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Adipose Derived Stem Cells Conditioned Media in Combination with Bioceramic-Collagen Scaffolds Improved Calvarial Bone Healing in Hypothyroid Rats

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Abstract

Background: Tissue engineering is an available treatment for large bone defects. The therapeutic effects of mesenchymal stem cell (MSC) are mostly attributed to secretion of many cytokines and growth factors. Many factors of MSC secretions accumulate in a conditioned medium and these factors recruit native cells into a defect site to generate new bone tissues.
Objectives: The aim of this study was to evaluate the influence of adipose tissue derived MSCs-conditioned medium (ADMSC-CM) on bone repair of rats with critical - size calvarial defect.
Methods: This experimental study was performed at Shahid Beheshti University of Medical Sciences, Tehran, Iran, from 2016 to 2017. Conditioned medium was collected from healthy rat adipose tissue derived MSC (ADMSC) at passage four. Calvarial bone defect was created in hypothyroid rats using a dental bur. Sampling was taken by the linear-mono-gram method to determine sample size (n = 6 per group). The rats were divided randomly into four groups based on graft material as follows: empty defect, scaffold (Bio-Oss / type I collagen gel), scaffold / ADMSCs, scaffold /ADMSC- CM. Evaluations were made at 4 and 8 weeks after surgery using stereological analysis.
Results: Histological analysis at 8 weeks indicated that the newly regenerated tissue almost covered the defect in the ADMSC-CM group. Stereological analysis showed that ADMSC-CM increased regenerated bone and numbers of osteocytes and osteoblasts compared with the defect and scaffold groups ($P < 0.05$). Also, bone regeneration was more effective in animals treated with ADMSC-CM than in those received ADMSC.
Conclusions: These results suggest an important role for ADMSC-CM in bone regeneration, through trophic impact of its cytokines and growth factors that induce native cell proliferation and migration into the defect. Thus, ADMSC-CM seems to have good potential for application in bone tissue regeneration, in the cases of hypothyroidism.

Keywords: Hypothyroidism, Mesenchymal Stem Cell, Conditioned Media, Bone Regeneration, Histology, Rats

1. Background

Thyroid hormones play an essential role in bone growth, development and turnover processes by regulating bone formation and resorption (1). In hypothyroidism, there is reduced bone turnover in trabecular and cortical bones and an increase in bone resorption that can lead to a negative calcium balance and result in bone loss (2-4). In children, hypothyroidism causes delayed skeletal maturation and growth arrest (5). In addition, treatment with thyroxine during childhood causes accelerated skeletal maturation and premature epiphyseal growth plate fusion and causes short stature (6). Some studies have shown that osteogenesis in the defect area is reduced in models with hypothyroidism (7, 8). This can be attributed to inhibited endochondral ossification in hypothyroidism (9). In general, it is expected that thyroid hormone deficiency leads to abnormal bone regeneration (10). Hypothyroidism can occur at all stages of life and is caused by number of factors including autoimmune disease, thyroid surgery, radiation therapy, medication and treatment for hyperthyroidism (2). Accordingly, introduction of a method to treat bone problems caused by hypothyroidism would be very useful. There are several clinical methods for reconstruction of bone defects caused by trauma, congenital deformity and tumor resection. The method of autogenous bone grafting is often considered as the "gold standard" for bone re-

pair, because of strong osteogenic characteristics and does not incur complications from rejection (11). However, autografting is associated with pain and morbidity at the donor site (12). Allografts and xenografts have serious limitations related to immunological reactions and infection (13, 14). Tissue engineering is an interesting and practical method for bone tissue regeneration (15). Mesenchymal stem cells (MSC), particularly that originated from bone marrow, are considered a suitable source for bone tissue engineering (16). However, MSC from adipose tissue (ADMSC) is a better choice for clinical application, because of minimally invasive procedure for fat harvesting, abundance and ease of MSC isolation and expansion in vitro (17). ADMSC is a multipotent cell that can differentiate into adipogenic, osteogenic, chondrogenic, myogenic, neurogenic and endothelial cells (18, 19). Treatment of calvarial defects with ADMSC transplantation has been demonstrated in the literature (20-22). Daei-farshbaf et al. reported that ADMSC seeded on a bioceramic scaffold promoted calvarial bone healing in hypothyroid rats (23). However, the use of stem cells in tissue engineering for bone regeneration had some problems such as expensive cell culture, complicated cell handling and invasive procedure for cell collection from patients (24). On the other hand, MSC secretes a wide range of bioactive molecules such as growth factors and cytokines that may repair and replace defective tissues and cells (25, 26). These factors accumulate in what is known as a conditioned medium (CM). MSC- CM improves angiogenesis and tissue regeneration and inhibits fibrosis, apoptosis and inflammation (27, 28). Since survival, differentiation and fate of transplanted MSC remains undetermined, it is suggested that paracrine effects of MSC secretion are the primary mechanism for tissue regeneration (29). Recent studies have demonstrated the effect of ADMSC-CM on tissue regeneration (17, 30, 31). Research has shown that MSC-CM contains large amounts of cytokines that are involved in bone regeneration such as insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and transforming growth factor- β1 (TGF-β1) (32). The IGF family is responsible for tooth development and growth and its expression is increased during osteogenic differentiation (33). VEGF is a known critical factor in development and regeneration of vascular tissue. Vascularization is very important in the healing process of bone fracture and it has been suggested for clinical application in nonunion bone fracture to accelerate the healing process (34). The TGF-β family has a critical role in expression of collagen as one of the most important proteins in the bone extra cellular matrix (ECM) (35, 36). Previous studies have reported that growth factors and cytokines secreted from MSC have high potential for bone regeneration. Osugi et al. demonstrated that conditioned medium derived from MSC could promote bone reconstruction by a cooperative effect between IGF-1 and VEGF that influenced osteogenesis and angiogenesis (24). Inukai et al. showed a positive effect of multiple cytokines contained in MSC-CM on alveolar bone and cementum regeneration (32). Also, Chang et al. reported that paracrine factors secreted from MSC under hypoxic condition could enhance healing of the calvarial defect in rats through increased endogenous stem cell migration via regulation of ICAM-1 (intercellular adhesion molecule-1) targeted microRNA-221 (37). However, most previous in vivo studies were performed on normal animal models and no investigation has been done to date using ADMSC-CM for bone healing in a hypothyroid animal model.

