The Effect of Undifferentiated Mesenchymal Bone Marrow Stem Cells on the Healing of Fresh Extraction Bony Sockets

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Abstract: Background: Mesenchymal Bone marrow stem cells (MBM SCs) have been shown to repair bone defects in various animals. Porous scaffolds for bone tissue engineering play an important role in both cell targeting and transplantation. They serve as carriers to transfer cells and bioactive materials to defect sites. The present investigation was undertaken to study the effect of polymer scaffolds seeded with mesenchymal bone marrow stem cells (MBM SCs) on the quantity and quality of bone formation in recently extracted bony socket. **Methods**: The present study was carried on 10 dogs divided according to the follow up period into two groups with 5 dogs in each group. Group (A) was followed for 1.5 months, while group (B) was followed for 3 months. MBM SCs from the femur's cortex were cultured and seeded on polymer scaffolds. After extraction of the lateral incisors, seeded polymer scaffolds were inserted in the left sockets which served as the experimental sites, while unseeded scaffolds were inserted in the right sockets which served as the control sites. At the end of the follow up period, the animals were sacrificed and sections stained for histological evaluation. **Results**: Polymer scaffolds seeded with MBM-SCs resulted in a significantly greater mean bone area percentage and showed more prevalence of mature lamellar newly formed bone than unseeded scaffolds after insertion in fresh extracted bony sockets at all evaluation intervals. **Conclusion**: Bone marrow stem cells provide an effective therapeutic approach for the regeneration of alveolar bone defects.

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Key words: Bone regeneration, stem cells, tissue engineering, alveolar sockets

1. Introduction

Bone maintenance after tooth extraction has a significant impact on the functional and esthetic outcomes of future treatment. [1,2] The attention of investigators has been directed to new technologies as bone tissue engineering, which has been emerging as a valid approach to the current therapies for bone regeneration. [3]

The basic concept of utilizing mesenchymal stem cells (MSCs) and autologous bone marrow (BM) aspiration to treat bone defects has several advantages. Osteoblast's differentiation from MSCs is very well described and standardized in many protocols. Mesenchymal bone marrow stem cells (MBM-SCs) can also be isolated by means of minimally invasive procedures from BM, requires only a small amount of tissue from the patient and this approach can restore bone defects without incurring donor site morbidity. [4,5] Stem cells are highly expandable in culture and have been used in the treatment of various tissues and organs. Their capacity to undergo extensive replication without losing their multi-potential capability makes them an attractive cell source for cell-based therapeutic approaches.[6]

In bone tissue engineering, material scientists attempt to make biocompatible and biodegradable scaffolds with appropriate porosity, mechanical strength and hydrophobicity comparable to native bone materials.[7] They also try to provide a framework for three-dimensional organization of the developing tissue. Scaffolds can deliver MSCs into a graft site, facilitate their retention and distribution into the new tissue area matrix and provide space in which vascularization, new tissue formation and remodeling can take place.[8] Polylactide-co-glycolide (PLGA) polymer scaffolds are suitable for tissue-engineering applications , [9,10] have been studied for drug delivery and have been approved by the Food and Drug Administration .[9-11] The polyglactin vicryl mesh combines glycolide and lactide at a ratio of 9:1. It is a co-polymer consisting of the rapidly degrading glycolic acid and the slower degrading and more hydrophobic lactic acid and it degrades by non-enzymatic hydrolysis creating by-products eliminated from the body in the form of carbon dioxide and water. The advantages of fiber meshes are a large surface area for cell attachment and a rapid diffusion of nutrients in favor of cell survival and growth. [12, 13]

The aim of the present investigation was to study the effect of polymer scaffolds seeded with MBM SCs on the quantity and quality of bone formation in recently extracted bony sockets.

