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Histological and histomorphometrical evaluation of adipose tissue and bone marrow-derived mesenchymal stem cells in regeneration of the cleft alveolus in dogs

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General Note

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ABSTRACT

Background: The study was designed to evaluate the therapeutic potential of adipose tissue-derived mesenchymal stem cells (AT-MSCs) compared to that of bone marrow-derived mesenchymal stem cells (BM-MSCs) toward the regeneration of surgically created cleft alveolus in dogs. *Methods:* A split mouth experimental study was performed on 12 healthy mongrel dogs divided into two groups, 6 each. In group A, on the experimental side (right side of the maxilla), AT-MSCs, scaffold, and growth factors were transplanted into the surgically created alveolus while in group B, BM-MSCs, scaffold, and growth factors were transplanted into the experimental side. On the control sides (left side of the maxilla), the surgically created alveolus received only scaffold and growth factors. Bone regeneration was evaluated histologically and histomorphometrically at 1.5 and 3 months following dog scarification. The data were evaluated using descriptive and t test methods (p = 0.05). *Results:* Transplantation of both types of stem cells (AT-MSCs or BM-MSCs) accelerated the healing and regeneration of the defected area as early as 1.5 and 3 months. *Conclusion:* AT-MSCs exhibited comparable effectiveness to that of BM-MSCs. Combined with their low cost, ease of harvesting, and safer procedure, our results support AT-MSCs as a preferred option for clinical application.

Keywords: AT-MSC, BM-MSC, Bone regeneration, Cleft alveolus, Transplantation, Animal model, craniofacial engineering, Cleft lip and palate, Collagen scaffold

1. BACKGROUND

Clefts of lip, alveolus, and palate constitute the most widespread craniofacial birth defects. As a therapeutic strategy, autogenous bone graft has been the gold standard of bone replacement for many years. However, despite its benefits, this method exhibits major disadvantages including limited amounts of bone along with donor-site morbidity such as post-operative pain, changes in sensation, and donor site infections and scars (Pourebrahim et al., 2013). In comparison, combined cell therapy and tissue engineering approaches can potentially avert these problems and develop safer and more effective therapies for such defects. Accordingly, such approaches may serve as a valid alternative to autogenous bone graft therapy (Moreau et al., 2007). Nevertheless, to reach optimal results, scientific orchestration of the three fundamental elements: stem cells, suitable scaffolds, and biochemical signals are required (Rosa et al., 2012). Historically, research attention has focused on bone regeneration using bone marrow derived mesenchymal stem cells (BM-MSCs) although more recently it has been reported that large amount of adipose tissue can be collected in with negligible morbidity compared to bone harvesting (Chen et al., 2005; Hibi et al., 2006). Moreover, adipose tissue mesenchymal stem cells; (AT-MSCs) can be isolated and differentiated into various cell lines including osteocytes, adipocytes, and myocytes depending on the culture conditions (Tsuji et al., 2014).

Animal models serve a valuable purpose for studies that cannot be performed on humans. Compared to other animal models, the dog is much more similar to human with regard to disease presentation in addition to body physiology (Starkey et al., 2005). Numerous veterinary studies have been performed on craniofacial engineering with MSCs on dogs, the great majority of which concluded that MSCs yielded better bone regeneration than that from the conventional bone substitute (Cruz et al., 2015; Yuanzheng et al., 2015; Alvira-González et al., 2016; Wang et al., 2017). The purpose of the present study was therefore to evaluate the curative potential of both AT-MSCs and BM-MSCs in regeneration of surgically created cleft alveolus in dogs over different time intervals (1.5–3 months).

2. METHODS

Study group and design

A split mouth experimental study design was established using 12 mongrel dogs. The dogs were treated in accordance with the Ethics of Animal Use in Research Committee authorized by the Faculty of Veterinary Medicine, Cairo University (Proposal number:

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Cu- vet/F/SAR/8/2015 and KAU Ethical Approval number: G-54-165-38). Inclusion criteria consisted of 12–18 months male dogs, orally and systemically healthy, vaccinated, with an average weight of 20 kg. The dogs were divided into two groups of six dogs each. In Group A, the right (experimental) side of the dog received AT-MSCs, growth factors, and scaffold. In Group B, the right (experimental) side received BM-MSCs, growth factors, and scaffold; whereas in each group the left (control) side received only growth factors and scaffold. Groups A and B were then divided into two subgroups, groups A1 and B1 were sacrificed after 1.5 months, whereas Group A2 and B2 were sacrificed after 3 months.

Stem cell preparation

AT-MSC isolation and culture

Atropine sulfate was injected subcutaneously at dosage of 0.5 mg/kg body weight 10–20 min prior to surgery. Under general anesthesia with pentobarbital anesthesia (40 mg/kg body weight; Abbott Laboratories, Abbott Park, IL, USA), excision of the adipose tissue was done from the inguinal fat pad and/or omentum of dogs and placed into a labeled sterile tube with 15 mL of phosphate buffer saline, (PBS; Gibco/Invitrogen, Grand Island, NY, USA) with 5% penicillin/streptomycin. Collagenase II (0.075%) (Serva Electrophoresis GmbH, Mannheim, Germany) in Hank's balanced salt solution was added to the tubes at 37°C for 60 min with shaking to promote enzymatic digestion, then filtered and centrifuged at 1400 rpm for 10 min. Removed of the erythrocytes was done by adding erythrocyte lysis buffer solution (Tomiyama et al., 2008). The remainder of the suspension was transferred into tissue culture flasks containing Dulbecco's modified Eagle medium (DMEM, Gibco/BRL, Grand Island, New York, USA) supplemented with10% fetal bovine serum (FBS; Gibco/Invitrogen, Grand Island, New York, USA). The culture flasks were incubated for 24h (37°C and 5% CO_2), then non-adherent cells were washed away by PBS. The adherent cells were transferred to tissue culture plates at a density of 1×10^6 cells per plate, containing DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1.25 mg/L amphotericin B (Gibco/BRL, New York, USA) and expanded in vitro with 5% CO_2 at 37 °C. When large colonies developed and reached 80–90% confluence the cell culture was washed twice with PBS and the cells were treated with 0.25% trypsin in 1mM ethylene diamine tetra acetic acid (EDTA; Gibco/BRL, New York, USA) for 5 min at 37°C. After centrifugation the cells were resuspended with serum-supplemented medium and incubated in Falcon culture flasks; the medium was changed every 3 days. The developed cultures were noted as first-passage cultures and expanded in vitro until passage three.