2. Objectives

Based on the role of CM bioactive molecules (such as IGF, VEGF and TGF-β) in osteogenesis and healing processes, it was hypothesized that application of ADMSC-CM could improve bone regeneration in cases of calvarial defect. Since hypothyroidism causes many problems in fracture healing, the effect of ADMSC-CM was evaluated on bone regeneration in a hypothyroid rat model. Bio-Oss and collagen type I was used as a mechanical substrate for CM, because one of the important items in bone grafting surgery is filling the cavity space.

3. Methods

3.1. Adipose Tissue Mesenchymal Stem Cell Extraction and Preparation of Conditioned Media

This experimental study was performed at Shahid Beheshti University of Medical Sciences, Tehran, Iran, from 2016 to 2017. Adipose tissue was harvested from testicular fat pads of healthy rats. The tissue was cut up into small pieces and ADMSCs were extracted by enzymatic digestion using collagenase type I (Sigma-Aldrich) solution, shaken for 30 minutes at 37°C. The digested tissue was centrifuged (Eppendorf 5702, Germany) and the cell pellet was collected. The pellet was then cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 mg/mL streptomycin and 100 U/mL penicillin (1% antibiotic, Gibco). At passage 4, when the ADMSCs had reached 70% - 80% confluence, the medium was replaced by serum free DMEM and incubated for 48 h. The conditioned media (ADMSC-CM) was then collected and concentrated 20-fold by filtration with 3-kD molecular cutoff filters (Amicon Ultra, Millipore, USA).

3.2. Characterization of Isolated ADMSC

Evaluation was made for the potential of ADMSCs to differentiate into osteogenic and adipogenic lineages. For osteogenic differentiation, ADMSCs were cultured under osteogenic differentiation media containing DMEM supplemented with 10 % FBS, 10^{-7}M dexamethasone (Sigma-Aldrich), 10 mM b-glycerophosphate (Merck), and 50 μg/mL ascorbic acid 2-phosphate (Sigma-Aldrich). After 3 weeks, alizarin red (Sigma-Aldrich) staining was performed to analyze mineralization. For adipogenic differentiation, cells were incubated in adipogenic medium. This medium consisted of DMEM supplemented with 10% FBS, 1 mM dexamethasone, 200 mM indomethacin (Sigma-Aldrich), 500 mM isobutyl-methyl xanthine and ascorbate 2-phosphate (Sigma-Aldrich). After 21 days, adipogenesis was evaluated by accumulation of lipid droplets in fat vacuoles of ADMSCs stained with oil red O (Sigma-Aldrich).

3.3. Scaffold Preparation

Collagen type I gel (obtained from rat-tail tendon) was prepared at the concentration of 5 mg/mL. A combination of Bio-Oss particles (Geistlich Pharma, North America) and collagen gel was used as a 3D scaffold for calvarial defect. For the preparation of the scaffold with ADMSCs, 5×10^5 third passage ADMSCs were seeded onto the scaffold. For scaffold preparation consisted of conditioned media, ADMSC-CM was used instead of PBS (phosphate buffered saline, sigma-Aldrich).

3.4. Animals

All animal experiments were performed in accordance with the guidelines of the ethical committee including observance of the ethics of working with laboratory animals of Shahid Beheshti University of Medical Sciences and health services, Tehran, Iran (Code.IR.SBMU.SM.REC.1394.36, Date.2015/10/3). Male Wistar rats (5 - 6 week old; 160 - 180 g) were used in this study. The animals (purchased from Razi Institute, Karaj, Iran) were kept individually at a constant temperature of 22°C with a 12-hour light/day cycle. Animals received a standard laboratory rat food and drinking water ad libitum. Hypothyroidism was induced by administration of 4mg Methimazole (Tehran, Iran hormone), dissolved in 100 cc water for 60 days. Blood was collected from the corner of the eye in each rat on the 60th day. Thyroid hormone level (TSH, T4) was measured by radioimmunoassay kit (TSH kit, Sunlong Biotec co, Korea; T4 kit, DiaPlus inc, USA) and absorbance was measured by ELISA reader (Sunrise, Tecan, Switzerland). According to hormone analysis and comparison of Methimazole treated with control healthy rats, T4 level below 1.7 μg/dl (2.751 $\pm$ 0.18 in control and 0.94 $\pm$ 0.146 μg/dL in Methimazole treated, $P < 0.001$) and TSH level above 2 μIU/mL (1.84 $\pm$ 0.029 in control and 2.644 $\pm$ 0.109 μ IU/mL in Methimazole treated, $P < 0.001$) considered evidence of hypothyroid condition.

