cells subsequently adhere and transmigrate across the endothelial layer into tissues [40]. Several migration factors, such as stromal cell-derived factor-1α (SDF-1α), receptor activator of NF-κB ligand (RANKL), platelet-derived growth factor (PDGF), interleukin-17 (IL-17), interferon gamma (INF-γ), insulin growth factor (IGF), transforming growth factor beta (TGF-β) and epidermal growth factor (EGF), are involved during the process of MSC migration and incorporation into the microenvironment of the damaged tissue or inflammation site. To respond to these factors, the presence of specific receptors expressed by MSCs is essential. These receptors include CCR4, RANK, IL-17 receptor, INF-γ receptor, IGF receptor, TGF-β receptor and EGF receptor, among others [41].

To date, Runx-2 (Cbfa1) remains the earliest of the known transcriptional regulators that serve critical roles in osteoblast differentiation and bone formation [42]. Recent investigations indicated that Runx-2 play a direct role in bone formation among the leader genes involved in the osteogenesis process [43]. The present study revealed that ovariectomy induced significant decrease in Runx-2 expression compared with the gonad intact control. This agrees with that of Cardenil et al. [44] who reported that ovariectomy affects not only the serum levels of bone remodelling-related proteins but also gene expression at the skeletal sites. Also, our result is in parallel with that of Dalle Carbonare et al. [45] who found down-regulation of Runx-2 in patients with osteoporosis and thus they support the hypothesis that in osteoporosis there is increased recruitment of osteoblastic precursors as a result of increased bone turnover [46], but these cells are not able to differentiate into mature osteoblasts because of an altered gene expression profile and this defect can contribute to the pathogenesis of osteoporosis. In the same context Benisch et al. [47] also indicated that the intrinsic alterations in stem cell biology are involved in the pathophysiology of osteoporosis. In addition this study revealed that disturbed bone homeostasis by inhibition of osteogenic regeneration is at least an equally important feature of osteoporosis besides enhanced bone resorption. In contrary, our result disagrees with that of Zhang et al. [48] who reported no difference in this key osteogenic factor between the ovariectomized and non ovariectomized rats. Besides, they suggested that the cellular response to ovariectomy, and the associated estrogen deficiency, is largely dependent on the bone type.

Concerning the regulatory network between Runx2 and the estrogen pathway that can contribute to skeletal development and bone homeostasis, Jeong and Choi [49] reported that aromatase, a key enzyme for estrogen synthesis from androgen, is a Runx-2 downstream target gene. In addition, local production of estrogen in bone may in part be due to the regulation of Runx-2 mediated aromatase gene expression. The estrogen production was elevated by forced expression of Runx-2 in several osteoblastic cells. In contrast, estrogen production was decreased in bone marrow stromal cells derived from Runx-2 heterozygous mice. Also aromatase expression in the perichondrial and periosteal area was drastically decreased in Runx-2 null mice. Therefore, aromatase gene expression was well correlated with the level of Runx-2 expression in osteoblastic cells [50].

The results of the different work concerning estrogen and its interaction with the OPG/RANKL pathway are not conflicting because estrogen has been shown to stimulate OPG secretion and also to downregulate the expression of RANKL. In addition the in vitro studies of human osteoblast revealed that estrogen induces OPG production.
Hence, in postmenopausal females as the production of estrogen is reduced, subsequently the production of OPG is hindered and postmenopausal osteoporosis sets in. This greatly supports our results that indicated significant decrease in serum OPG levels in the osteoporotic group compared with the intact control. Dalle Carbonare et al. [45] reported similar results, indicating a lower OPG levels in osteoporotic patients in respect to the normal.