BM-MSC isolation and culture

Under general anesthesia each dog was punctured with a 14gauge needle in the cortex of the tibia. Then, 10 mL of bone marrow was collected. The extracted BM-MSCs were suspended in 30 mL of DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 500 ng/mL amphotericin B (culture medium) and were seeded at a density of 2×10^8 cells/100mm tissue culture dish. After 3 days floating cells were removed leaving only the adherent cells. When large colonies reached 80–90% confluence, the cells were harvested using0.02% EDTA and 0.05% trypsin, washed twice with PBS, and seeded at 5×10^3 cells/cm^2 in100mm dishes with culture medium. Every 3 days the medium was changed. The resulting cultures corresponded to first-passage cultures that were then expanded until passage three (El-Menoufy et al., 2010).

Surgical procedures

All surgical procedures were conducted after three weeks from the initial stem cell surgery and performed under aseptic condition and general anesthesia. Clindamycin (10–22 mg/kg body weight) was administrated orally, and 2% chlorohexidine solution was topically applied. All the surgical procedures were performed by one operator who was blinded to the method of treatment performed. Surgery was initiated using a #15 scalpel blade along the buccal vestibule. A muco-periosteal labial flap was made at the area between the lateral and canine teeth. Standardized alveolar bone defects were created using a carbide bur with a straight hand piece. The defect size was 0.8×0.5×0.5 mm. The experimental (right) site was transplanted with scaffold (SURGISPON®, Aegis Life Science, India) and injected with AT-MSCs (1 × 107 viable cells/cm2) in group (A) and with the equivalent amount of BM-MSCs in group(B) along with growth factors: 10 ng/mL vascular endothelial growth factor; 100 ng/mL basic fibroblast growth factor; 50 mg/mL nerve growth factor; and 100 ng/mL bone morphogeneticprotein-7 (R&D, Minneapolis, MN, USA). Alternatively, the control (left) site was transplanted with scaffold and growth factors without MSCs. The defects were sutured with a layered fashion technique using resorbable suture (AssuCryl®, Assurt sutures of Switzerland) (Figure 1). Vetrocin® (Gibco/BRL, New York, USA) was added locally in all the defect sites to guarantee post-operative healing without any infection. All animals were observed daily and followed up for any post-surgical edema or infection, or adverse or allergic reactions owing to the placement of the stem cells. Intramuscular antibiotic (10% gentamycin) was administered with dosage of 0.5 mL/kg twice daily for seven successive days.

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Analgesic therapies were applied for 3 days. Regular application of 2% betadine solution during the recovery period was performed to clean and remove any debris around the wound, twice daily. The dogs were fed concentrated commercial diet (pellet) for one week then transitioned to soft diet. They were housed in separate cages and observed for any wound complication and delayed reactions until the time of euthanasia.

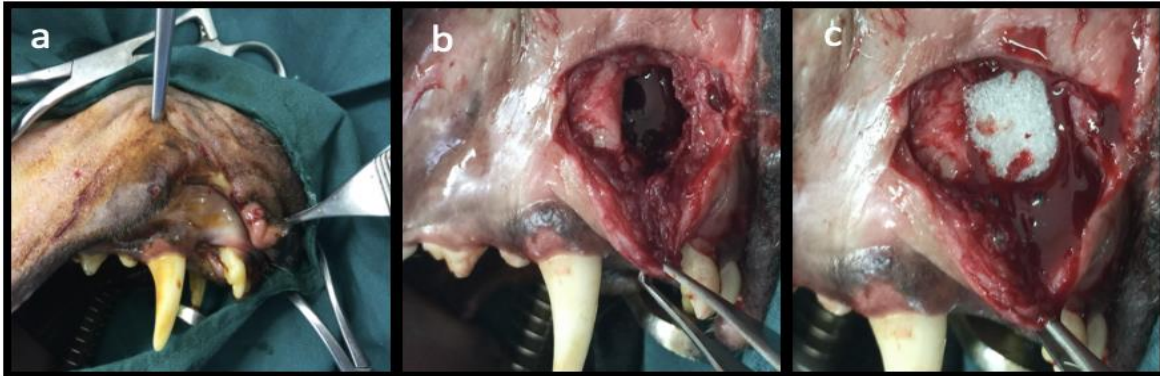

Figure 1 A. The surgical site was covered with sterile surgical towels. B. A standardized alveolar bone defect defects (0.8Å~0.5Å~0.5 mm). C. The defect filled with a scaffold (Surgispon®).