2. Materials and Methods

I-Study group and design:

The present study is a split mouth experimental study performed on ten 9 months old mongrel dogs, weighing 7-9 kg, in an orally and systemically health condition. The dogs were treated according to the guidelines approved by the Institutional Animal Care and Use Committee of Cairo University and the animal research protocol was approved by the Ethics Committee of the National Research Center, Cairo, Egypt. The ten dogs were divided according to the follow up period into two groups (A) and (B) with five dogs in each group: Group (A) was followed up for 1.5 month, and Group (B) for 3 months. In both groups, the left and right upper lateral second incisors were extracted. Each animal received two scaffolds. The left socket side was chosen to be the experimental site, where a polymer scaffold seeded with MBM-SCs was inserted while the right side served as the positive control site, where an unseeded polymer scaffold was inserted. At the end of the follow up period the animals were sacrificed by IV injection of concentrated sodium thiopental for histological evaluation.

II-Stem Cell Preparation:

a- Bone marrow (BM) Aspiration and MSCs Expansion : [14]

Under general anesthesia by IM injection of a compound of ketamine (22 mg/kg b.wt.) and xylazine (0.2 mg/kg b.wt.), a 14-gauge needle was used to penetrate the cortex of the femur of each dog, and about 10 ml of BM was drawn in a syringe containing 1500 U of heparin. BM aspirate was collected into a 50 ml tube, containing 30 ml Dulbecco's Modifed Eagle's medium (DMEM, Sigma, Australia) and heparin (100U/mL), The mixture was centrifuged at 400 g without acceleration or brake for 35 min at 20ºC on density gradient media (Ficoll-Paque; GE Healthcare, Waukesha, WI) and the top layer of fat containing plasma was discarded. Cells located at the interface between the BM sample and gradient media were collected, washed with Dulbecco's phosphate-buffered saline (DPBS), (Invitrogen, Carlsbad, California, USA) and re-centrifuged at the same speed. After determination of cell viability and the number of viable cells by trypan blue staining, the cells were re-suspended in DMEM, supplemented with 10% fetal bovine serum (FBS), (USDA, Gibco, Grand Island, NY, USA) and antibiotics (penicillin 10 000 U ⁄ ml, streptomycin 10 000 ug ⁄ ml, amphotericin B 25ug ⁄ ml). The nucleated cells were plated in tissue culture flasks at 2.5 X 105 ⁄ cm^2 and incubated at 37°C in a humidified atmosphere containing 5% CO_2. On the fourth day of culture, the non-adherent cells were removed along with the change of medium. The culture medium was changed every 3 days until the outgrown cells reached 90% confluence. On the 18th day, the adherent colonies of cells were trypsinized, counted and sub-cultured at 90% confluence. Cells were passaged to the subculture 2 so that sufficient number of cells was available to continue the experiment described below. Cells were identified as being MSCs by their morphology which was fusiform, their adhesiveness, and their power to differentiate into osteocytes and neurocytes . [15, 16] . Kinetic quantitative determination of alkaline phosphatase was carried out in the medium of differentiated osteocytes using a commercial kit provided by Stanbio laboratory, Boerne, TX, USA. Differentiation into neurocytes was confirmed by detection of nerve growth factor gene expression in cell homogenate. [17]

b- Seeding Procedure: [18]

Second passage dog MBM SCs were used for the seeding procedures. Prepared polymer scaffolds were sterilized by soaking in 95% ethyl alcohol for 30 minutes. The polymer scaffold used was polyglactin 910 vicryl mesh, which features a weight of 53.6 g/m² and has an average pore size of 500 micrometers (Ethicon Inc, J&J, Sommerville, NJ, USA). Scaffolds were then transferred to 24 well tissue culture plates, one scaffold in each well. All scaffolds were washed with DPBS for 1 hour, and the DPBS was changed every 15 to 20 minutes. All DPBS were aspirated and scaffolds were pre-wetted with 2 ml supplemented culture media.

For the experimental scaffolds, 500 µL of media containing MBM SCs (9.4 x 106 cells/mL) was added to each well, 1 scaffold per well. The following day, the media and unattached cells were aspirated, then 500 µL of fresh culture medium was added to each well and the number of unattached cells was calculated. This procedure was repeated every 2 days until the seventh day. The seeded scaffolds were monitored daily with a phase contrast microscope. On the seventh day, scaffolds were used for the animals after adherence of cells to the mesh scaffold was completed. As for the control scaffold, culture media without BM-MSCs were added to the control scaffolds.