3.5. Surgical Procedure

A critical sized rat calvarial defect was created according to the protocol reported in our previous study (38). All rats were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Rotex Medica, Tritteu, Germany) with 5 mg/kg diazepam (Caspian, Rasht, Iran). Rat scalps were shaved and then skin and periosteum were raised to expose the calvarial bones. Circular bone defects (full-thickness, 5mm in diameter) were then created in parietal bones using a dental bur (terminal: 5 mm Diameter, Medesy, Italy; Micro Motor, Strong, Korea) and were irrigated constantly with saline. Dura mater was kept intact because of its osteoinductive effect. Defects were then randomly filled with implants and skin incisions were closed with 04 nylon sutures (Supa, Iran) (Figure 1). Aseptic povidone iodine (Tolidaru, Tehran, Iran) was used before and after surgery. The hypothyroid rats were divided into groups as follows, based on implanted materials: (1) empty defect; (2) defect filled with scaffold (Bio-Oss and type I collagen gel); (3) defect filled with scaffold /ADMSCs; (4) defect filled with scaffold / ADMSC- CM. The rats were sacrificed at 4 or 8 weeks after transplantation and calvaries with grafts were harvested (n = 6 per group).

3.6. Histological and Stereological Analyses

Surgical sites were dissected, fixed in 10% formaldehyde (Merck) and then decalcified in 14% EDTA (Sigma-Aldrich) solution for 21 days. After the decalcification process, explants were dehydrated by gradual exposure to alcohol, cleared in xylol (Merck) and embedded in paraffin (Merck). For light microscopy study, each specimen was serially cut into sections of thickness 10 μm (Leica microtome, Germany). The sections were then stained with hematoxylin and eosin (H and E) (Merck), and then 10 sections were selected from each sample for stereological analysis. All instruments used for ADMSC-CM preparation, hormone analysis, surgery and histology were calibrated.

3.6.1. Volume Measurement of Bone and Connective Tissue

For stereological analysis, a light microscope connected to a digital color camera (Nikon, Germany) was applied to estimate new bone volume (mm^3) and connective tissue volume (mm^3), using unbiased Cavalieri method. This method considered the product of summed section areas and distances between sections. Stereological software was used to determine volume of each interesting

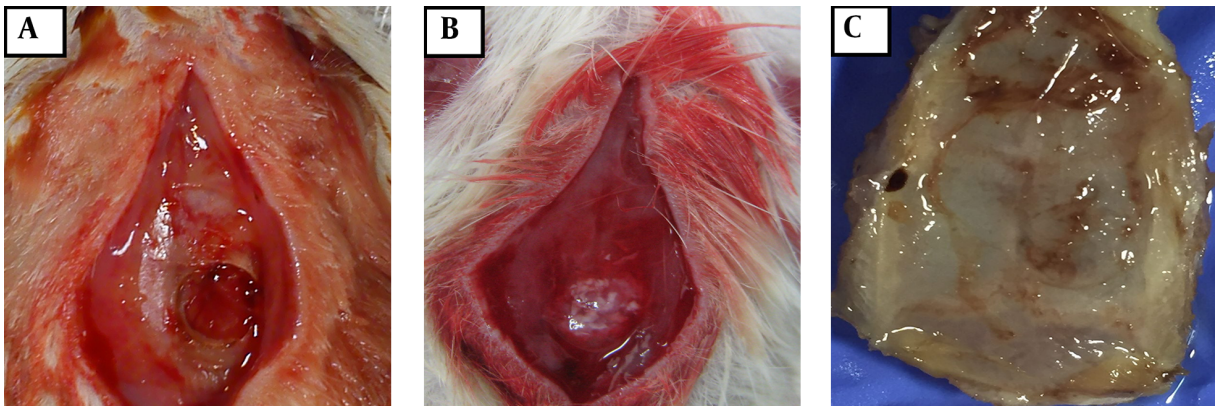

Figure 1. Rat Surgery and Implant Placement. After Exposing the Calvarial Bones, Critical Size (5-mm) Defect Was Created in Rat Calvaria (A). Defect Was Left Empty or Was Filled with one of the Following Materials: Scaffold, Scaffold/ADMSCs, Scaffold/ADMSC-CM (B). The Graft Area of Calvaria at 8 Weeks After Transplantation Indicated that the Newly Regenerated Tissue Almost Covered the Defect (C)

area in all sections and was calculated with following formula (39):

Volume = t × ΣA

where "ΣP" was the total number of points hitting the bone tissue sections, "a/p"(ΣA = ΣP × a/p) was the area associated with each point and "t" was distance between the sampled sections.

3.6.2. Measurement of Number of Bone Cells

Numerical density (N_V) of osteocytes and osteoblasts were estimated using an optical dissector method. Microscopic images obtained from calvarial defects using 40 × magnification were transferred to a monitor. Then, an unbiased counting frame was super-imposed onto a live image of each section (Figure 2). Also, the optical distance through the specmen in the Z axis was measured with microcator (Heidenhain, Germany).