Detection of gene expression of healing markers using real time PCR (RT–PCR)

Real time PCR for detection of healing markers (vascular endothelial growth factor [VEGF], collagen, transforming growth factor [TGF], and alkaline phosphatase [ALP]) was performed as previously described (Alamoudi et al., 2014). Briefly, according to manufacturer's instruction, total RNA was isolated from tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA). The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically, then assessment of concentration of the RNA was done using the OD 260/280 ratio. The integrity of the RNA was studied by using gel electrophoresis on a 1% agarose gel, containing ethidium bromide. First-strand cDNA synthesis was performed with the cDNA synthesis kit (Qiagen -USA) by mixing 2 µg total RNA with 0.5 µg of oligo (dT)12-18 primer in a total volume of 12µL. After the mixture was heated at 70°C for 10 min, a solution containing 50 mmol/L Tris•HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 0.5 µL RNase inhibitor, and 200U 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)

Five 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, which is shown in table 1. 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 the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). The comparative threshold cycle method was used to calculate the relative expression of studied genes. All values for the GAPDH genes were standardized.

Table 1 Primer sequences for the studied genes in the work

Gene	Primers sequence	Gene bank Accession number
VEGF	F:5- CAG GCG TAT GCA GGC AAA GA-3 R:5-GAG GTG GCT TGT GCT GGT GT-3	XM540047
Collagen type1	F:5- GTGTGTACAGAACGGCCTCA -3 R:5- TCGCAAATCACGTCATCG -3	AF056303
TGF-b	F: TTGATGTCACTGGAGTCGTGA R: GGAACTGAACCCGTTAATGTCTA	AB042265
ALP	F: GGCCTGAACCTCATCGACAT R: GCGGTTCCAGACGTAGTGAGA	XM_005617214.1
GAPDH	F: 5 - TAT CGTGGAAGGACTCA-3 R: 5 - GCAGGGATGATGTTCTGGA-3	NC_006621.3

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Histological Examination

The dogs were euthanized at 1.5 months in groups A1 and B1 and at 3 months in groups A2 and B2. On both sides (experimental and control) the 3 mm trephine drill (Cizeta Surgical, Bologna, Italy) was used to extract the bone core. The slides stained with hematoxyl and eosin were prepared in accordance with the standardized protocol (IHC Research AID Laboratory, Cairo, Egypt) (Lillie and Fullmer, 1976).

Histomorphometrical analysis

On slides stained with hematoxyl and eosin, and by osteonectin immunostain, the surface area of spongy bone sections and the width of compact bone sections were measured. A Bio Imagine Scanner (Roche, Roswell, GA, USA) scanned all the slides digitally. Multiple photographs [snapshots] were produced at various magnifications from x10 to x400 from the virtual slides of the cases. Morphometric study of the virtual slides obtained from the slide scanner was carried out using the Image Viewer Software (Roche), which provided the micron morphometric results. At very low magnification [x2], the entire surface area in addition to the sponge width and the compact bone are measured on the digital slides. When a large area of the slide was formed by the sponge and compact bone area, more than one field was selected and the entire surface area of the sponge and compact bone along with the maximum width was detected, the summation of these areas was calculated to detect the entire surface area of the sponge and compact bone in the entire slide (Figures 2 and 3). A pathologist carried out a blind assessment of the histomorphometric assessment. The intra-examiner agreement on the histomorphometric analysis of the specimens was developed using the Kappa statistics and was considered excellent (K = 0.94).

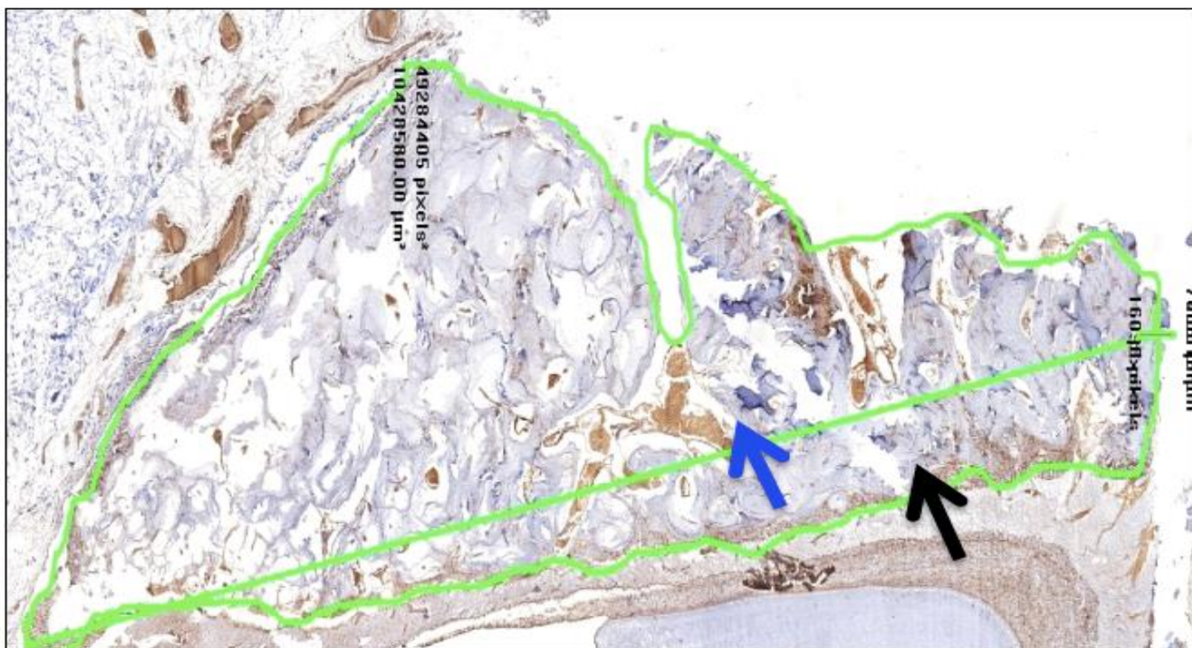

Figure 2 A section from the bone marrow stem cell group in the experimental site after 1.5 month. Morphometric study of the virtual slide was performed including the surface area of the whole new formed bone (black arrow) and the largest diameter (blue arrow) [in micron].