III-Dental surgical procedure:

The surgical procedures were performed under general anesthesia by IM injection with a compound of Ketamine (22 mg/kg b.wt.) and xylazine (0.2 mg/kg b.wt.).The systemic anesthesia was complemented with infiltration anesthesia to ensure local hemostasis. Prophylactic IM antibiotics were administrated and 2% chlorohexidine solution was topically applied. After dental extraction scaffolds were inserted, then labial flaps were raised to cover the alveolar sockets and the scaffolds. The flaps were then sutured using 4-0 black silk suture. Postsurgical management included IM

administration of antibiotics, pain killers, a soft diet and a daily application of 2% chlorohexidine solution during the healing period. Observation of the surgical sites with regard to oral health, maintenance of the suture line closure and edema was done every day until suture removal and at least twice weekly thereafter. [19, 20]

IV. Histological evaluation:

Specimens from both control and experimental sites were taken and fixed in 10% formalin for one week, then decalcified and processed according to a standardized protocol (IHC Research AID laboratory, Cairo, Egypt). Sections were cut (5 um thick) and stained with H&E and Masson Trichrome and examined with Olympus CX20 microscope attached to a camera and computer. All the stained sections were analyzed by image analyzer computer system using the Image J software (NIH version v1.45e, USA), capable of performing high speed digital image processing for the purpose of tissue measurements. A millimeter scale was also photographed at the same magnification of the captured photo-micrographs (10 x) to allow further conversion of the measurements obtained from the camera in pixels into micrometers. Image J software was calibrated and the image opened on the computer screen for pre-analysis adjustments. For histomorphometric analysis the most representative five fields were captured and the surface area percentage of bone was measured.

Histomorphometric readings were compared in both groups of experimental and control sites and analyzed using SPSS version 18. Data were presented as mean and standard deviation (SD) values. Student t test was carried out to compare between the mean bone area percentage which represents the area of trabecular bone including both mineralized and osteoid tissue expressed as percentage of the total tissue area. P value < 0.05 was considered significant.

3. Results

I-Histological findings:

The structural bony changes were studied in both groups (A) and (B) at experimental and control sites

A-Analysis of sections taken from experimental sites in group (A) i.e. after 1.5 months:

Most cases showed more prevalence of bone than that seen at the same time interval in the control group. This bone ranged from thick interconnected trabeculae to mature lamellar bone. Osteoblastic rimming showed the active engagement in bone formation. Widened osteocytic lacunae were seen as well as irregular marrow spaces. Congested dilated blood vessels and angiogenesis increased markedly reflecting the persistent attempt of the tissues to heal. Mild inflammatory response was detected in most cases. Finally, signs of remodeling were reflected by the mild osteoclastic activity in the form of few sporadic osteoclasts (Figures 1, 2).

B- Analysis of sections taken from experimental sites in group (B) i.e. after 3 months:

After three months interval there was an increase in the thickness of bone. Larger areas of regular concentric lamellae could be seen. Osteoblastic rimming was still clearly demarcated. Remodelling was still distinguished from the osteoclastic activity. Mild to moderate inflammatory response could be seen. Extravasated blood cells and congested dilated blood vessels were the main vascular hallmarks. Masson Trichrome stain revealed that most of the cases showed calcified bone rather than osteoid tissue (Figures 3, 4).

C- Analysis of sections taken from control sites in group (A):

The main picture was that of compactly packed granulation tissue composed of newly formed collagen fibers, fibroblasts and blood vessels. Unconnected spicules of bone were interspersed within the granulation tissue. Moderate inflammatory response was verified. Blood vessels were dilated. Evidence of bone formation was detected. Areas of woven bone and osteoblastic rimming showed in some cases. Angiogenesis increased markedly reflecting the persistent attempt of the tissues to heal (Figures 5, 6).

D- Analysis of sections taken from control sites in group (B):

The main picture showed increased thickness of woven bone showing and larger regions of lamellar bone organization could be detected. Irregular marrow spaces were seen with osteoblastic rimming and wide osteocytic lacunae were seen. Extensive fibrous and mesenchymal activity were also demonstrated (Figures 7, 8).