The OPG/RANKL ratio is considered to better reflect the bone remodeling environment signs. A high ratio represents bone formation while a low ratio favors bone resorption [52]. Evidence that OPG acts as an inhibitor of osteoclastogenesis has emerged from experiments with transgenic mice, in which over expression of OPG led to severe osteoporosis and reduced the number of mature osteoclasts. In contrast, mice lacking the gene for OPG are osteoporotic [45]. The biological effects of OPG include inhibition of the terminal stages of osteoclast differentiation and suppression of mature osteoclast activation [53] by blocking the RANKL/RANK interaction which activates the osteoclast formation [54]. So as the competitive inhibition by OPG is stalled, the production of RANKL is increased which leads to the formation of RANKL-RANK complex, as part of the counterbalancing phenomenon. Finally this leads to greater bone resorption and decrease in bone mineral density [52]. Studies performed on ovariectomized rats' models lend credibility to this theory. As ovariectomy is known to disturb the balance between bone formation and bone resorption, in addition the serum biomarkers have been comparatively analyzed as indications of osteoporosis [44]. More in detail Ominsky et al. [55] have reported that ovariectomy in rats was associated with high levels of serum RANKL and also showed that OPG reduced osteoclast surface and prevented ovariectomy-associated bone loss in femur.

NF-κB signaling plays essential roles in certain aspects of osteoclast, osteoblast activities and activated in a number of pathologic conditions affecting the skeleton, including postmenopausal osteoporosis [56]. Our results indicated a significant increase in NF-κB expression in ovariectomized rats compared with control group. This finding comes in line with previously reported data that the local expression levels of NF-κB increased significantly in a time dependent manner in the bone tissue of osteoporotic rats, and it could play a role in the pathogenesis of osteoporosis [33]. Also it agrees with Chang et al. [57] who showed an increase in NF-κB activity in ovariectomized and evidences that estrogen regulates NF-κB in osteoblasts in vivo. The activation of NF-κB due to estrogen deficiency not only promotes osteoclast activation, osteoclast survival and bone resorption, but simultaneously inhibits osteoblast function [58]. Historically, NF-κB has not been considered as a key mediator of osteoblast signaling, but several studies have shown that NF-κB inhibits both osteoblast differentiation and activity. Yamazaki et al. [59] demonstrated that NF-κB can interact with the Smad1-Smad5 complex in the nucleus and disrupt its binding to target promoters. NF-κB blocks the induction of Runx-2 by SMAD, resulting in the inhibition of osteoblast differentiation [60].

Our results indicated significant increase in MCP-1 level in ovariectomized female rats in the respect to that of the control. Similar results have been reported by Abu-Taha et al. [61] in studying the MCP-1 levels in the rat model of estrogen depletion by ovariectomy. Also Aggarwal et al. [62] data revealed a marked increase in MCP-1 levels in the osteoporotic mice while MCP-1 level plummeted after the stem cell transplantation in osteoporotic mice and this came parallel with the improvements in bone.

The major role of MCP-1 in bone is to facilitate bone resorption by recruiting osteoclasts and their precursors to the remodeling site to initiate the process of bone remodeling. At the same time, MCP-1 facilitates osteoclastogenesis by facilitating the fusion of the pre-osteoclast lasting immature osteoclasts through a paracrine mode [63, 64]. The expression of MCP-1 (CCL2) is temporally and spatially associated with the recruitment of monocytes in both oosseous inflammation and during developmentally regulated bone remodeling [63]. Both CCL2 and its receptor CCR2 (G protein-coupled receptor) have been demonstrated to be induced and involved in various diseases [65] including the mediation of bone loss in the absence of estrogen [66].

Our cell therapy data indicated significant improvement in osteoporosis following the MSCs infusion. This finding agrees with Ocario et al. [67] who indicated that intra-bone injection of differentiated MSCs into femurs of osteoporotic females rats results in improvement in the treated group as compared to untreated group. After 2 months, the femurs of treated rats showed trabecular bone percentage almost similar to the femurs from control healthy rats. In contrast Gasser [68] reported the failure of MSCs to home in the bone tissue in osteoporosis models, as the local environment does not provide the required stimulus to the MSCs for their differentiation down the osteoblast lineage.