Statistical analysis

The data was analyzed using version 18 (IBM, Chicago, IL, USA) of the SPSS / PC Statistics software package. Descriptive statistics were applied in quantitative variables in the form of the mean ± standard deviations (SD). In the form of qualitative variables, percentages and ratios are applied. To detect differences between study groups, an inferential statistical test was used. $P \leq 0.05$ was considered as a statistically significant level.

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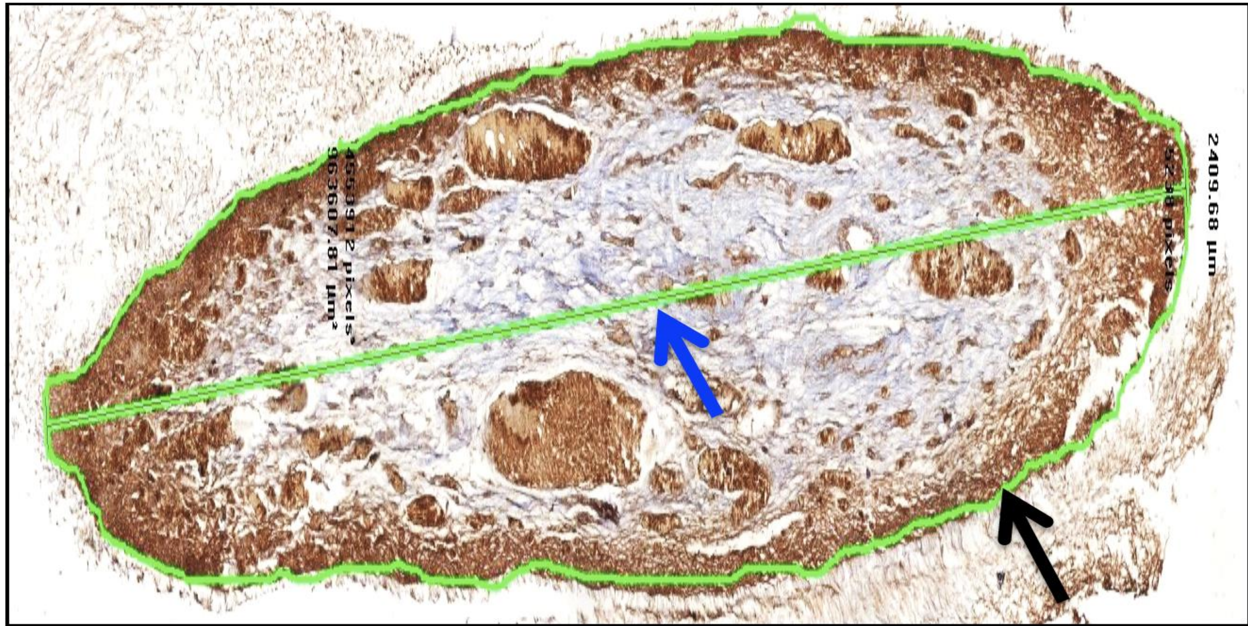

Figure 3 A section from the Adipose tissue stem cell group in the experimental site after 3 months. Morphometric study of the virtual slide was performed including the surface area of the whole new formed bone (black arrow) and the largest diameter (blue arrow) [in micron].

