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Adipose Tissue and Bone Marrow-Derived Mesenchymal Stem Cells Role in Regeneration of Cleft Alveolus in Dogs

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ABSTRACT

Aim: This study was designed to evaluate the therapeutic potential of adipose tissue-derived mesenchymal stem cells (AT-MSCs) compared to bone marrow-derived mesenchymal stem cells (BM-MSCs) on the regeneration of surgically created cleft alveolus in dogs. Methods: Split mouth experimental study was performed on twelve healthy mongrel dogs. The dogs were divided into two groups (A and B): Ingroup A, the surgically created alveolus was transplanted with AT-MSCs, scaffold and growth factors at the experimental side (right side of the maxilla). Ingroup B, the surgically created alveolus was transplanted with BM-MSCs, scaffold and growth factors at the experimental side (right side of the maxilla). In the control side (left side of the maxilla), the surgically created alveolus was transplanted with scaffold and growth factors only. The flaps were replaced and sutured with resorbable sutures. Bone regeneration was evaluated clinically and radiographically after 1.5 and 3 months following dogs' scarification. The data were evaluated with descriptive and t-test methods (p=0.05). Results: Stem cells whether AT-MSCs or BM-MSCs accelerate the healing and regeneration of the defected area by increasing the bone width and surface area; providing the bone quantity and quality as early as 1.5 and 3 months. Conclusions: AT-MSCs and BM-MSCs are attractive tools in bone regeneration. AT-MSCs in experimental studies showed that their effectiveness is comparable to BM-MSCs, in addition to its low cost, ease of harvesting and safer procedure to obtain stem cells as well as less risk of infection.

Key words: *Mesenchymal Stem Cells, Regeneration, Cleft Alveolus, Dogs*

INTRODUCTION

Clefts are the most common craniofacial abnormalities in newborns [1]. The effective alveolar graft is important to allow correct eruption of the lateral incisor or canine eruption through the cleft and to close the oronasal fistula [2]. The most common site for bone grafting is the iliac crest of bone. However, it showed several disadvantages such as the limited amount of harvested bone, requires second surgical site, and it causes donor site morbidity [3]. To avoid adverse effects, regenerative medicine has been proposed. It deals with replacing or arranging the damaged tissues and organs, with a suitable scaffold, mesenchymal cells, and growth factors to fully restore the structure and function of the damaged tissue or organ [4]. This mixture is often denoted as the tissue engineering triad [5].

The more predominant cells used for healing of bones are BM-MSCs (bone marrow mesenchymal stem cells), and lately, AT-MSCs (adipose tissue mesenchymal stem cells) are the most common cells used in bone regeneration trials because of their abundance, minimal morbidity and lack of ethical concerns [4]. These cells have the abilities of proliferation, differentiation to different cell lines, and immune suppression [6].

Animal models are significant in researches that cannot be done on humans. The dogs are much more like the human in disease presentation as well as in the body physiology when compared to other animal models [7]. There are masses of animal experiments that were carried out on craniofacial engineering using mesenchymal stem cells (MSCs) on canines. They all postulated that MSCs is considered as the best tool for regeneration of bones than the traditional methods for bone substitution [8, 9].

There is a lack of evidence comparing the bone regenerative potential between BM-MSCs and AT-MSCs. Therefore, the purpose of this study was to evaluate the curative potential of both AT-MSCs and BM-MSCs in the regeneration of surgically created cleft alveolus in dogs at different time intervals (1.5 and 3 months).

MATERIALS AND METHODS

Study group and design:

The present study is a split-mouth experimental study implemented on twelve healthy and vaccinated mongrel dogs.

Inclusion criteria: 1) Orally and systemically healthy and vaccinated dogs, 2) Male dogs, 3) Age range of 12-18 months, and 4) Average weight of 20 kgs. Exclusion criteria: any criteria that did not match the above-mentioned inclusion criteria. The dogs were grouped into two main groups (six dogs in each group). Group A (experimental): the right side received AT-MSCs, growth factors and scaffold. Group B (experimental): the right side received BM-MSCs, growth factors and scaffold; while the left side (control) in both experimental groups received growth factors and scaffold. Groups A and B were further divided into two subgroups (three dogs each) based on the scarification time. Subgroups A1 and B1 dogs were sacrificed at 1.5 months, while subgroups A2 and B2 dogs were sacrificed at 3 months. The dogs were euthanized by over-dosage of 10% thiopental sodium injected directly through the cephalic vein.

Animal care prior to surgery:

The animals were hosted, and quarantined in separate cages at a temperature of 27-28 °C, with humidity of 55±10 for one week prior to surgery for acclimatization to the housing and diet. All animals were examined prior to surgery to ensure that they are free from any disease.