Numerical density of cells was evaluated by the following formula:

$$N_v = \left[\frac{\sum Q-}{\sum P \times a/f \times h} \times \frac{t}{BA}\right] \quad (1)$$

where "ΣQ-" was the number of the nuclei coming into focus and counted bone cells, "ΣP" was the total number of frames counted in all fields, a/f (μm^2) was the area per frame, "h" was the height of the dissector, "t" was the real section thickness measured using the microcator when Q- was counted and BA was the block advance of the microtome. The total number of bone cells was estimated by multiplying numerical density (N_V) by the V (total volume) (40):

$N = N_V \times V_{total}$

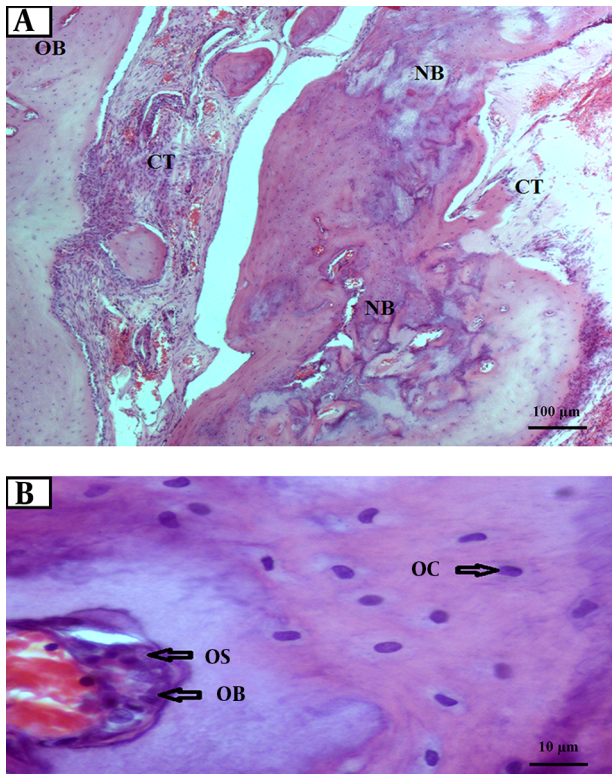

Figure 2. Light Micrographs of Newly Regenerated Tissue in Graft Area After Transplantation, Stained with H&E for Stereological Examination. OB, Old Bone; NB, New Bone; CT, Connective Tissue (A). OC, Osteocyte; OB, Osteoblast; OS, Osteoclast (B)

3.7. Statistical Analysis

The Shapiro-Wilk statistics method was used to test for normal distribution of data. Descriptive statistics were extracted using the usual and bootstrap methods

and expressed as Mean ± SD. Moreover, one-way analysis of variance (ANOVA) and Kruskal-Wallis test (under non-parametric assumption) were applied to illustrate the results. The LSD approach was used to detect any probability of difference between the experimental groups. The results were considered statistically significant for P value < 0.05. For comparisons of the groups in this study, the sampling- linear-mono-gram method, introduced by Day and Graham, was used to determine sample size (41). We found that, in each group, the optimum sample size 6 with type one error 0.05 and approximate power of 0.9 was determined as the best. We also used the simple random sampling (SRS) method for random allocation of the experimental units to each group. Median and IQR were added to statistical methods.

4. Results

4.1. Characterization of Isolated ADMSCs

Isolated stem cells from adipose tissue were characterized based on their morphology and differentiation potential. ADMSCs showed fibroblast-like and spindle-shaped morphology. The multipotency of the ADMSCs was confirmed using osteogenic and adipogenic differentiation assays. Osteogenic differentiation of ADMSCs was confirmed by red-colored calcium depositions that were positively stained using alizarin red. Moreover, After 21 days of adipogenic differentiation, lipid droplets were clearly visible by oil red O-staining (Figure 3).

4.2. Stereological Results

Stereological analysis of new bone formation, connective tissue and bone cell numbers were performed in all experimental groups, separately, at 4 and 8 weeks after implantation.

4.2.1. Volumes of New Bone and Connective Tissue

After 4 weeks, the total bone volume (mm^3) and connective tissue (mm^3) in the defect group were significantly lower than those in the treatment groups (LSD test, P < 0.001). The results showed significantly greater bone volume in the ADMSC-CM group compared with the scaffold group (P = 0.003). Although the results determined that bone volume was inferior in the ADMSC group compared to the ADMSC-CM group, but the difference was not statistically significant (P = 0.06). Also, results of connective tissue volume (mm^3) showed no significant difference among three groups ADMSC-CM, ADMSC and the scaffold group.

After 8 weeks, this relative difference between the ADMSC-CM and the other treatment groups was stronger, such that the ADMSC-CM group had significantly higher bone volume compared with the scaffold and ADMSC groups with evaluations of P = 0.002 and P = 0.02, respectively. Moreover, the defect group exhibited a significantly lower evaluation for bone volume in comparison to the other groups (P < 0.001). Furthermore, evaluations for connective tissue volume (mm^3) were significantly lower in the defect group compared to the scaffold (P = 0.002), ADMSC (P < 0.001) and ADMSC-CM (P = 0.001) groups, whereas there was no significant difference among three last groups at level 0.05 (Figure 4).

4.2.2. Total Numbers of Osteocytes, Osteoblasts and Osteoclasts

After 4 weeks, the total number of osteocytes (10^6) and osteoblasts (10^6) in the defect group showed average values significantly lower than those in the other groups (P < 0.001). Additionally, total numbers of osteocytes and osteoblasts in the ADMSC-CM were significantly increased compared with those in the scaffold group (P = 0.005 and P = 0.004, respectively). However, ANOVA showed no significant difference in numbers of osteocytes and osteoblasts between ADMSC-CM and ADMSC groups (P > 0.05) (Figures 5A, 6A). Moreover, results showed that total number of osteoclasts (10^3) in the defect group was significantly lower than that in scaffold (P = 0.002), ADMSC (P < 0.001) and ADMSC-CM (P < 0.001) groups. Statistical analysis confirmed no significant difference between treatment groups (P > 0.05).