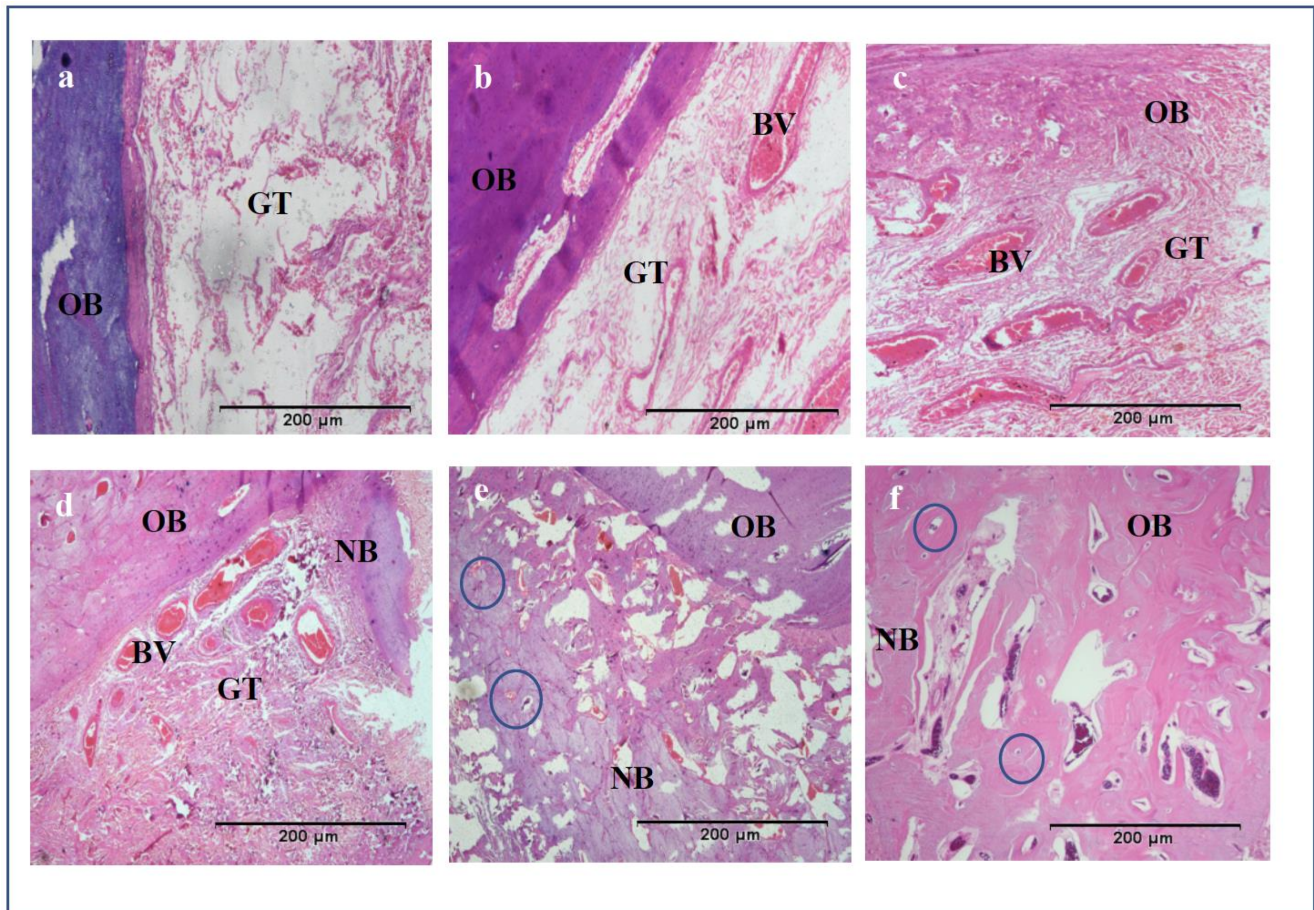

Figure 4 a. The surgically created defect alveolus transplanted with scaffold and growth factors (control group) at 1.5-month time period showing old bone (OB) and the defect filled with granulation tissue (GT). H&E stain, original magnification X 100. b. The surgically created defect alveolus transplanted with (ATMSCs), scaffold, and growth factors at 1.5-month time period showing OB

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and the defect filled with granulation tissue (GT) containing large blood vessels. H&E stain, original magnification X 100. c. The surgically created defect alveolus transplanted with (BM-MSCs), scaffold, and growth factors at 1.5-month time period showing OB and the defect filled with granulation tissue (GT) containing numerous large blood vessels. H&E stain, original magnification X 100. d. The surgically created defect alveolus transplanted with scaffold and growth factors (control group) at 3-month time period showing OB and the defect filled with granulation tissue (GT) containing blood vessels and newly formed bone (NB). H&E stain, original magnification X 100. e. The surgically created defect alveolus transplanted with AT-MSCs, scaffold, and growth factors at 3-month time period showing OB and the defect filled with newly formed bone (NB). The two circled areas show the newly formed osteons of lamellar bone. H&E stain, original magnification X 100. f. The surgically created defect alveolus transplanted with BM-MSCs, scaffold, and growth factors at 3-month time period showing OB and the defect filled with newly formed bone (NB). The two circled areas show the newly formed osteons of lamellar bone. H&E stain, original magnification X 100).

3. RESULTS

MSC isolation and surgical outcome

On the basis of their spindle-shaped (fusiform) morphology after the third passage, isolated bone marrow or adipose tissue cells were known as MSCs. Such cells had the ability to differentiate into different cell lines (osteogenic, adipogenic and chondrogenic), which had been confirmed previously (Alamoudi et al., 2014). In addition, in our study, characterization of mesenchymal stem cells (BMSCs and ADMSCs) was previously performed using cell surface marker flow cytometry detection (CD34, CD73, CD105, CD44 or CD90) (Alamoudiet al., 2018). All the animals have been healthy throughout the study. In the dogs, there were no foreign body reactions, inflammation, or infections.

Histological findings

Most of the experimental sites showed a greater tendency in both experimental groups to regenerate the bone than the control site at the same time. Compared to the control sites, the integrity of the cleft alveolus was substantially established (Figure 4).

Statistical analysis of histomorphometrical findings in the surgically created cleft alveolus

Table 2 shows the statistical analysis of histomorphometrical findings at 1.5 months between the experimental group A1 (AT-MSC)-seeded site versus the control site. Significant increase in bone surface in addition to bone width as evinced by micron was observed in A1 compared to the control site (p =0.050). Similar results between the experimental B1 (BM-MSC)-seeded site versus the control site were observed, as shown in Table 3 (p = 0.050). Table 4 shows statistical analysis of histomorphometrical findings between A1 (AT-MSC) and B1 (BM-MSC)-seeded sites after1.5 months. Notably, the results of both groups were comparable (Figure 5).

Table 2 Statistical comparison of histomorphometrical findings at 1.5 months between experimental A1 (AT-MSC)-seeded versus control site

Variables	Groups	Mean±SD	P-Value
Bone surface/Micron	A1	116710184±199999998.0	0.050*
	Control	9494743.00±200001.00	
Bone width/Micron	A1	17843.67±2093.420	
	Control	6024.00±200.000	0.050*

*Significant at $P \leq 0.050$

A1 =Adipose Tissue Derived Mesenchymal Stem Cells at 1.5 months.

Table 3 Statistical comparison of histomorphometrical findings at 1.5 months between experimental B1 (BM-MSC)-seeded versus control site

Variables	Groups	Mean±SD	P-Value
Bone surface/Micron	B1	105710184±19999998.0	0.050*
	control	12961409.7±2274497.25	
Bone width/Micron	B1	15844.67±1908.509	

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	control	2924.00±200.000	0.050*

*Significant at $P \leq 0.050$

B1 = Bone Marrow Derived Mesenchymal Stem Cells at 1.5 months.