Stem cell preparation:

a. AT-MSCs isolation and culture [10]:

Under general anesthesia (GA) with pentobarbital (40 mg/kg body weight; Abbott Laboratories), the surgical site was cleaned and swabbed with betadine. Adipose tissue was excised from the inguinal fat pad and/or omentum of each dog. After that, the wound was irrigated with normal saline and sutured. The dogs were then sent to the cage for post-operative recovery and care. The excised tissue was placed into a labelled sterile tube with 15 ml of phosphate buffered solution (PBS; Gibco/Invitrogen, Grand Island, New York, USA) mixed with 5% penicillin/streptomycin and transferred to the cell culture lab in an ice-box. The tissues were digested by adding 0.075% Collagenase II (Serva Electrophoresis GmbH, Mannheim, Germany) with Hank's Balanced Salt Solution to the tubes for 60 minutes at 37 ^{o}C with shaking to promote digestion. The digested tissue was filtered and centrifuged at 1400 rpm for 10 minutes. The erythrocyte lysis buffer solution was added to remove the erythrocytes. The remaining cells were transferred into tissue culture flasks containing Dulbecco modified Eagle medium (DMEM, Gibco/BRL, Grand Island, New York, USA) with a supplement of 10% fetal bovine serum (FBS; Gibco/BRL, Grand Island, New York, USA). After 24-hour incubation (37^{o}C and 5% CO_2), the non-adherent cells were eliminated by PBS wash. The adherent cells were transferred to tissue culture plate; at a density of 1×10^6 cells per plate; which contain DMEM media supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco/BRL, Grand Island, New York, USA), and 1.25 mg/L amphotericin B (Gibco/BRL, Grand Island, New York, USA) in 5% CO_2 at 37 ^{o}C and expanded in vitro. The medium was changed every 3 days. The cell culture was washed twice with PBS when large colonies developed and reach 80-90% confluence. The cultured cells were treated with 0.25% trypsin in 1 mM ethylene diamine tetraacetic acid-EDTA (Gibco/BRL, Grand Island, New York, USA) for 5 minutes at 37 ^{o}C with shaking. The cells were centrifuged and then resuspended in serum-supplemented medium and incubated in 50 cm^2 culture flasks

(Falcon, BD Biosciences, Two Oak Park, Bedford, MA, USA), and the medium was changed every 3 days. The developed cultures were noted as first-passage cultures and expanded in vitro until passage three.

b. BM-MSCs isolation and culture [11, 12]:

Under GA with pentobarbital (40 mg/kg body weight; Abbott Laboratories), each subject was punctured with a 14-gauge needle in the cortex of the tibia. Ten ml of bone marrow was aspirated in a syringe containing diluted heparin (5000 U/ml) in 2 ml PBS. The extracted BM-MSCs were maintained in tissue culture dishes containing 30 ml of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Biowest), 100 U/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich), and 500 ng/mL amphotericin B (Invitrogen). The cells were seeded at a density of 2 x 10^8 cells/100-mm. Floating cells were discarded after 3 days and the attached cells were subsequently expanded. Passages were performed when the cells reached 70-80% confluence.

The cells were trypsinized with 0.05% trypsin and 0.02% EDTA, washed with PBS, resuspendedat $5x10^3$ cells/cm^2 in 100-mm dishes, and maintained with a serum-supplemented medium. The resulting cultures were referred to as first passage cultures. The medium was changed every 3 days. Cells at the third passage were used in experiments as dog MSCs.

Characterization of expanded cultures:

The isolated stromal cells from adipose tissue and bone marrow were characterized according to the guidelines proposed by the International Society for Cellular Therapy (ISCT) [13], which includes three main criteria: Morphological characteristics, ability to differentiate, and immunophenotyping by flow cytometry.

Immunophenotyping by flow cytometry:

At passage 2-3, the adherent cells were collected by trypsinization and suspended in PBS with 2% FBS for 45 minutes. The cells were then initially obstructed with 10 µl monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against CD34, CD73, CD105, CD44 or CD90, and incubated for 1 hour at 4 ºC in the dark. The negative control was from the same species isotype (Millipore Corp, Temecula, CA, USA) incubated for 45 minutes on ice. The cells were washed twice, and the immunoreactivity for each CD marker was assessed by flow cytometry using a Beckman Coulter FC500 flow cytometer (Beckman Coulter, San Jose, CA, USA). The data were analyzed using CXP Software version 2.2 (Beckman Coulter, San Jose, CA, USA).