After 8 weeks, the results indicated significant difference in the total number of osteocytes (10^6) and osteoblasts (10^6) in the defect group compared with the treatment groups (P < 0.001). Furthermore, total numbers for osteocytes and osteoblasts in the ADMSC-CM group were significantly higher compared with the scaffold group (P = 0.01 and P = 0.008, respectively). Although it seems that numbers of osteocytes and osteoblasts were higher in ADMSC-CM than ADMSC group, these values were not statistically significant (P > 0.05) (Figures 5B and 6B). However, significant difference was determined between the number of osteoclasts (10^3) in defect group compared to scaffold (P = 0.002), ADMSC and ADMSC-CM groups (P < 0.001). On the other hand, no significant difference determined between ADMSC-CM with the other two treatment groups (P > 0.05). Tables 1 and 2 show these results in more detail. To provide more details of our data for all variables considered in this research, Tables 3 and 4 gives their median and IQR.

5. Discussion

Extensive large calvarial defects do not heal spontaneously; supportive therapeutic strategies are needed to

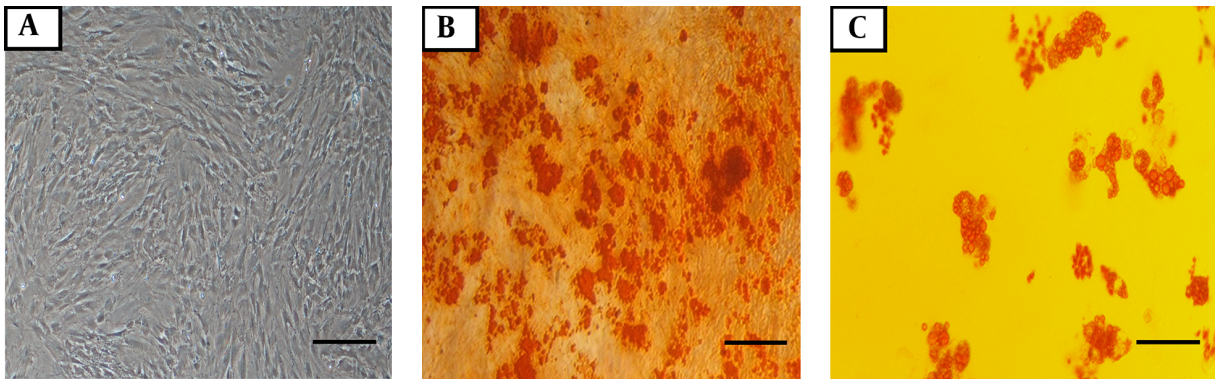

Figure 3. Morphology of ADMSCs Under Basal Medium (A). Morphology of ADMSCs After 21-Days Under Osteogenic Induction Medium. Calcium Deposition Can Be Seen After Staining with Alizarin Red (B). Morphology of ADMSCs After 21-Days Under Adipogenic Induction Medium. Small Lipid Vacuoles in ADMSCs Stained Positively Using Oil Red Solution (C). Bar 100 μm

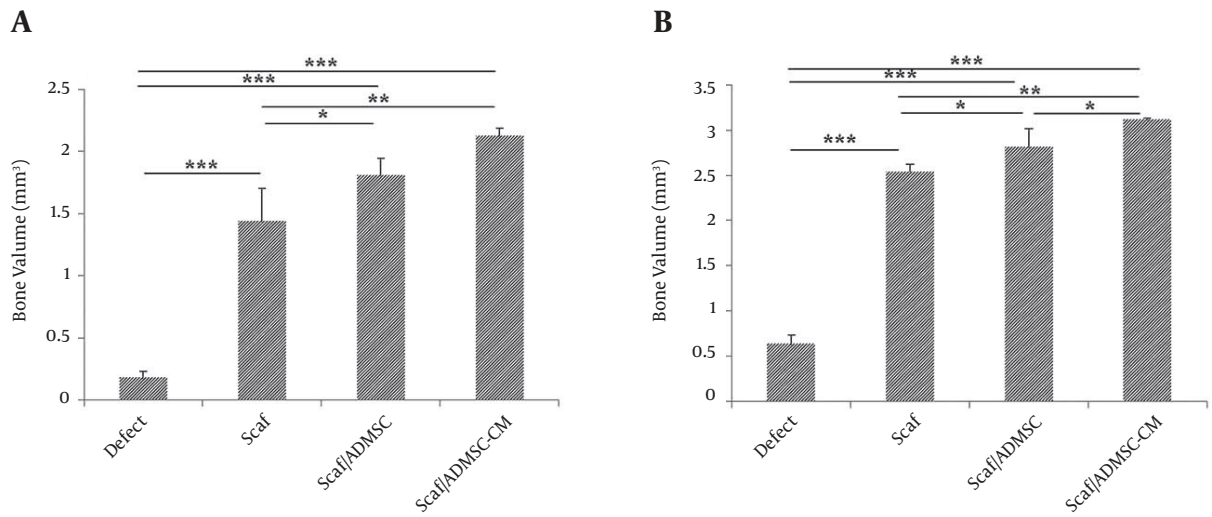

Figure 4. Newly Formed Bone Volume (mm^3) from Stereological Analysis in Defects at 4 Weeks (A), and 8 Weeks (B) After Transplantation. According to the Graphs There Were Significant Differences Between the Bone Volume (mm^3) of the ADMSC-CM and that of the Other Groups Both at 4 and 8 Weeks Except Between the ADMSC-CM and ADMSCs Groups at 4 Weeks. The Groups Compared by the LSD Test. *P < 0.05, **P < 0.01, ***P < 0.001

increase the chance of new bone formation (42). This problem is more severe in patients with hypothyroidism. MSC is an appropriate candidate for bone regeneration (43). Although, clinical application of MSCs have shown beneficial effects in the treatment of various diseases, implanted MSCs do not survive for long after transplantation (44). Also, evidence has shown that allogeneic MSCs can elicit immune response against the transplanted cells and immunological issues present an obstacle for tissue engineering (45). However, MSCs secrete trophic factors that can modulate a large number of cellular responses such as cell survival, proliferation, migration and gene expression (28).

