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BONE MARROW MESENCHYMAL STEM CELLS- LOADED CHITOSAN/BIOGLASS SCAFFOLD FOR RECONSTRUCTION OF SEGMENTAL BONE DEFECT

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ABSTRACT

Objectives: To reconstruct a critically sized segmental bony defect using chitosan/ bioactive glass scaffold seeded with osteogenically differentiated stem cells with the evaluation of the engineered bone tissue. **Subjects and methods:** Eighteen adult male mongrel dogs were selected to be used in the present study. The experimental protocol was divided in to five stages; A. Teeth extraction. B. Bone marrow aspiration. C. Stem cells isolation and culturing. D. Scaffold preparation and seeding of BMSCs. E. Implantation of the engineered construct in the defect site. F. Evaluation of the newly formed osseous tissue. **Results:** Radiographic evaluation of the constructed tissue revealed the highest bone density (259.23 +/- 52.42 %) at week 16 by group I_2. Histomorphometric analysis revealed the increase in areas of mature newly formed bone by increasing time intervals. The highest mean bone area fraction value (47.72+/-1.94%) was recorded by group I_2 at 16week time interval. **Conclusion:** This study suggests that chitosan/ bioglass tissue engineering scaffolds seeded with BMSCs could be a reliable treatment modality for reconstruction of critically-sized bone defects.

KEYWORDS: Chitosan; Bioglass; Bone marrow stem cells; segmental defect

INTRODUCTION

Although the nature of bone enables the regeneration of minor defects, most large sized bony defects need surgical intervention [(1)]. Mandibular segmental defects could result from tumor therapy, severe traumatic injury or infectious diseases that result in loss of the vitality of the mandibular bone requiring its debridement. Treatment modalities have been changed significantly over the last years with the progress in the introduced techniques and technologies. Several treatment options have been attempted for the aim of bone reconstruction such as bone grafts. Autogenous bone graft is considered as the gold standard among graft types due to its osteogenic properties and absence of immunological reactions as the bone is harvested from the patient himself. However, limitations of autografts as limited availability, possible harvesting morbidity and the need for second operation had been reported[(2-4)]. Allograft is the second bone grafting technique where the bone graft can be taken from cadavers. Allogeneic bone is available in various forms, including demineralized bone matrix, cancellous chips, corticocancellous and

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cortical grafts, and osteochondral and whole-bone segments. However, the risks of immunoreactions and infection transmission limited its use. Besides, the lack of cellular components results in reduced osteoinductivity. The limitations of such treatment modalities directed the attention of maxillofacial surgeons towards tissue engineering area [5,6].

Tissue engineering (TE) was defined by Langer and Vacanti[7] as ''an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function''. One key feature, is the use of a scaffold that acts as a matrix for stem cells to proliferate and differentiate in to osteoblast that can lay down bone tissue at the same rate of scaffold degradation. Bone engineering scaffolds should be made of porous biodegradable materials with satisfactory mechanical, osteoconductive and osteoinductive properties. Extensive efforts in the field of tissue engineering through the past years have introduced several materials to be used as bone tissue engineering scaffolds [8-10].

Chitosan, natural biodegradable polymer, has recently been one of the commonly used tissue engineering scaffold material due to its biocompatibility, biodegradability, non-antigenicity, anti-tumor activity, anti-inflammatory effect, bio adhesive properties and wound healing ability [11,12]. In addition, chitosan played an important role in the attachment and differentiation of stem cells into osteoblasts [13,14]. However, chitosan is a polymer of low strength and high degradation rate that limits its use alone as a bone tissue engineering scaffold [15]. Several types of bioceramics were proposed to be used in conjunction with chitosan in attempts to overcome the draw backs of polymers. Chitosan/bioglass composite scaffolds have emerged recently to mix the flexibility of polymers with the stiffness, strength and bioactive character of the bioactive glass [16- 20].

Tissue engineering comprises two main approaches; the first approach involves using a scaffold upon which cells are seeded in vitro; cells are then directed to lay down matrix to produce the engineered tissue for transplantation. The second approach involves the use of scaffold being combined with growth factors without cells [21- 24].

Despite all the progress that has been made in tissue engineering field, clinical use remains limited due to some outstanding problems. Therefore, this study was performed to evaluate the effect of using bioactive glass/chitosan scaffold seeded with in vitro culture expanded, osteogenically induced BMSCs for reconstruction of mandibular segmental defects in dogs.

SUBJECTS AND METHODS

Grouping of the animals

Eighteen adult male mongrel dogs were selected and divided in to two groups; (I_1) control group that comprised dogs with segmental defects augmented with unseeded scaffolds, while (I_2) experimental group that comprised dogs with segmental defects augmented with BMSCs seeded scaffolds. Each group was further subdivided into 3 subgroups according to euthanize time intervals; A (8 weeks), B (12 weeks) and C (16 weeks) as presented in **table 1**.