Surgical procedure (Figures 1-6):

All surgical procedures were conducted after three weeks from the initial stem cell surgery. The second surgery was done under aseptic condition and GA. The surgical site was shaved, washed with water and soap, swabbed with betadine, and covered using sterile surgical towels. The perioral tissues and gingiva were sterilized with povidone iodine. Clindamycin (10-22 mg/kg body weight), a prophylactic antibiotic, was administrated orally, and 2% chlorhexidine solution was topically applied. All the surgical procedures were done by one operator who was blinded to the method of treatment performed. Surgery was initiated using #15 scalpel blade along the buccal vestibule. A mucoperiosteal labial flap was made at the area between lateral and canine teeth. Standardized alveolar bone defects were created using carbide bur with a straight handpiece. The defect size was 0.8×0.5×0.5 mm, the experimental (right) side was transplanted with scaffold (Surgispon®, Aegis Lifesciences, India), injected with AT-MSCs ($1x10^7$ viable cells/cm^2) in group A and with BM-MSCs in group B as well as growth factors: 10 ng/mL vascular endothelial growth factor (VEGF-2); 100 ng/mL basic fibroblast growth factor (bFGF); 50 mg/mL nerve growth factor (NGF); and 100 ng/mL bone morphogenetic protein-7 (BMP7). On the other hand, the control (left) side was transplanted with scaffold (Surgispon®) and growth factors: 10 ng/mL vascular endothelial growth factor (VEGF-2); 100 ng/mL basic fibroblast growth factor (bFGF); 50 mg/mL nerve growth factor (NGF); and 100 ng/mL bone morphogenetic protein-7 (BMP7). The defects were sutured in a layered fashion technique with resorbable suture (AssuCryl® [Pully-Lausanne, Switzerland]). The bleeding in all surgical procedures was controlled by applying steady, direct pressure using a gauze. Intramuscular (IM) Vitrocin® (Gibco/BRL, New York, USA) was added locally in all the defect sites to guarantee postoperative healing without any infection. The wounds were cleaned after surgery.

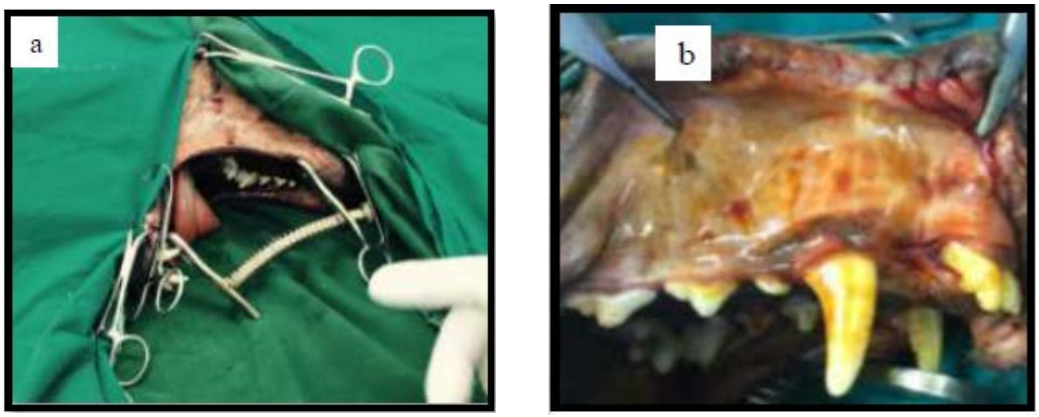

Figure 1. (a) The surgical site was covered with sterile surgical towels. (b) It was washed with betadine and chlorhexidine.

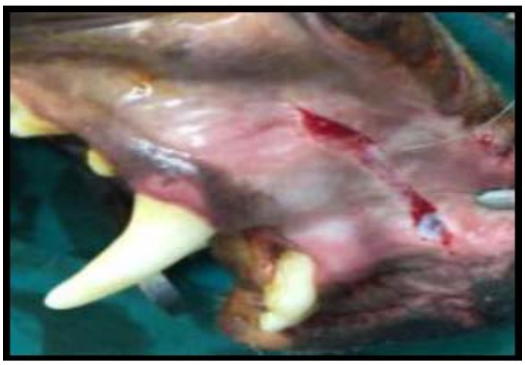

Figure 2. The mucoperiosteal labial flap was made at the area between lateral and canine teeth.

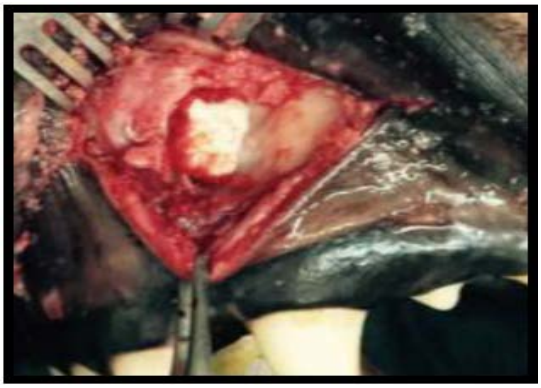

Figure 3. The defect filled with a scaffold (Surgispon®).