Based on this information, we hypothesized that ADMSC-CM will promote healing of calvarial defect. To confirm this hypothesis, we filled 5-mm calvarial defects with ADMSC-CM, and Bio-Oss /collagen I as a delivery system. Volumes of new bone and connective tissue were analyzed by stereological methods. We used a combination of Bio-Oss and type I collagen gel as carrier for the conditioned medium. Bio-Oss is a deproteinized bovine bone mineral used to treat bone defects, dental implant therapy and healing for alveolar process defects. We also showed, in two previous studies, that Bio-Oss could significantly increase osteogenic differentiation potential of MSCs in vitro and bone regeneration in vivo (23, 46). Bio-

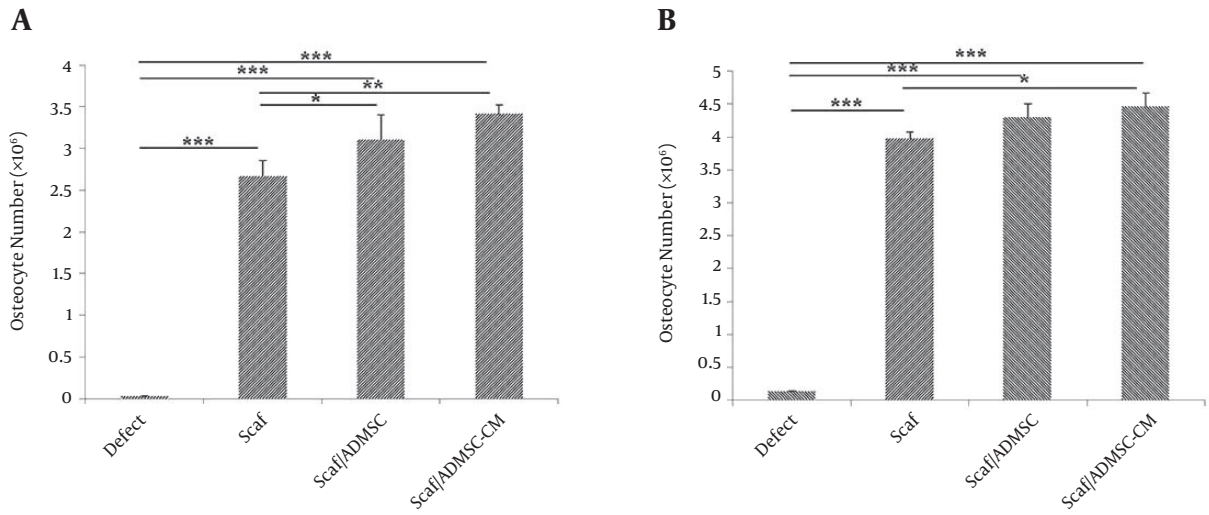

Figure 5. Osteocytes Total Numbers (10^6) from Stereological Analysis in Defects at 4 Weeks (A), and 8 Weeks (B) After Transplantation. Total Numbers of Osteocytes in the ADMSC-CM group were significantly Higher Compared with the Defect and Scaffold Groups Both at 4 and 8 Weeks. The Groups Compared by the LSD Test. * P < 0.05, **P < 0.01, ***P < 0.001

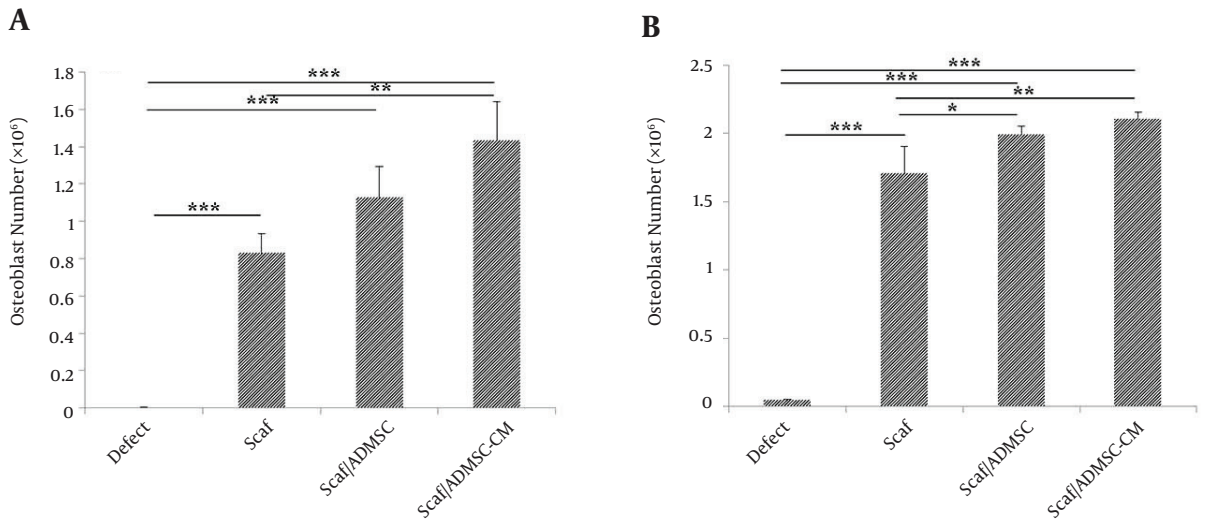

Figure 6. Osteoblasts Total Numbers (10^6) from Stereological Analysis in Defects at 4 Weeks (A), and 8 Weeks (B) After Transplantation. Total numbers of Osteoblasts in the ADMSC-CM group were Significantly Higher Compared with the Defect and Scaffold Groups Both at 4 and 8 Weeks. The Groups Compared by the LSD Test. * P<0.05, **P < 0.01, ***P < 0.001