Statistical Comparison between experimental and control sites in the same group revealed the following:

In Group (A) after 1.5 months, the mean area percentage of the newly formed bone areas was 52.346 % in the experimental sites, while it was 22.908% in the control sites. There was a very significant increase in the mean area percentage of the newly formed bone in the experimental sites when compared with the control sites. (p=0.0028) (Table1).

In Group (B) after 3 months, the mean area percentage of the newly formed bone was 61.768 % and 44.244% in the experimental and control sites respectively. There was a significant increase in the mean area percentage of the newly formed bone in

the experimental sites when compared with the control sites (*p*=0.0144), (Table 1).

Statistical Comparison between experimental sites in group (a) & (b) and control sites in group (a) & (b) revealed the following:

There was no significant difference in the mean area percentage of newly formed bone between group (A) experimental sites at 1.5 months and group (B) experimental sites at 3 months, (*p*=0.194), while there was a significant increase in the mean area percentage of newly formed bone at group (B) control sites at 3 months when compared to group (A) control sites at 1.5 months (*p*=0.038), (Table 2).

Table 1: Comparison of the histometrical mean value of bone area percentage between group (A) experimental sites and control sites (at 1.5 month) and between group (B) experimental sites and control sites (at 3 months).

	Time	Group	N	Mean	±S.D.	*P*
Group A	***1.5month***	Experimental	5	52.35	17.65	0.003*
		Control	5	22.91	19.19	
Group B	***3 Months***	Experimental	5	61.77	11.09	0.014 *
		Control	5	44.24	11.73	

*Significant at *P* <0.05

Table 2: Comparison of the histometrical mean value of bone area percentage between group (A) experimental sites and group (B) experimental sites and between group (A) control sites and group (B) control sites

Group		N	Mean	±S.D.	*P*
Group A:	Experimental	5	52.35	17.65	0.194
Group B:	Experimental	5	61.77	11.09	
Group A:	Control	5	22.91	19.19	
Group B :	Control	5	44.24	11.73	0.038*

*Significant *P*< 0.05

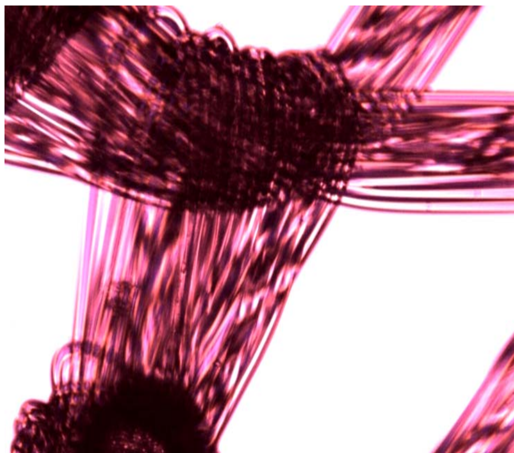

Figure 1. Polyglactin vicryl mesh in culture medium without MBM SCs under a phase contrast microscope

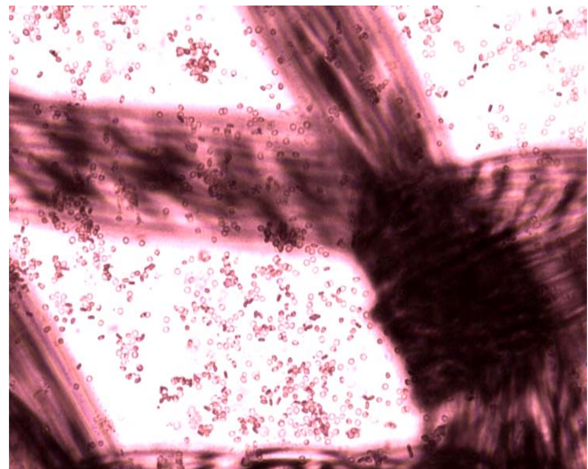

Figure 2. Polyglactin mesh seeding in culture medium containing MBM SCs for 2 days under a phase contrast microscope.