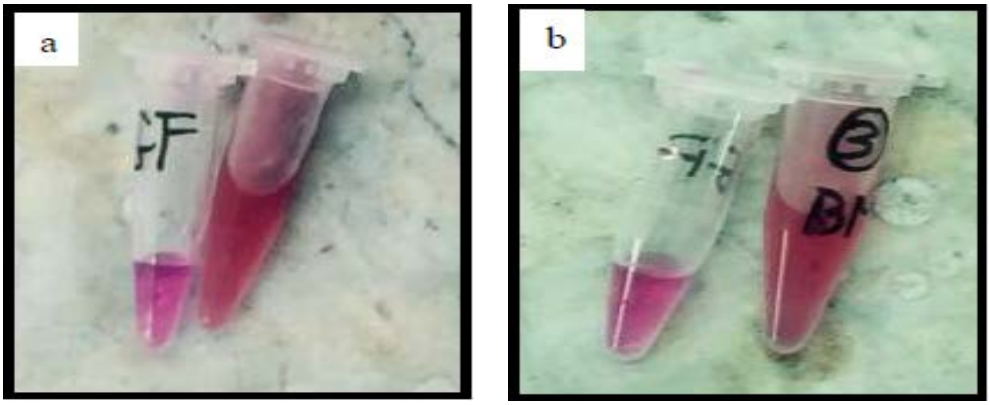

Figure 4. (a) AT-MSCs ($1x10^7$ viable cells/cm^2) and growth factor, (b) BM-MSCs ($1x10^7$ viable cells/cm^2) and growth factor.

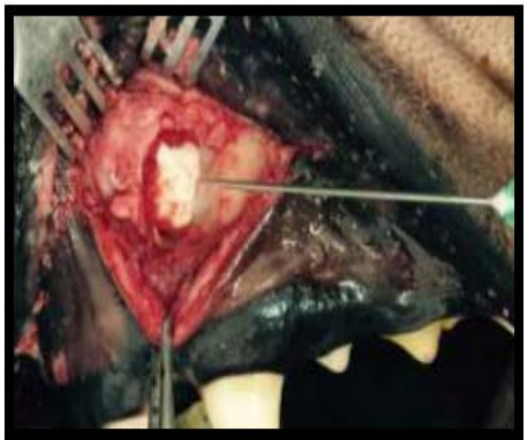

Figure 5. BM-MSCs or AT-MSCs injected in the scaffold.

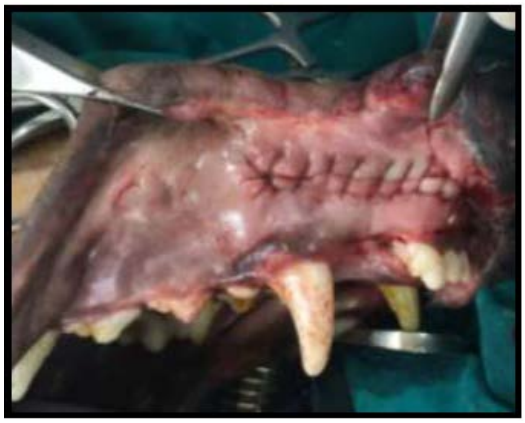

Figure 6. Flap sutured with a sterile resorbable suture (AssuCryl®).

Investigations:

a. Clinical observation:

All the animals were observed daily and followed up for any post-surgical edema or infection, adverse or allergic reactions due to the placement of the stem cells. Ten % IM gentamicin antibiotic (Gibco/BRL, New York, USA) was given with a dosage of 0.5 ml/kg twice daily for seven successive days. Analgesic therapies were applied for 3 days. Regular application of 2% betadine solution during the recovery period was done to clean and remove any debris around the wound, twice daily. The dogs were fed concentrated commercial diet (pellet) for one week and transitioned to a soft diet. They were housed in separate cages and observed for any wound complication and delayed reactions until the time of euthanasia.

b. Radiologic examination:

A series of standardized 6 radiographs of dogs' maxilla were taken for each subgroup; at 1.5 months (A1 and B1) and at 3 months (A2 and B2) post-operatively. The aim of this step was to evaluate the quality and quantity of bone regeneration. The radiographs were evaluated by two examiners who were PhD students at King Abdulaziz University (KAU), Jeddah. Inter-examiner reliability test was performed to assure the accuracy with repeated analysis of the radiographs, using Kappa statistics and was considered excellent (Kappa=0.92).