Table 4 Statistical comparison of histomorphometrical findings betweenA1 (AT-MSC) and B1 (BM-MSC)-seeded sites after 1.5 months

Variables	Groups	Mean±SD	P-Value
Bone surface/Micron	A1	116710184±199999998.0	0.127
	B1	105710173±19987998.0	
Bone width/Micron	A1	17843.67±2093.420	0.513
	B1	15844.67±1908.509	

*Significant at $P \leq 0.050$

Table 5 shows significant increase in bone surface by micron measurements in A2 seeded sites than the control sites with p value=0.050. Also, the results between the experimental B2 (BM-MSC)-seeded site versus the control site, revealed significant increase in bone surface in B2 seeded sites than the control sites, as shown in table 6. In comparison, no significant difference was observed between A2 (AT-MSC) and B2 (BM-MSC)-seeded sites after 3 months (Table 7). The statistical analysis of histomorphometrical findings between A1 (AT-MSCs) at 1.5 month and A2 (AT-MSCs) at 3 months interval seeded sites revealed no significant difference in bone surface or bone width (Table 8). Similarly, in table 9, there was no statistical differences of histomorphometrical findings between B1 and B2 (BM-MSCs) seeded sites (Figure 5).

Table 5 Statistical comparison of histomorphometrical findings between A2 (AT-MSCs) seeded site versus control site after 3 months

Variables	Groups	Mean±SD	P-Value
Bone surface/Micron	A2	1455615041±2999996.00	0.050*
	Control	35867751.0±19078784.9	
Bone width/Micron	A2	19593.00±2003.00	0.127
	Control	11646.00±2003.00	

*Significant at $P \leq 0.050$

A2 =Adipose Tissue Derived Mesenchymal Stem Cells at 3 months

Table 6 Statistical comparison of histomorphometrical findings between B2 (BM-MSCs) seeded site versus control site after 3 months

Variables	Groups	Mean±SD	P- Value
Bone surface/Micron	B2	125867751±9999999.00	0.050*
	control	13561504.0±2000004.0	
Bone width/Micron	B2	16277.00±997.385	0.513
	control	7339.00±1997.037	

*Significant at $P \leq 0.050$

B2 =Bone Marrow Derived Mesenchymal Stem Cells at 3 months.

Table 7 Statistical comparison of histomorphometrical findings between A2 (AT-MSC) and B2 (BM-MSC)-seeded sites after 3months

Variables	Groups	Mean±SD	P-Value
Surface/Micron	A2	1455615041±2999996.00	0.127
	B2	125867751±9999999.00	
Bone width/Micron	A2	19593.00±2003.00	0.513
	B2	16277.00±997.385	

*Significant at $P \leq 0.050$

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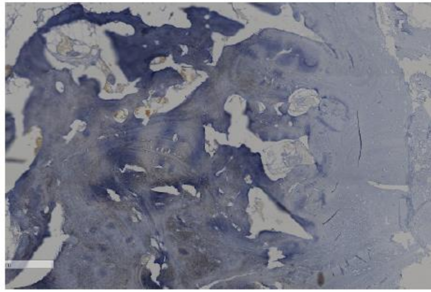
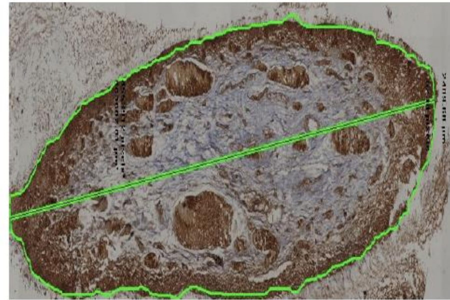
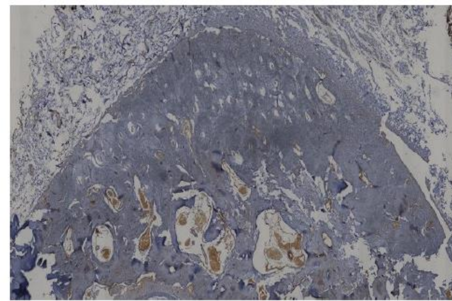
Adipose stem cell group





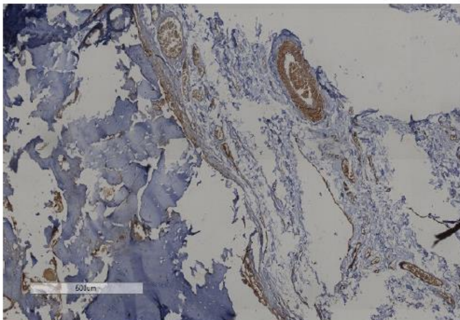
1.5 months 3 months

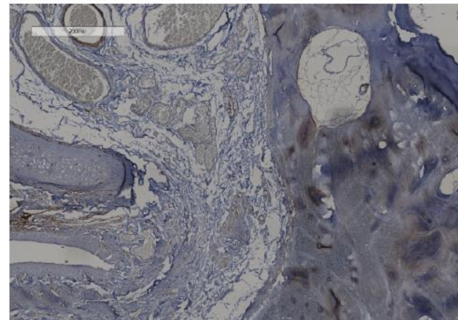
Bone marrow stem cell group



1.5 months 3 months

Control group





1.5 months 3 months

Figure 5 Sections from different tested groups show increase in bone surface and bone width measurements by pixel/micron in both experimental sites (AT-MSCs &BMSCs) than the control sites at 1.5 and 3months.