Oss has a highly porous structure (75% - 80 % of the total volume) that increases surface area of the scaffold (47). We added Bio-Oss particles in to collagen type-I gel, since collagen is biodegradable, biocompatible and has mechanical properties, pore structure and permeability that provide a suitable scaffold for tissue engineering; such that collagen type I can support osteoblast, osteoclast and chondrocyte attachment, proliferation and differentiation (48). The results of stereological analysis showed that ADMSC-CM significantly increased new bone volume, connective tissue volume and number of bone cells at 4 and 8 weeks after surgery. Interestingly, bone formation following transplantation of the ADMSC/scaffold was less than ADMSC-CM/scaffold transplantation. In agreement with our results, Osugi et al. reported a higher evaluation of new bone regeneration in the MSC-CM group compared with the control and the MSC transplanted groups after 4, and 8 weeks (24). In another study, Wang et al. demonstrated

Table 1. After 4 Weeks, Mean ± SD and Their Corresponding Bootstrap Intervals of Total Bone Volume (mm^3), Total Connective Tissue Volume (mm^3), Total Number of Osteocytes (10^6), Total Number of Osteoblasts (10^6) and Total Number of Osteoclasts (10^3)

Variables	Groups			
	Defect	Scaffold	Scaffold/ADMSC	Scaffold/ADMSC-CM
Total bone volume, mm^3				
Mean ± SD	0.173 ± 0.060	1.433 ± 0.266	1.803 ± 0.140	2.126 ± 0.064
Bootstrap mean ± SD	0.173 ± 0.045	1.433 ± 0.216	1.803 ± 0.126	2.126 ± 0.059
Total connective tissue volume, mm^3				
Mean ± SD	0.433 ± 0.095	1.996 ± 0.242	2.126 ± 0.178	2.423 ± 0.291
Bootstrap mean ± SD	0.433 ± 0.069	1.996 ± 0.220	2.126 ± 0.149	2.423 ± 0.253
Total number of osteocytes, 10^6				
Mean ± SD	0.030 ± 0.005	2.666 ± 0.185	3.103 ± 0.294	3.416 ± 0.104
Bootstrap mean ± SD	0.030 ± 0.003	2.666 ± 0.170	3.103 ± 0.261	3.416 ± 0.095
Total number of osteoblasts, 10^6				
Mean ± SD	0.005 ± 0.002	0.830 ± 0.104	1.126 ± 0.168	1.433 ± 0.207
Bootstrap mean ± SD	0.005 ± 0.001	0.830 ± 0.080	1.126 ± 0.090	1.433 ± 0.182
Total number of osteoclasts, 10^3				
Mean ± SD	0.896 ± 0.896	50.843 ± 18.628	69.466 ± 13.867	80.433 ± 15.824
Bootstrap mean ± SD	0.896 ± 0.666	50.843 ± 17.274	69.466 ± 12.027	80.433 ± 13.252

Table 2. After 8 Weeks, Mean ± SD and Their Corresponding Bootstrap Intervals of Total Bone Volume (mm^3), Total Connective Tissue Volume (mm^3), Total Number of Osteocytes (10^6), Total Number of Osteoblasts (10^6) and Total Number of Osteoclasts (10^3)

Variables	Groups			
	Defect	Scaffold	Scaffold/ADMSC	Scaffold/ADMSC-CM
Total bone volume, mm^3				
Mean ± SD	0.626 ± 0.104	2.536 ± 0.085	2.81 ± 0.178	3.113 ± 0.021
Bootstrap mean ± SD	0.626 ± 0.076	2.536 ± 0.078	2.81 ± 0.344	3.113 ± 0.019
Total connective tissue volume, mm^3				
Mean ± SD	0.82 ± 0.069	1.746 ± 0.312	2.156 ± 0.421	1.963± 0..042
Bootstrap mean ± SD	0.82 ± 0.050	1.746 ± 0.210	2.156 ± 0.346	1.963 ± 0.034
Total Number of Osteocytes, 10^6				
Mean ± SD	0.127 ± 0.012	3.973 ± 0.099	4.296 ± 0.208	4.46 ± 0.211
Bootstrap mean ± SD	0.127 ± 0.008	3.973 ± 0.078	4.296 ± 0.173	4.46 ± 0.177
Total number of osteoblasts, 10^6				
Mean ± SD	0.043 ± 0.010	1.706 ± 0.196	1.993 ± 0.062	2.103 ± 0.054
Bootstrap mean ± SD	0.043 ± 0.007	1.706 ± 0.100	1.993 ± 0.055	2.103 ± 0.048
Total number of osteoclasts, 10^3				
Mean ± SD	4.58 ± 3.966	76.066 ± 27.485	94.873 ± 21.299	113.766 ± 13.136
Bootstrap mean ± SD	4.58 ± 2.930	76.066 ± 22.740	94.873 ± 19.025	113.766 ± 11.302

that MSC-CM delivered in gelatin sponge increased angiogenesis and bone regeneration in a diabetic rat model (49). We applied a different approach for quantitative comparison between studied groups using three dimen-