Statistical analysis:

The data were processed with the SPSS/PC Statistics software package (IBM, Chicago, version 18). Descriptive statistics were applied in the form of means ± standard deviation(SD) in quantitative variables. Percentages and proportions were applied in the form of qualitative variables. An inferential statistical test was used to detect differences between study groups. The 0.05 level was used to indicate statistical significance.

Ethical considerations:

Ethical approval was obtained from the Research Ethics Committee, Faculty of Dentistry, KAU, Jeddah, Saudi Arabia. Proposal number was 042-15. Dogs were treated in accordance with the Ethics of Animal Use in

Research Committee (EAURC) authorized by the Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt. Proposal number was Cu- vet/F/SAR/8/2015.

RESULTS

Throughout the study, all the animals were healthy, and no major problems occurred at the time between adipose and bone marrow harvesting and stem cells implanting. Clinically, there was neither any foreign body reaction nor inflammation or infections were seen in dogs. All 12 dogs were available for examination at each of the 1.5 and 3-month evaluations. Intra-examiner reliability test indicated perfect agreement (Kappa=0.94).

Cells were identified as MSCs based on their morphology. Isolated MSCs exhibited a spindle-shaped (fusiform shape) morphology after the third passage. It means that the isolated MSCs from inguinal region and femur of the dog had the features of MSCs and there was no population of hematopoietic lineage cells, as well as have the potential to differentiate into different cell lines (osteogenic, adipogenic, and chondrogenic).

Immunophenotypic characteristics of AT-MSCs:

By flow cytometry analysis, the AT-MSCs demonatrate immunopositivity for MSC specific cell-surface antigens, where CD105 and CD73 were recorded 96.2% and 92.1% cells positive, respectively. However, 4.2% of cells were CD34 negative. It means that AT-MSCS had the features of MSCs and there was no population of hematopoietic lineage cells (Figure 7).

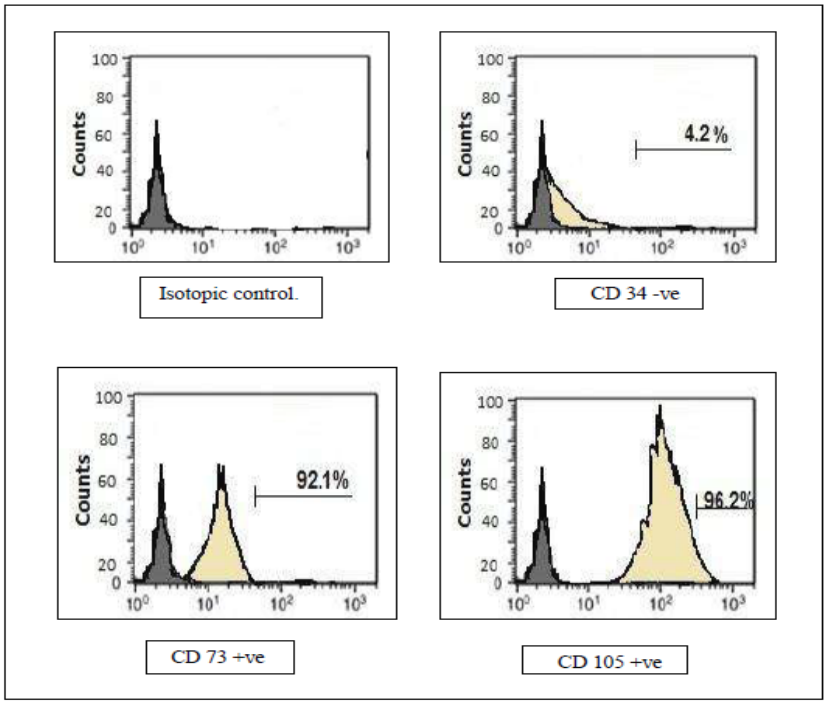

Figure 7. Flow cytometry results for adipose tissue-derived mesenchymal stem cell (AD-MSC) markers: isotopic control, CD34 negative, CD73 positive and CD105 positive.

Immunophenotypic characteristics of BM-MSCs:

The BM- MSCs as well demonstrated immunopositivity for MSC antigens specific cell-surface, where CD34, CD44 and CD90 recorded a rate of 98.1% and 94.6% for CD44 and CD90 positive, respectively, while CD34 recorded 1.4% cells (negative). It means that BM-MSCS had the features of MSCs and there was no population of hematopoietic lineage cells (Figure 8).