Table 8 Statistical comparison of histomorphometrical findings between A1and A2 (AT-MSC)-seeded sites

Variables	Time Interval (Months)	Mean±SD	P-Value
Bone surface/Micron	1.5	116710184±1999999980	
	3	175561504±2999996.0	0.127

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Bone width/Micron	1.5	17843.67±2093.420	
	3	19593.00±2003.00	0.513

Significant at $P \le 0.05$

Table 9 Statistical comparison of histomorphometrical findings between B1and B2 (BM-MSC)-seeded sites

Variables	Time Interval (Months)	Mean±SD	P-Value
Bone surface/Micron	1.5	105710184±19999998.0	
	3	125867751±9999999.0	0.827
Bone width/Micron	1.5	15844.67±1908.509	
	3	16277.00±2003.00	0.513

*Significant at $P \le 0.050$

Statistical analysis of healing gene marker expression in the simulated cleft alveolus

Table 10 showed significant increases in the levels of healing markers (VEGF, collagen, TGF, and ALP) was observed in the group A1 experimental site compared the control site. The same results regarding group A2 experimental site compared to the control site were shown in table 11.Similarly, significant increases in healing marker levels were shown in the experimental sites versus control sites for both groups B1 and B2 (Tables12 and 13), respectively. In table 14, group A1 showed a trend toward increased healing marker levels compared to those in group B1, whereas collagen was significantly higher in experimental group B1 compared to that in group A1. In comparison, all marker levels were increased, albeit not significantly, in Group A2 versus group B2 at 3 months post-surgery (Table 15). Furthermore, over time, group A exhibited a significant ($P \le 0.050$) increase in the levels of all healing markers except TGF as the difference did not reach significance (Table 16). In comparison, all healing marker levels significantly increased in group B over time ($P \le 0.050$) (Table 17).

Table 10 Statistical comparison analysis of healing gene marker expression of A1 (AT-MSCs) after 1.5 month between experimental and control sites

Variables	Site of surgery	Mean ± SD	*P*-value
VEGF	A1	7.833± 1.872	
	Control	1.093± 0.179	0.050*
Collagen	A1	9.6733± 0.854	
	Control	0.997 ± 0.114	0.050*
TGF	A1	12.833± 2.676	
	Control	0.987 ± 0.049	0.050*
ALP	A1	20.533 ± 9.286	0.046*
	Control	1.023 ± 0.040	

*Significant at $P<0.05$

BM-MSCs = Bone Marrow Derived Mesenchymal Stem Cells

VEGF = Vascular Endothelial Growth Factor

TGF = Transforming Growth Factor

ALP = Alkaline Phosphatase

Table 11 Statistical analysis of healing gene marker expression of A2 (AT-MSCs) after 3 months between experimental and control sites

Variables	Site of surgery	Mean± SD	*P*-value
VEGF	A2	10.767± 1.343	0.050*
	Control	1.053± 0.042	

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Collagen	A2	14.633± 0.416	0.050*
	Control	1.343± 0.658	
TGF	A2	17.467± 3.707	0.046*
	Control	1.167± 0.289	
ALP	A2	42.033± 8.127	0.050*
	Control	1.137± 0.148	

*Significant at *P*<0.05

Table 12 Statistical comparison of healing gene marker expression of B1 (BM-MSCs) after 1.5 month between experimental and control sites

Variables	Site of surgery	Mean ± SD	*P*-value
VEGF	B1	6.167 ± 0.751	0.050*
	Control	1.0200± 0.070	
Collagen	B1	12.100 ± 0.819	0.046*
	Control	1.307 ± 0.335	
TGF	B1	10.067 ± 2.732	0.050*
	Control	0.937 ± 0.065	
ALP	B1	19.667 ± 8.501	
	Control	1.020 ± 0.025	0.050*

*Significant at *P*<0.05

Table 13 Statistical comparison of healing gene marker expression of B2 (BM-MSCs) after 3 months between experimental and control sites

Variables	Site of surgery	Mean± SD	*P*-value
	B2	10.000±0.265	0.050*
VEGF	Control	1.1100± 0.168	
Collagen	B2	14.500± 1.600	0.050*
	Control	1.233± 0.497	
TGF	B2	14.633± 1.909	0.050*
	Control	1.443± 0.520	
ALP	B2	37.100±2.787	0.046*
	Control	1.030±0.052	

*Significant at *P*<0.05

Table 14 Statistical comparison of healing gene marker expression between A1 (AT-MSCs) seeded sites and B1 (BM-MSCs) seeded sites after 1.5 month

Variables	Experimental Group	Mean±SD	*P*-value
VEGF	A1	7.833 ± 1.871	0.275
	B1	6.167± 0.751	
Collagen	A1	9.673 ± 0.854	0.050*
	B1	12.100 ± 0.819	
TGF	A1	12.833± 2.676	0.275
	B1	10.067 ± 2.731	
ALP	A1	20.533± 9.286	0.827
	B1	19.667 ± 8.500	

*Significant at *P*<0.05

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Table 15 Statistical comparison of healing gene marker expression between A2 (AT-MSCs) seeded sites and B2 (BM-MSCs) seeded sites after 3 months

Variables	Experimental Group	Mean±SD	*P*-value
VEGF	A2	10.767 ±1.343	0.376
	B2	10.000 ±0.265	
Collagen	A2	14.633± 0.416	1.000
	B2	14.500±1.600	
TGF	A2	17.467±3.707	0.184
	B2	14.633 ±1.909	
ALP	A2	42.033±8.127	0.513
	B2	37.1000±2.787	

*Significant at $P<0.05$

Table 16 Statistical comparison of healing gene marker expression of A (AT-MSCs) seeded sites after 1.5 month and 3months interval

Variables	Time Interval	Mean±SD	P-Value
VEGF	1.5	7.833 ±1.872	0.050*
	3	10.767±1.343	
Collagen	1.5	9.673 ±0.854	0.050*
	3	14.633 ±0.416	
TGF	1.5	12.833±2.676	0.127
	3	17.467 ±3.707	
ALP	1.5	20.533 ±9.286	0.050*
	3	42.033±8.127	

*Significant at $P<0.05$

Table 17 Statistical comparison of healing gene marker expression of B (BM-MSCs) seeded sites after 1.5 month and 3months interval