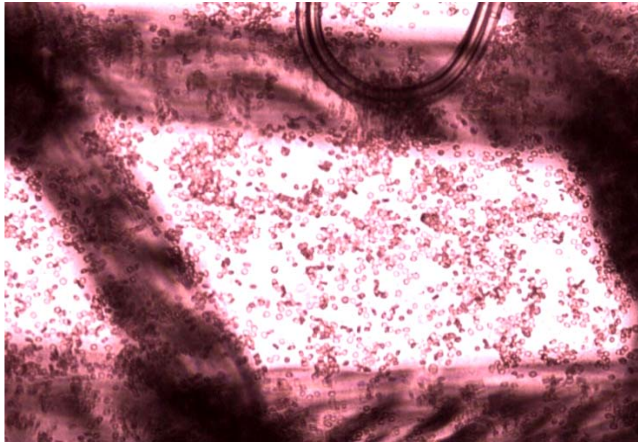

Figure 3. Polyglactin mesh after seeding in culture containing MBM SCs for 7 days under a phase contrast microscope

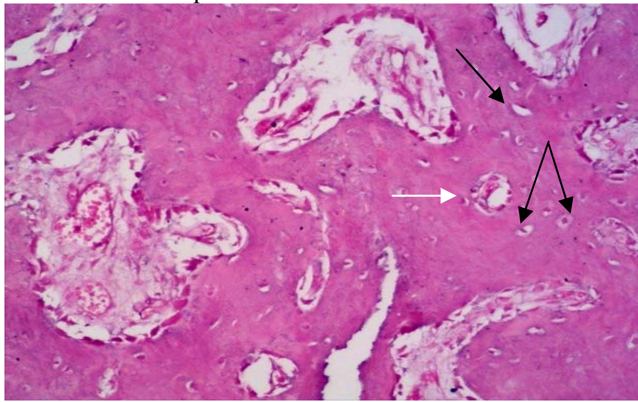

Figure 4. A photomicrograph of group (A) experimental specimen showing osteoblastic rimming of marrow cavities, congested blood vessels, wide osteocytic lacunae (black arrows) and lamellar organization of osteocytes (white arrow). H&EX200

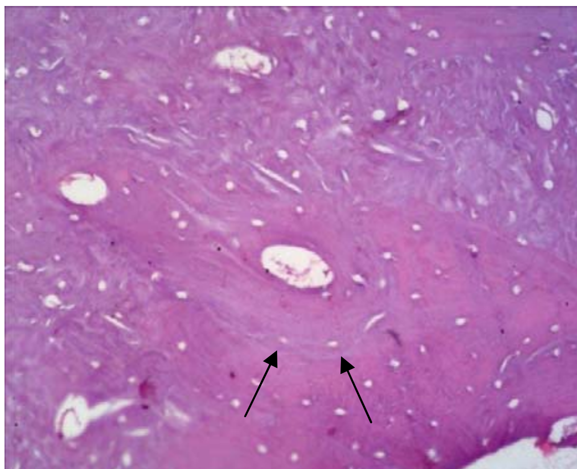

Figure 5. A photomicrograph of group (A) experimental specimen showing a mixture of woven bone and the more regular concentric lamellar bone (arrows) (Masson trichrome x200)

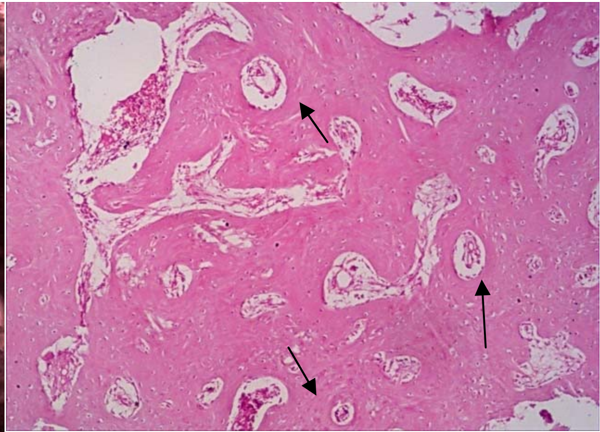

Figure 6. A photomicrograph of group (B) experimental specimen, showing arrangement of lamellae in concentric manner around bone marrow cavities (arrows) H&EX100