TABLE (1) Factorial design of the subgroups:

Group	Subgroup (A)	Subgroup (B)	Subgroup (C)	Total number of dogs
I_1	I_1A	I_1B	I_1C	Nine
I_2	I_2A	I_2B	I_2C	Nine
Total	Six	Six	Six	Eighteen

A. Teeth extraction:

The dogs were anaesthetized using a mixture of xylazine HCL 1mg/kg body weight and ketamine HCL 5mg/kg via 20 gauge I.V cannula through the cephalic vein. Anesthesia was maintained by injection of sodium thiopental 25 mg/kg body

weight via the cannulated cephalic vein in a dilution of 0.5/0.5 liter saline solution. The right first molar in the lower jaw of each dog was extracted using an elevator and chisel.

B. Bone marrow aspiration:

The skin of the inner side of the dogs' tibia was shaved, washed and painted with betadine. A biopsy needle containing heparin (Cal-Heparine 5000 I.U/ Amoun/ Egypt) was introduced through the tibial tuberosity down for about 1.5 cm depth and the sample was aspirated. The samples were transferred immediately in an ice box to the Tissue Culture laboratory, Pathology Department, National Cancer Institute, where the tissue culture procedures were performed.

C. Stem cells isolation and culturing:

The isolation of MSCs was performed under strict aseptic conditions as follows: Dulbecco's Modified Eagle's Medium- low glucose with 1000 mg/L glucose, supplemented with 10% fetal bovine serum (FBS) and antibiotics (100U/ml penicillin and 100µg/ml streptomycin) was added to bone marrow sample and then centrifuged. The formed cellular pellet was resuspended in an expansion medium, and the cells were seeded at density of 1.8x 105 per cm2. The flasks were incubated at 10% CO_2 concentration, 95% humidity and a temperature of 37°C. After 3 days, the non-adherent cells were removed, the MSCs were expanded until 80% confluence and the medium was changed every 3 to 4 days for 10 days. The subcultured cells were then, centrifuged and the formed cell pellet was resuspended in 10 ml of osteogenic medium and the medium was changed every 3-4 days for 21 days.

D. Scaffold preparation and seeding of BMSCs:

Bioactive glass/chitosan (B/C) composite scaffolds (Nanostreams company, Egypt), with dimensions of (20x12x30mm) were effectively sterilized using 70% ethanol followed by 3% antibiotic (penicillin /streptomycin / amphotericin B). Half of the scaffolds (I_2) were seeded with osteogenically differentiated stem cells, while the other half (I1) was left unseeded.

E. Implantation of the engineered osseous tissue.

A segmental defect of almost 20x12x30 mm was created at the healed extraction site in each experimental dog. The dogs were prepared and anesthetized for the surgery the same way as for the extraction. The operative field was shaved and scrubbed with betadine where an 8 cm longitudinal incision was carried out using a number 24 Bard Parker blade along the inferolateral border of the mandible, deep fascia was dissected, and then the periosteum was reflected from the buccal and lingual surfaces to expose the cortical bone of the mandible. The proposed defect site and size was marked with a sterile permanent marker at mesial and distal ends by two vertical lines. At the mesial and distal peripheries of the defect, two vertical parallel cuts were made through the cortical bone on the buccal and lingual surfaces with a no. 6 fissure bur under copious irrigation with saline. Chisel and mallet were used to complete the bicortical segmental defect by creating two osteotomies along the preformed bony cuts until bone fracture. In the experimental group of dogs (I_2), C/B scaffolds seeded with differentiated BMSCs were implanted while in the control group of dogs (I_1), unseeded scaffolds were implanted. Two fixation plates were then placed over the defect and screwed tightly. The wound was then thoroughly irrigated using sterile saline solution and closed in two layers with a subcutaneous continuous 00 vicryl suture and interrupted 000 black silk skin sutures. The dogs were housed in separate cages and observed for any postoperative complications until the time of euthanasia.

According to the time period of each subgroup, the dogs were euthanized by hyper dosage of Thiopental Sodium in a concentration of 10% directly injected through the cannulated cephalic vein.

F. Evaluation of the newly formed osseous tissue:

1. Radiographic assessment

Digora software version 1.51 Sroedex Finndent was used for bone density measurement in this study. Digital images of radiographs were obtained using HP Scanjet scanner. Images were saved as BMP format with a resolution of 180 and 1800 X 1300 pixles.