Table 3. After 4 Weeks, Median and IQR of Total Bone Volume (mm^3), Total Connective Tissue Volume (mm^3), Total Number of Osteocytes (10^6), Total Number of Osteoblasts (10^6) and Total Number of Osteoclasts (10^3)

Variables	Groups			
	Defect	Scaffold	Scaffold/ADMSC	Scaffold/ADMSC-CM
Total bone volume, mm^3				
Median	0.180	1.530	1.830	2.100
IQR	0.09	0.38	0.21	0.09
Total connective tissue volume, mm^3				
Median	0.450	1.950	2.120	2.280
IQR	0.14	0.36	0.27	0.40
Total number of osteocytes, 10^6				
Median	0.031	2.650	2.980	3.475
IQR	0.01	0.28	0.41	0.16
Total number of osteoblasts, 10^6				
Median	0.006	0.820	1.050	1.500
IQR	0.01	0.16	0.23	0.30
Total number of osteoclasts, 10^3				
Median	0.001	51.700	72.600	85.100
IQR	2.0175	27.92	20.40	22.95

Table 4. After 8 Weeks, Median and IQR of Total Bone Volume (mm^3), Total Connective Tissue Volume (mm^3), Total Number of Osteocytes (10^6), Total Number of Osteoblasts (10^6) and Total Number of Osteoclasts (10^3)

Variables	Groups			
	Defect	Scaffold	Scaffold/ADMSC	Scaffold/ADMSC-CM
Total bone volume ,mm^3				
Median	0.630	2.520	2.750	3.120
IQR	0.16	0.13	0.30	0.03
Total connective tissue volume ,mm^3				
Median	0.820	1.850	1.960	1.950
IQR	0.11	0.37	0.58	0.06
Total number of osteocytes ,10^6				
Median	0.127	3.980	4.340	4.380
IQR	0.02	0.15	0.31	0.30
Total number of osteoblasts ,10^6				
Median	0.040	1.730	2.030	2.100
IQR	0.02	0.29	0.08	0.08
Total number of osteoclasts ,10^3				
Median	6.770	63.40	90.500	121.100
IQR	5.23	37.80	31.44	17.25

sional stereological analysis to determine more reliable results. Two dimensional histological section analysis may fail to recognize small islands of newly formed bone in areas of defect. The increased amount of new tissue and

bone cell numbers in the ADMSC-CM group could be explained by the fact of MSCs secretions such as IGF-1, TGF-β1, VEGF, HGF (Hepatocyte growth factor), BMP-1 (Bone morphogenetic protein-1), IL-6 (Interleukin), IL-3 (32, 50) regulate osteogenesis process as well as osteoblast proliferation and differentiation (17, 51). Conditioned medium, in addition to stem cell mobilization into the injured site, induces stem cell differentiation into several lineages of mesenchymal tissue (24). IGF-1 is present in bone tissue and induces osteoblast proliferation. Furthermore, IGF-1 induces stem cell migration via the PI-3-kinase (PI3K) signaling pathway (51, 52). VEGF is known as the main regulator of angiogenesis and it cooperates in the osteogenesis process (53). Ando Y et al. demonstrated that the conditioned medium of MSC improved new bone formation. MSC-CM induced mobilization of endogenous bone marrow stem cells and endothelial progenitor cells through MCP-1/-3 (Monocyte Chemoattractant Protein-1, 3) and IL-3/-6 signaling, respectively. Therefore, they suggest that MCP-1/-3 and IL-3/-6 could enhance bone callus formation (54). Recently, Katagiri et al. reported that MSC-CM promoted early bone regeneration in rabbit sinus cavities through increased endogenous stem cell mobilization, proliferation and tissue vascularization (55). In general, the results of previous studies are consistent with results of this study and support the hypothesis that MSC-CM promotes bone reconstruction. In the present study, we evaluated the ability of ADMSC and their conditioned medium to repair critical-size calvarial defects in hypothyroid rats. Our data showed that ADMSC-CM had high potential for osteogenesis. The clinical use of CM could reduce several difficulties of stem cell application, such as problems of cost, expense, time, safety and immunological reactions. MSC-CM can recruit endogenous stem cells, without the need for stem cell transplantation.

5.1. Weak Points and Limitations

There was a small sample size (n = 6) for each experimental group.

5.2. Strong Points

We used ADMSC conditioned medium to improve bone repair. This novel regenerative medicine provided a new therapeutic approach for bone regeneration without necessity for stem cell implantation. This method has various benefits for clinical application, such as simple CM storage and transportation and low cost. Cytokines and growth factors enhance cell proliferation and induce differentiation of endogenous cells. Strength of this study was in its findings and its methodology.

5.3. Conclusions

Our results demonstrated that ADMSC condition media enhanced bone regeneration in a hypothyroid rat model, and the amount of new bone was greater when paracrine factors of ADMSC were used instead of ADMSC. These data suggest that ADMSC conditioned media in combination with bioceramic- collagen can be used effectively for bone repair in hypothyroid patients suffering from decreased bone-regenerating potential.

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Footnotes

Authors' Contribution: Tayebe Sanchooli, contributed to designing the project, the materials and methods section, data collection and writing the manuscript. Abdolreza Ardeshirylajimi, Mohammad Amin Abdollahifar, Hamid Nazarian, contributed to the materials and methods section. Seyed Kamran Ghoreishi, performed statistical analysis. Mohsen Norouzian and Abbas Piryaei designed and conducted the project.

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