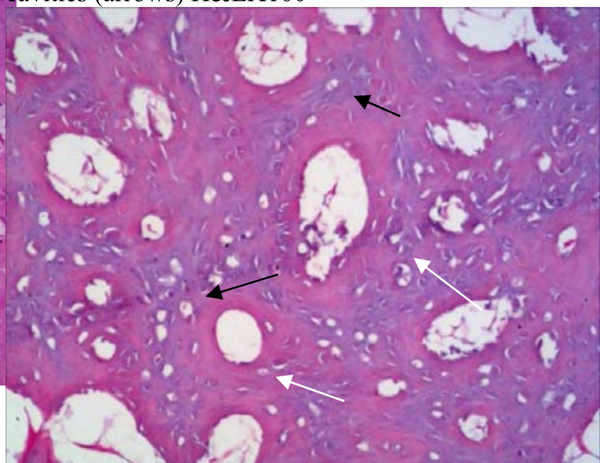

Figure 7. A photomicrograph of group (B) experimental specimen showing mature lamellar bone (RED) (white arrows) surrounding marrow cavities and less organized woven bone (BLUE) (black arrows). Masson Trichrome X 200.

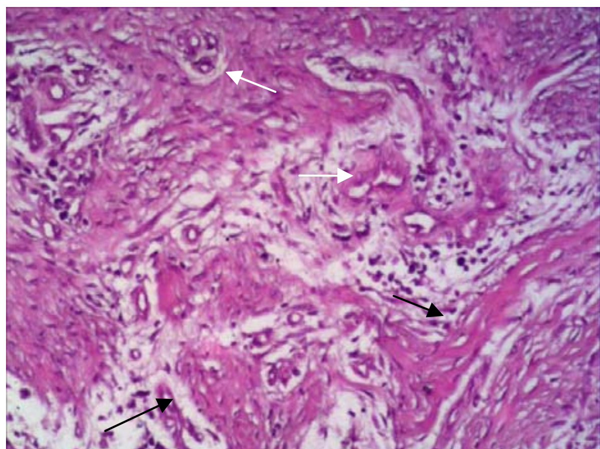

Figure 8. A photomicrograph of group A control specimen showing unconnected spicules of woven bone (black arrows) interspersed within the granulation tissue. Note the angiogenic activity (white arrows) and moderate inflammatory response.(H&E X200)

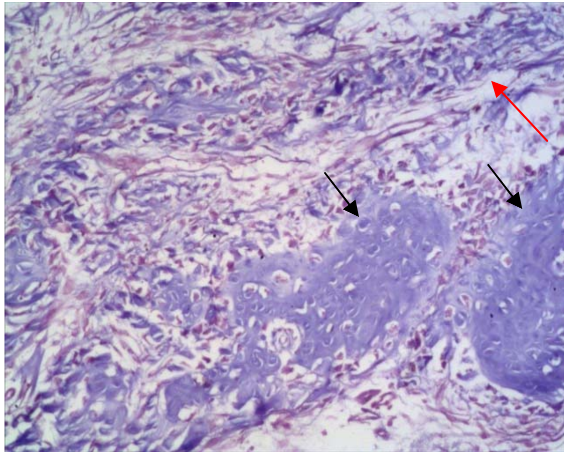

Figure 9. A photomicrograph showing irregular woven bone spicules rimmed by osteoblasts (black arrows), as well as densely packed collagen fibers (red arrows), (Masson Trichrome x200)

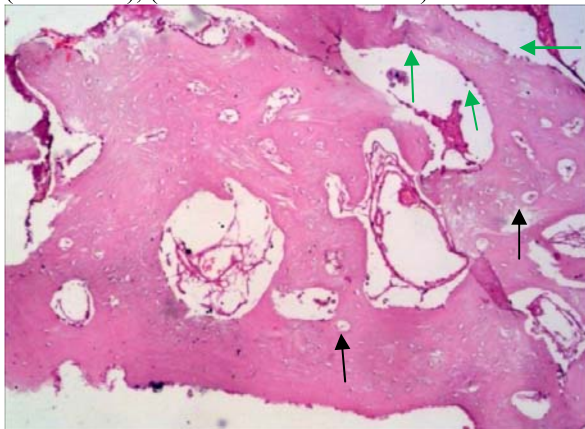

Figure 10. A photomicrograph of a specimen of group (B) control specimen showing woven bone with wide osteocytes (black arrows) and irregular marrow spaces. Note the osteoblastic rimming (green arrows) and sporadic lamellar organization (red arrows). (H&EX100)