2. Histomorphometric analysis

Histomorphometric analysis was performed using Masson trichrome stain where three microscopic fields were selected for each GTC-stained section. Photomicrographs were captured at original magnification of 20X. using digital camera (EOS206, Cannon, Japan) which was mounted on a light microscope (BX60, Olympus, Japan). Images were then transferred to the computer system where immunohistochemical assessment were carried out using Image J, 1.41a, (NIH, USA) image analysis software. The area fraction (AF) of the red/orange GTC-stained osteoid was measured automatically and represented the percentage of the newly formed osteoid to the total area of the microscopic field.

Statistical analysis

The obtained data was tabulated using the statistical Package for Social Science (SPSS 15.0) Software. The statistical tests included ANOVA and Post Hoc test for comparison of means. The results were considered significant when the P value was ≤0.05. Graphs were performed using Microsoft power point Software (Microsoft Office 2007).

RESULTS

1. Radiographic evaluation:

The Density of the newly formed bone at the defect site in the tested groups I_1 and I_2 at various time intervals was evaluated radiographically and presented in **table 2, figure 1**. Statistical analysis revealed significantly higher bone density for group I_2, at the end of each time interval; week 8, 12 and 16, than group I_1. The highest mean bone density value (259.23 +/- 52.42 %) was recorded by subgroup I_2C.

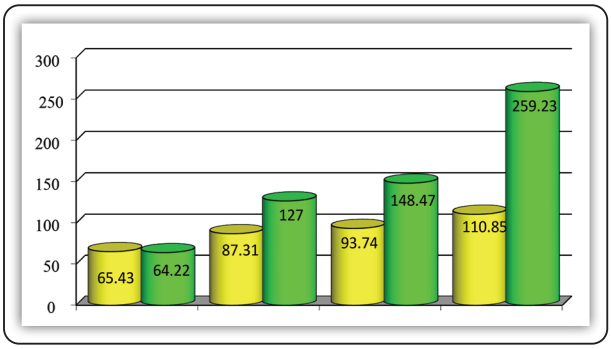

FIG (1) Mean values of bone density in both groups throughout the follow up period.

TABLE (2) Mean bone density (%) values of newly formed bone in the two tested groups at the end of each time interval

Subgroups	Group I_1		Subgroups	Group I_2		Probability
	Mean	SD		Mean	SD	
Immediate	65.43^{aA}	1.07	*Immediate*	64.22^{aA}	1.44	0.154 NS
I1A	87.31^{bA}	1.72	I2A	127.00^{bB}	2.25	0.00001*
I1B	93.74^{cA}	5.29	I2B	148.47^{cB}	5.93	0.0001*
I1C	110.85^{dA}	14.45	I2C	259.23^{dB}	52.42	0.005*
P value						

2. Histomorphometric analysis results:

Sections from the engineered bone constructs were stained by Masson trichrome stain that can detect the formation of new bone and differentiation between mature and immature newly formed bone, **figure 2**. Red stained areas represent the osteoid of immature newly formed bone while mixed greenish red areas denote the maturation of the formed bone. The red stained areas were larger in size with focal areas of bone maturation in the experimental group (I_2) seeded with stem cells than control unseeded group (I_1). Moreover, at longer time periods; mixed greenish red areas indicating the maturation of the newly formed bone were formed. These areas of matured bone were higher in subgroup I_2C that revealed the trabecular organization of the formed bone indicating further bone maturation. In addition, Statistical analysis showed that the highest mean bone area fraction value (47.72+/-1.94%) was recorded by group I_2 at 16 week time interval, while the lowest mean bone area fraction value (9.25+/-2.31%) was recorded by group I_1 at 8 week time interval, table 3 figure 3.