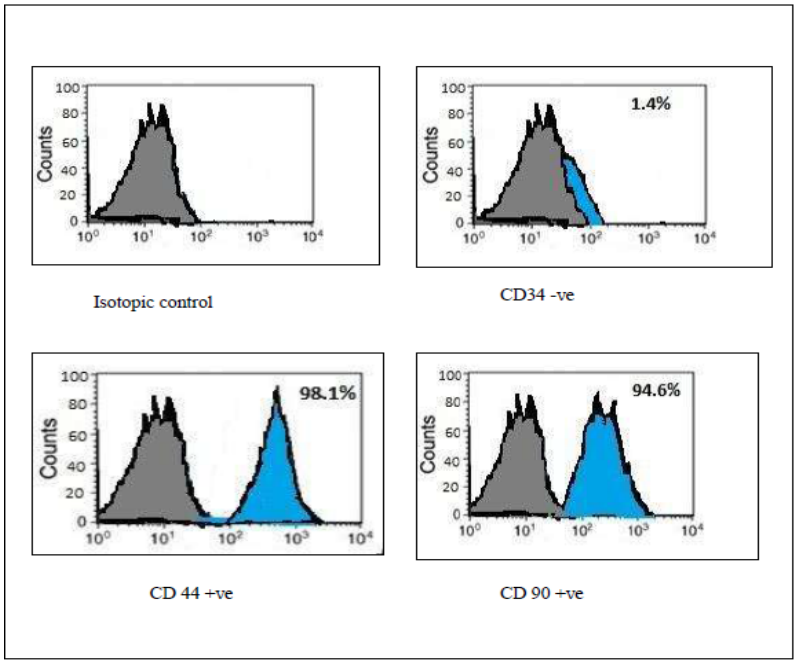

Figure 8. Flow cytometry results for bone marrow-derived mesenchymal stem cell (BM-MSC) markers: isotopic control, CD34 negative, CD44 positive, CD90 positive.

Radiographic findings, the images were measured with ImageJ software (NIH versionv1.45e, USA) by tracing the defect taking the cementoenamel junction (CEJ) as a reference point to the end of the apex of third incisor [14] (Figures 9-11). They were rated by two examiners; the inter-examiner reliability test indicated excellent agreement (Kappa=0.94).

Regarding bone density of surgically created cleft alveolus in experimental side A1 (AT-MSCs), versus control side at 1.5 months, there was a significant increase in the bone density in experimental sides than the control with $P \leq 0.050$ at 1.5 months (Table 1).

Table 1. Statistical analysis of bone density after 1.5 months in A1 seeded sides versus control sides.

Variables	Groups	Mean±SD	Median	P-Value
Bone Density	A1	123.236±1.557	123.409	0.050*
	Control	76.102±1.932	76.505	

*Significant at $P \leq 0.050$ A1: AT-MSCs at 1.5 months

Table 2 shows the bone density of surgically created cleft alveolus in experimental side A2 (AT-MSCs), versus control side at 3 months. There was a significant increase in the bone density in experimental sides than the control with $P \leq 0.050$ at 3 months.

Table 2. Statistical analysis of bone density after 3 months in A2 seeded sides versus control sides.

Variables	Groups	Mean ± SD	Median	P-value
Bone Density	A2	135.547± 1.129	135.841	0.050*
	Control	97.441± 0.924	97.022	

*Significant at $P \leq 0.050$ A2: AT-MSCs at 3 months

Concerning bone density of surgically created cleft alveolus in experimental side B1 (BM-MSCs), versus control side at 1.5 months, there was a significant increase in the bone density in experimental sides than the control with $P \leq 0.050$ (Table 3).

Table 3. Statistical analysis of bone density after 1.5 months in B1 seeded sides versus control sides.

Variables	Groups	Mean ± SD	Median	P-value
Bone Density	B1	116.720± 3.562	135.841	0.050*
	Control	63.215± 1.513	97.022	

*Significant at $P \leq 0.050$ B1: BM-MSCs at 1.5 months

Table 4 shows the bone density of surgically created cleft alveolus in experimental side B2 (BM-MSCs), versus control side at 3 months. There was a significant increase in the bone density in experimental sides than the control with $P \leq 0.050$.

Table 4. Statistical analysis of bone density after 3 months in B2 seeded sides versus control sides.

Variables	Groups	Mean ± SD	Median	P-value
Bone Density	B2	138.904± 1.884	139.601	0.050*
	Control	88.276± 1.466	88.527	

*Significant at $P \leq 0.050$ B2: BM-MSCs at 3 months

Regarding the comparison of the bone density of surgically created cleft alveolus between A1 (AT-MSCs) and B1 (BM-MSCs) seeded sides at 1.5 months, the mean percentage in A1 was 123.236% (±1.557), while in B1 was 116.72% (0± 3.565), but none were significant (Table 5).

Table 5. Statistical analysis of bone density after 1.5 monthsin A1 versus B1 seeded sides.