The present study showed significant increase in Runx-2 expression with the MSCs treatment. Runx-2 revealed the existence of osteoblast progenitors [69]. That Runt domain-containing transcription factor, Runx-2, promotes the osteoblastic differentiation of mesenchymal stem cells [70]. As indicated by Benisch et al. [47], osteoporosis causes distinct transcriptional changes, which differ from age-related changes in the non-osteoporotic donors. This indicates the intrinsic deficiency in self-renewal and differentiation potential in osteoporotic stem cells. This point of view may explain the up-regulation of Runx-2 expression after the injection of MSCs as they could participate partially in the supporting and replenishing of endogenous altered osteoporotic stem cell. Runx-2 activates and regulates osteogenic differentiation by two independent signaling pathways via transforming growth factor-beta1 (TGF-β1) and RANK/RANKL pathway in bone remodeling [71]. Consistently Sowa et al. [72] reported a close interaction between Runx-2 and BMP2 to induce osteoblast specific gene expression in mesenchymal stem cells. After the commitment to osteoblast lineage, Runx-2 maintains the osteoblasts in an immature stage, during which immature bone forms, and triggers the expression of major bone matrix protein genes [73].

In addition to the induction of osteogenic differentiation, Runx-2 inhibits the differentiation of MSCs towards the adipogenic lineage [71]. In osteoporosis, osteoblast differentiation from MSCs is compromised, associated with increased marrow adipogenesis, leading to bone loss [74]. In consistent Aggarwal et al. [62] found that the loss of trabecular bones spicules and loss of cortical bones in the osteoporotic mice accompanied by concurrent increment of adipocytes. In addition the application of stem cells induced significant regression of osteoporosis, by increment of bone formation presumably by osteoblasts and concomitant decrease in number of adipocytes and osteoclasts.

Our observation regarding the altered level of OPG following the injection of MSC in the osteoporotic rats may be explained by the direct influence of mesenchymal stromal cells in bone homeostasis, both by the rate at which they create osteoblasts and by the production of factors affecting the osteoblast/osteocyte balance such as RANKL and OPG. As this bone homeostasis is lost, under certain conditions, leading to reduced bone strength and/or poor bone healing. Indeed, a loss of functional MSC may be the key factor behind these conditions, which may be amenable to cellular therapy with MSC [75]. Also reporter assays indicated that MSCs influence host-derived osteoclastogenesis via the production of OPG which increased the OPG/RANKL ratio and thus decreased osteoclast differentiation and activation [76].

The role of RANKL in osteoclastogenesis has been established as accumulated evidences have suggested that osteoclast differentiation is principally stimulated by an increase in the ratio of RANKL to OPG in bone [77, 78]. RANKL mediated NF-κB activation via both the classic and alternative pathways leading to increased osteoclast differentiation and survival and decreased osteoblast maturation and function [60]. Also, the expression of MCP-1 at elevated levels of RANKL and regulated by NF-κB and TGF-β1. Taken together, these findings support the hypothesis that MSCs could affect osteosteatogenesis via the RANKL/ RANKL/ OPG signal transduction pathway that regulates osteoclast formation. In addition, given the known immunosuppressive effect of MSCs, it is possible that immunosuppressive soluble mediators contribute to inhibition of osteostealogenesis [71]. Moreover, MSCs are known for their strong anti-inflammatory functions, as they often transplanted into inflammatory environments where they able to
survive and modulate host immune responses [79]. Consequently, this intriguing property might explain the useful therapeutic application of MSCs in osteoporosis as various lines of evidence have indicated that inflammation exerts significant influence on bone turnover in osteoporosis [80].

Our study indicated a novel approach utilizing injectable biphasic calcium phosphate composite constituted of HMP and BCP granules, with undifferentiated BM-MSCs as a therapeutic application of osteoporosis. The improvement in the osteoporotic rats comes parallel with a significant up-regulation of Runx-2 gene expression, while a significant down-regulation of NF-kB gene expression. In addition to significant increase in serum OPG level associated with significant decrease in serum MGP-1 towards the normal values after the infusion of MSGs and IBS. These results are in accordance with the principal that bone formation is an intricate and ordered cascade reaction between relevant stem cells and biomaterials in a continuously renewed internal environment, the procedure of which is regulated by growth factors [81].