Variables	Time Interval	Mean± SD	P-Value
VEGF	1.5	6.167 ± 0.751	0.050*
	3	10.000± 0.265	
Collagen	1.5	12.100 ± 0.819	0.050*
	3	14.500 ± 1.600	
TGF	1.5	10.067± 2.732	0.050*
	3	14.633 ± 1.909	
ALP	1.5	19.667 ± 8.501	0.050*
	3	37.100± 2.787	

*Significant at $P<0.05$

4. DISCUSSION

The application of tissue engineering in dentistry especially in craniofacial anomalies obviates the problems associated with autogenous bone grafts and raises expectations for the biological replacement of craniofacial bones, faster healing, and complete functional and esthetic regeneration along with early management of the defect to allow bony and dental development. In tissue

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engineering, stem cells scaffold, and growth factors are considered as a novel triad for successful bone repair and natural bone healing potential. For craniofacial regeneration, the most popular adult stem cells used comprise BM-MSCs and AT-MSCS. Both cell types share the same fibroblast-like structure, having the potential to differentiate into different cells lines, and carry the same immunophenotypic cell marker expression (Alamoudi et al., 2014; Alamoudi et al., 2018; Rebelatto et al., 2008).

Moreover, large animal models such as canines offer a valuable experimental model and have been utilized by numerous studies that mainly focused on regeneration-based bone repair using MSCs. Numerous earlier studies evaluated the use of BM-MSCs in defected bone in dogs and published successful results in bone regeneration (Yuanzheng et al., 2015; Wang et al., 2017; Abdel Ghaffar et al., 2012; Liu and Ma, 2004). More recently, several studies have utilized AT-MSCs in defected bone in dogs and published valuable results regarding new bone formation and defect repair (Pourebrahim et al., 2013; Cruz et al., 2015; Alvira-González et al., 2016; Haghighat et al., 2011). Nevertheless, to our knowledge no studies have been reported that compare the results of BM-MSCs and AT-MSCs in terms of regeneration of bone in a single experiment. Thus, the aim of the present study was to compare the curative potential between BM-MSCs and AT-MSCs for the regeneration of bone in surgically created cleft alveolus in dogs over different time intervals (1.5–3 months). The curative potential of AT-MSCs and BM-MSCs was assessed histomorphometrically.

The present study results showed that MSCs harvested from both the bone and inguinal fat of the canine shared the same characteristics with regard to cell morphology and ability to differentiate; in addition, flow cytometric analysis established that membrane protein markers were expressed, confirming the mesenchymal origin of the cells. These data are consistent with the results of previous reports (Alamoudi et al., 2018; Rebelatto et al., 2008; Hattori et al., 2004). Furthermore, considering that collagen scaffolds are an excellent tool for supporting stem cell differentiation and proliferation(George et al., 2006; Sumanasinghe et al., 2009) and that skeletal restoration requires mechanical and structural support such as provided by scaffolds (Arinzeh, 2005; Mastrogiacomo et al., 2006; Kanczler and Oreffo, 2008). In the current study we used SURGISPON® as a scaffold for the respective MSCs. Many previous studies concluded that alveolar cleft repair and new bone formation in dogs depended on both a suitable scaffold and stem cells (Yuanzheng et al., 2015; Wang et al., 2017; Liu and Ma, 2004; Yamada et al., 2004; Chung et al., 2011; Vahabi et al., 2012; Tanimoto et al., 2015; Wei et al., 2016). Two studies revealed sound bone formation along with healing of the sockets without signs of infection when collagen scaffolds were used (Gupta et al., 2013 and Jimson et al., 2015).

In histomorophometrical analysis, parallel results were obtained between AT-MSCs and BM-MSCs in terms of bone surface and bone width in micron measurements, with these cells filling all the defected area across 1.5-month intervals. Moreover, both types of stem cells demonstrated significant results versus the control, in which bone formation was only confirmed at the periphery whereas in the central region the sponge bone was still not filled with mature bone by 3 months. In comparison, various studies have reported that MSCs accelerate healing of the craniofacial area within 4- or 6-week intervals (Wang et al., 2011; Castro-Govea et al., 2012; Khojasteh et al., 2013; Inukai et al., 2013; Li et al., 2014; Lin, 2014; Higgins, 2015). These data support our findings, in which the bone regenerated within 1.5 months. Furthermore, these findings were confirmed histomorphometrically by the significant increase in the bone width and surface as evidenced by micron measurements.

Notably, this assessment technique has not been used in previous studies examining cleft alveolus repair. Histomorophometrical analysis has several advantages over that using traditional microscopy. Although both techniques share the same objective magnification, in the present study the virtual slides were captured by a camera connected to the computer, to which the virtual slides were saved. This simplifies uploading the images and sharing them with others. In addition, the expansive system capacity allows the acquisition of measurements and magnification from very low magnification (x2) up to x400 of a selected area without affecting the actual size and resolution of the virtual slides. Accordingly, this technique is considered to provide an excellent solution for education between doctors and students, archiving original slides, teleconsultation, and research across different fields (Higgins, 2015).

5. CONCLUSION

The results demonstrated that MSCs, regardless of whether sites were seeded with AT-MSCs or BM-MSCs, yielded significant regeneration and healing of the cleft alveolus compared to that of the control site at a relatively short time interval (1.5 months). Moreover, both stem cell types exhibited comparable results in term of bone quality and quantity. Combined with their low cost, ease of harvesting, and safer procedure for obtaining stem cells, along with lower risk of infection, our results support AT-MSCs as a preferred option for clinical application.

Abbreviations

ALP Alkaline phosphatase

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AT-MSCs	Adipose tissue-derived mesenchymal stem cells
BM-MSCs	Bone marrow-derived mesenchymal stem cells
FBS	Fetal bovine serum
DMEM	Dulbecco's modified Eagle medium
DSR	Deanship of Scientific Research
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

Conflict of Interest

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

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