Variables	Groups	Mean±SD	Median	P-value
Bone Density	A1	123.236 ± 1.557	123.409	1.000
	B1	116.720± 3.565	118.160	

*Significant at $P \leq 0.050$ A1: AT-MSCs at 1.5 months B1: BM-MSCs at 1.5 months

Table 6 shows the bone density of surgically created cleft alveolus in A2 (AT-MSCs), versus B2 (BM-MSCs) seeded sides at 3 months. The mean percentage in A2 was 135.547% (±1.129), while in B2 was 138.904 % (± 1.884) but none were significant.

Table 6. Statistical analysis of bone density after 3 months in A2 versus B2 seeded sides.

Variables	Groups	Mean±SD	Median	P-value
Bone Density	A2	135.547 ± 1.129	135.547	1.000
	B2	138.904± 1.884	138.904	

*Significant at $P \leq 0.050$ A2: AT-MSCs at 3 months B2: BM-MSCs at 3 months

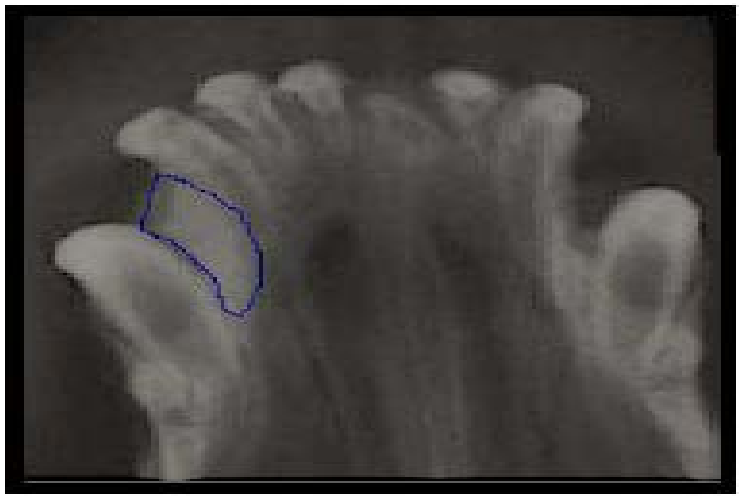

Figure 9. ImageJ software tracing the study area, taking the CEJ as a reference point to the end of the apex of third incisors.

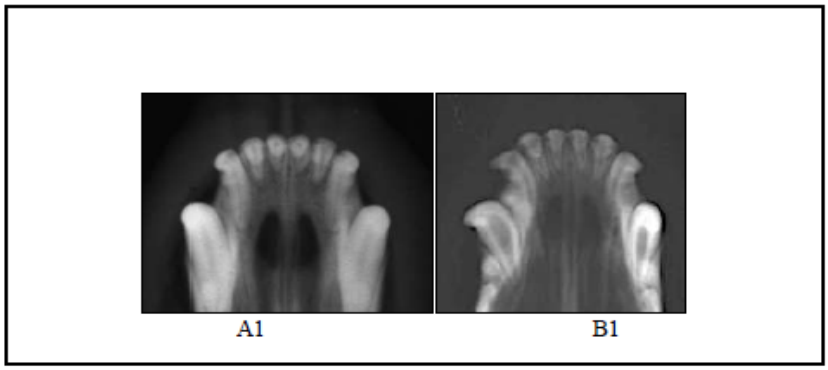

Figure 10. At 1.5 months, the experimental sides (right) A1, B1 showed radiopaque area covering the defect, while the control side (left) showed areas of radiolucency.

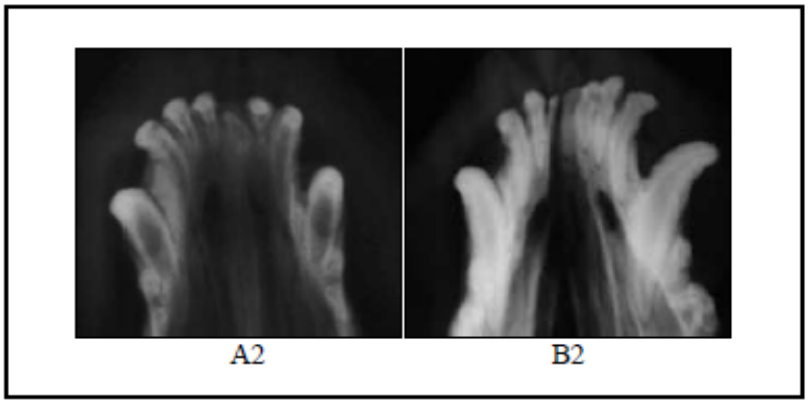

Figure 11. At 3 months, the experimental sides (right) A2, B2 showed radiopaque area covering the defect, while the control side (left) showed areas of radiolucency.