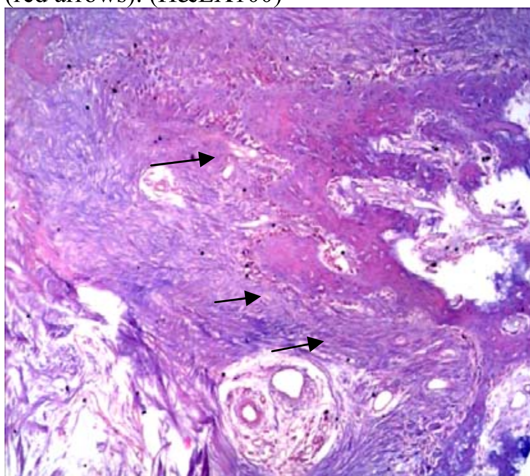

Figure 11. A photomicrograph of group (B) control specimen showing woven bone with wide osteocytes forming thickened irregular trabeculae. Note the widespread osteoblastic rimming (black arrows) and the dense fibrous background. (Masson Trichrome x100)

4. Discussion

Tissue engineering has been emerging as a valid approach to the current therapies for bone regeneration. Its approaches have recently proven to be very effective in bone regeneration and the successful repair of bone defects has been demonstrated in large animals like canines, goats and sheep. In particular stem cell-based therapies have shown great promise in regenerative medicine and continue to generate wide interest in future clinical applications.[21, 22 , 3]

In the current study, MBM SCs seeded on polymer scaffolds were inserted in fresh extracted bony sockets of upper lateral incisors in the experimental sites in mongrel dogs to demonstrate the ability of these cells to enhance bone healing and increase the amount of formed bone. The results were compared with unseeded polymer scaffolds inserted in fresh extracted bony sockets of upper lateral incisors in the positive control sites. Several studies have highlighted the potential for choosing alveolar socket defect models when histologically evaluating bone tissue-engineered constructs. The surgical procedure is simple, with limited risk of infection, and a similar intervention by grafting is advocated clinically.[23] De Kok *et al.* [19] stated that an alveolar socket model may be an appropriate model for initial clinical investigation of MSCs-mediated bone repair.

In the present research a polyglactin vicryl mesh was used as a polymer scaffold on which MBM-MSCs were seeded. This type of scaffold has been used for many years as substitute for natural tissue, as it is absorbable over 45 -60 days and highly biocompatible. [24, 25]

The histological results showed more prevalence of mature lamellar newly formed bone in the experimental sites than in the control sites in both groups. This denotes that the addition of MSCs to the PLGA scaffold resulted in more formation of better bone quality when placed in fresh extracted alveolar bony sockets than using the scaffold alone, which demonstrates the success of these cells in regenerating the bone defect. These findings are in line with Yang *et al.* [26] who investigated the effect of MBM SCs in enhancing bone regeneration in critical-sized rat calvarial defects and showed that stem cells' group produced more and higher quality mature lamellar bone compared to the control group.

One of the most important capabilities of MSCs is their migration capacity in response to signals produced by an injured bone. [27] In 2009, Granero-Molto *et al.* [28] stated that at the injury site, MSCs could help in repair in two ways; first by differentiating into tissue cells in order to restore lost morphology and function and second by secreting a wide spectrum of bioactive factors that help creating a repair environment owing to their anti-apoptotic and immune-regulatory properties by stimulating the proliferation of endothelial

progenitor cells. Hence, we can deduce that the local microenvironment and surrounding tissues may have provided the nutrients, growth factors and extracellular matrices necessary to support differentiation of the transplanted MSCs. This was also concluded by Krause *et al.* [29]

On the other hand one can attribute the improved results observed in the experimental sites of both groups in the present study solely to the bone forming capacity of MSCs, a conclusion that was also drawn by Pieri *et al.* [30] who showed that MSCs in combination with platelet-rich plasma-fluorohydroxyapatite (PRP-FHA) enhanced the amount of newly formed bone in the minipig mandible compared with PRP-FHA alone, with a similar effect to autogenous bone graft.