In cell based approaches, several reports demonstrated that calcium phosphate bioencasmes combined with MSCs [82] and/or growth factor [83] could induce ectopic bone formation. In the same context, Trojani et al. [24] showed that injectable biphasic calcium phosphate with than grimated HPMc (Si-HPMC) hydrogel composite, loaded with undifferentiated MSCs has osteoinductive properties in ectopic sites. These evidences greatly supported with the recent study of Chai et al. [76] who indicated that stem cell-based strategies for bone regeneration, which use calcium phosphate (CaP)-based biomaterials in combination with developmentally relevant progenitor populations, have significant potential for clinical repair of skeletal defects. As seeding a threshold amount of stem cells into CaP-based biomaterial is a key determinant for CaP-driven ectopic bone formation. Regarding the mechanism of osteoinduction by biomaterials, it was hypothesized that biomaterials in vivo could adsorb endogenous growth factors from the body fluids, which in turn would facilitate the recruitment and homing of relevant pluripotent stem cells to form new bone [84]. However, there is no evidence to prove this mechanism. Another proposed mechanism suggests that osteoinductive biomolecules, such as bone morphogenetic protein 2 (BMP-2), are adsorbed on the surface of CaP after implantation, and then these adsorbed biomolecules involve in bone formation, which appears as osteoinduction that reflects the intrinsic osteoinductivity of CaP [85]. Moreover Cheng et al. [86] reported that both CaP and BCP powders induce osteogenic because of Ca ions existed in these materials. Meanwhile, in vivo, both CaP and BCP powders could induce osteogenic differentiation of hMSCs, suggesting that chemical composition was the necessary condition of osteogenesis. Also recently, Song et al. [36] suggested that the BCP granules with a HA/P-TCP ratio of 5/1, are likely successful in adsorbing endogenous growth factors from the body fluids in vivo, which is manifested by imparting an osteoinductivity to the BCP to achieve ectopic bone formation. Thus, the ability of such implants to recruit distant MSCs and induce osteogenic differentiation and bone formation is promising for applications in regenerative medicine.

A lot of attention has been paid to injectable calcium phosphate cements (i-CPCs) in bone augmentation due to the high reactivity of these materials, since the reaction products lead to crystallization into an apatite-like phase, similar to the inorganic component of bones in vertebrate species. In addition, the CPC being integrated into the tissue by the same processes active in remodeling healthy bone [87]. Our results for the IBS group indicated the inability of this composite alone to contribute to osteoporosis improvement. This result agrees with those of Hickok et al. [88] who observed osteoid formation in 80% of HA-TCP implants loaded with MSC cells implanted subcutaneously in mice, while in the control HA-TCP implanted without cells, the osteoid formation was not observed. The differences in the experimental protocols could be attributed to the shortage effect of IBS in management of osteoporosis. As the injection model used in the present study contrast the direct implantation model used in various reports. However, these studies demonstrated a positive effect on using the biomaterials for local treatment of bone diseases, but they require more invasive surgical interventions. Boulin et al. [23] reported that HMPC /BCP composite implanted in osteoporotic rats induces bone formation. This IBS was injected in the holes drilled in the femurs cortex and X-ray examination ensures the corrected implantation of the biomaterials. Soon after implantation, disappearance of the polymer is followed by bone inlantation of the bone site by cells and remodeling leading to the deposit of woven bone around BCP granules. Also, extensive studies indicated that the first generation IBS composites, which consists of a water soluble 3% cellulose polymer (like HPMC), needed the improvement of its properties by grafting silane to HPMC. This Si-HPMC hydrogel have been set up to make it easy to manipulate in vitro and to inject in vivo [24].

Concerning the effect of calcitin in treatment on osteoporosis, calcitonin antiresorptive activity is particularly mediated through an inhibition of osteoclastic bone resorption [89]. Calcitonin reduces osteoclast acid secretion [90] and inhibits bone resorption by binding to receptors on osteoclasts, which induces flating of their ruffled borders and their detachment from bone [91]. Calcitonin may also prevent osteolast and osteocyte apoptosis [92]. However, the therapeutic utility of sCT to treat those conditions with current formulations of commercially available sCT is severely restricted by the short half-life of sCT (16.9–57.3 min) due to rapid systemic clearance and enzymatic degdation via proteolysis in kidneys, liver and blood. These contribute to the poor and variable bioavailability of sCT [93].

In conclusion, the present results clearly demonstrated the importance of MSCs in management of osteoporosis. In addition the combination of MSGs implantation with osteoinductive material such as the injectable calcium phosphate composite may be useful to achieve the significant therapeutic effects.

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