DISCUSSION

The use of the stem cells has increased recently over the past in regenerative medicine, these cells haveaunique ability to self-renewal and differentiate into various cell lineage as osteoblasts, adipocytes, and chondroblasts [15, 16]. Stem cells aspirated from bone marrow and recently stem cells from fat tissues are the most important and widely used cells in bone tissue engineering [17, 18]. Both share the same fibroblast-like structure as well as having the potential to differentiate into different cells lines (osteogenic, adipogenic, and chondrogenic) and both had immunophenotypic characteristics of MSCs.

Regeneration of bone defects as cleft alveolus remains a clinical challenge. Bone tissue engineering is considered as a promising substitute to bone grafting, autogenous and allogenic grafting [19]. Highest bone regeneration was achieved by a combination of three important elements which are MSCs, scaffold and growth factors [20]. In term of bone healing and regeneration, several studies in dogs were done and showed successful results whether using BM-MSCs or AT-MSCs [7, 9, 20]. Still, there is no study comparing BM-MSCs and AT-MSCs in term of regeneration of bone in one experiment. Thus, our study aimed to compare the curative potential between BM-MSCs and AT-MSCs in the regeneration of bone in surgically created cleft alveolus in dogs, at different time intervals (1.5 and 3 months).

The curative potential of AT-MSCs and BM-MSCs were assessed clinically by observation and radiographically by ImageJ software (Image Analyzer Computer System) tracing the defect taking the CEJ as a reference point to the end of the apex of the third incisor, then the results were analyzed in form of mean, median, standard deviation and P value.

This study showed that both MSCs harvested from the bone and inguinal fat of the canine respectively shared the same characteristics in cell morphology and ability to differentiate. Also, flow cytometric analysis established that membrane protein markers were expressed, confirming the mesenchymal origin of the cells. These data coincide with a previous report by Morcos et al. [21].

In the present study, surgical defects were done in the alveolus (surgically created cleft alveolus) in both sides in the maxillary region of dogs. Experimental sides received either AT-MSCs or BM-MSCs with scaffold and

growth factors, while the control side received scaffold and growth factors only. Radiographically, the experimental sides showed radiopaque areas filling the defects but the control side showed radiolucent areas indicating incomplete bone maturation.

Skeletal restoration requires mechanical and structural support provided by a suitable scaffold that can fill the three-dimensionally complicated defects, that allow withstanding the load and the resistance [22]. In stem cell differentiation and proliferation, collagen scaffolds showed excellent results [23]. The results of this study were supported with previous studies which concluded that alveolar cleft repair and new bone formation in dogs depend on both suitable scaffold and stem cells rather than scaffold alone or stem cells alone [7, 24-26].

In the current study, we selected the surgispon® as a scaffold. From the achieved results, collagen scaffolds have provided an optimal support for MSCs in cell-guided regeneration. The radiological findings revealed complete resorption of the collagen scaffolds as there were no residuals evident. Furthermore, during clinical observation there were no signs of infection and complete healing. These results were in accordance with Jimson et al. [27], who revealed sound bone formation and healing of the sockets without signs of infection when the collagen scaffolds were used.

Our study results showed a proper triangular relationship, through complete bone regeneration, scaffold degradation and vasculature. These results matched the study done by Wu et al. [28], who evaluated the effect of different vascular carrier pattern on angiogenesis and osteogenesis. They stated that angiogenesis and osteogenesis, as well as scaffold degradation, are critical steps in bone repair by tissue engineering.

The alveolar cleft repair accompanied different phases of dentition and necessitated further orthodontic treatment, a suitable timing of correction can allow more maxillary growth and decrease future craniofacial abnormalities [29]. Various researches have reported that MSCs accelerate the healing of craniofacial area within 4- or 6-weeks intervals [30-34]. These data support our findings, as regenerated bone was healed within 1.5 months. Radiographically, the critical size defects were replaced by a radiopaque area indicating mature bone development. Our results can be of interest to the craniofacial orthodontist to use stem cells therapy which will lead to rapid healing and regeneration with a high quality of bone; consequently, reducing the treatment line for cleft alveolar patients. In other words, bone regeneration can be started as early as canine eruption or before.

This study has some limitations that should be considered. First, the age of the dogs (puppies) to simulate the age of the child, could not be used due to the aggressiveness of the procedure, multiple defects in each dog, and high risk of morbidity. Second, the number of dogs in each group, could not be increased due to the high expenses of dogs, animal house, postoperative care and follow-up. Finally, the availability of 3D CT scan would have resulted in more accurate and representative images.

CONCLUSION

AT-MSCs and BM-MSCs are an attractive tool in bone regeneration. AT-MSCs in experimental studies showed that their effectiveness is comparable to BM-MSCs, in addition to its low cost, ease of harvesting and safer procedure to obtain stem cells as well as less risk of infection.

Conflict of Interest

The authors did not have any conflict of interest related to this study.

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