From the obtained results, it could also be assumed that polyglactin scaffolds may have provided an optimal support for MSCs in cell-guided regeneration. This was also suggested by other investigators who used polyglactin mesh for cell transplantation therapy and engineering of various tissues.[31-33] According to other studies, the surface of the polymer scaffold may also serve as a site on which various bioactive molecules from the wound site become concentrated, including growth factors and adhesion molecules matrix.[34,35] The complete closure of all alveolar bone sockets filled with unseeded polyglactin vicryl mesh in control sites in group (B) indicated that it did not hamper the physiologic bone healing response. The histological findings of our study suggest the complete resorption of the polymer scaffolds as there were no residuals evident in the histological sections.

Our results are also supported by Holy *et al.* [36] who showed that it is possible to induce bone regeneration, by combining cells isolated from the bone marrow with PLGA biodegradable scaffolds. Their results have shown significant bone regeneration in MSC-based PLGA scaffolds compared with PLGA alone in 1.2 cm bone defects in a rabbit femur. Moreover reported enhanced bone formation was reported in MSCs-containing transplants as early as 6 weeks after implantation in a mouse mandible compared with MSCs-free transplants.[36]

While some authors stated that engineered bone tissue can be used to repair clinical alveolar cleft bone defects, [38] others suggested that tissue-engineered bone may be sufficient for predictable enhancement of bone regeneration around dental implants when used simultaneously with implant placement. [39]The higher percentage of new bone obtained in the MSCs treated sites in the present study is consistent with previous studies, who used MSCs in enhancing bone regeneration in various animal models.[21,40,41] The findings of the current study also confirm previous reports in which the use of a cell transplantation approach combining different types of scaffolds with osteogenic cells could repair surgically created defects with comparable effect to autologous bone graft. [42,43]

On the contrary, the results of the present study do not agree with Henkel *et al.* [44] who grafted minipig mandibular defects with a bioactive matrix (60% hydroxyapatite and 40% β-tricalcium phosphate) alone or mixed with MSCs and found that the addition of MSCs did not enhance new bone formation after an implantation period of 5 weeks. The authors observed that the nutrition of the cultured osteoblasts seeded in the carrier material was insufficient for complete ossification to occur. Also in contrast to our study, Simsek *et al.* [45] found no significant difference in the amount of alveolar bone formation in class II furcation defects in dogs in the three groups involved in their study, where one group was treated with both MSCs and platelet rich plasma (PRP), another group treated with PRP alone and a third group treated with autogenous bone transplantation.

On the other hand Nasiff *et al.* [46] stated that MBM SCs possess osteogenic features *in vitro* and *in vivo* in conjunction with polymer scaffolds which can have diverse clinical application in maxillofacial bone regeneration. In addition, it was found that using nano-fibered scaffold puramatrix seeded with MBM SC in dogs' mandible can give higher bone implant contact around dental implants than using the puramatrix scaffold alone. [47] MSC transplantation was also shown to promote peri-implant bone regeneration and it was suggested to use this approach in clinical settings to enhance bone regeneration and healing in patients with poor bone quality.

Since our histological findings showed that there was no significant difference in the mean area percentage of bone between experimental sites after 1.5 months interval and those after 3 months, we may postulate that the application of MBM SCs to enhance bone regeneration could allow the insertion of dental implants as soon as 1.5 month postoperatively and thereby improving the psychological state of the patients as well as enhancing dental implant treatment outcomes in terms of bone quality and quantity.

Conclusion

Our histological findings show that implantation of PLG scaffolds seeded with MBM SCs immediately after extraction resulted in increase in the amount of the newly formed bone in fresh extraction alveolar bone sockets. MSC may therefore provide an effective therapeutic approach for the regeneration of alveolar bone defects. The procedure is efficient, exhibits low morbidity of the collection site, and is free from diseases incurred by transmission of pathogens. The conduction of further clinical trials on a larger scale with the

application of additional diagnostic tools are recommended.

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