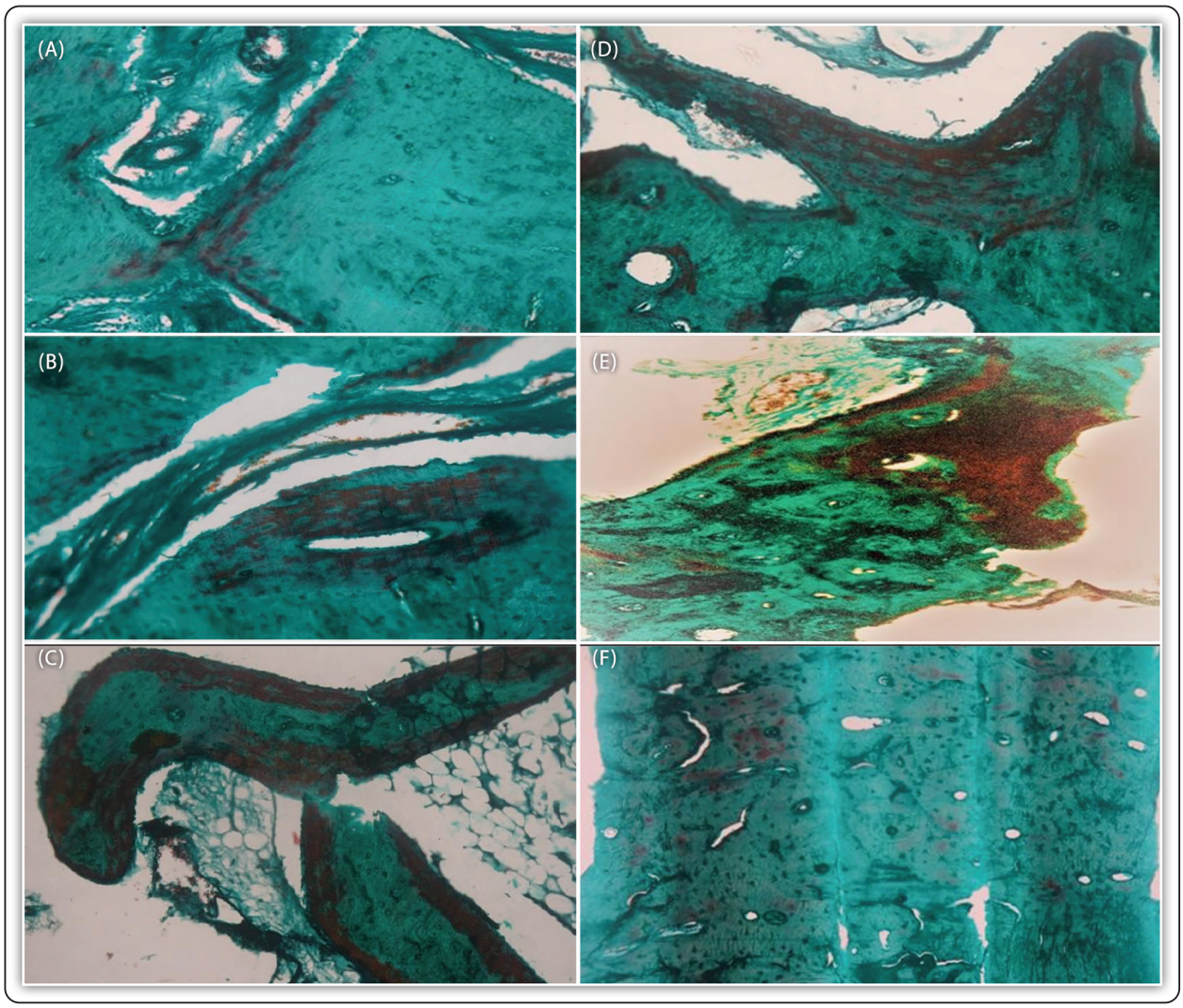

FIG (1) Photomicrographs A, B & C represent control subgroups I1A, I1B & I1C, respectively while photomicrographs D, E & F represent experimental subgroups I2A, I2B & I2C, respectively.

TABLE (3) Mean area fraction (%) values of newly formed bone in the two tested groups at the end of each time interval

Subgroups	Group I_1		Subgroups	Group I_2		Probability
	Mean	SD		Mean	SD	
I_1A	9.25^{aA}	2.31	I_2A	38.17^{aB}	2.82	<0.001
I_1B	13.96^{bA}	1.78	I_2B	42.93^{bB}	3.7	<0.001
I_1C	18.64^{cA}	1.61	I_2C	47.72^{cB}	1.94	<0.001
P value		<0.001			<0.001	

SD: standard deviation *Means are significant at P≤ 0.05*

Similar capital subscript letters in same raw indicate no significant difference, and similar small subscript letters in same column indicate no significant difference.

DISCUSSION

Since bone is a 3- D tissue, therefore it's beneficial to culture cells in a 3D scaffold instead of a 2D culture plate. In the present study, eighteen experimental dogs were used to evaluate the osteogenic activity of chitosan/bioglass composite scaffolds to regenerate critically sized bony defects. A segmental defect with dimensions 20 x 12 x 30 mm was created in the mandibular molar area of each dog. The scaffolds, either loaded (I_2) or unloaded with stem cells (I_1), were fitted in the defect sites and two fixation plates were placed over the defect. The choice of bioglass/chitosan composite scaffold for bone regeneration was based on the interesting characteristics of this composite that was reported by previous studies, in terms of porosity, degradation, strength and bioactivity[21-23]. Several studies[25-27] have demonstrated the high proliferation and differentiation capabilities of bone marrow derived mesenchymal stem cells. In addition, bone marrow MSCs directly contribute to becoming osteoblasts and osteocytes. Therefore, in the current study, mesenchymal stem cells were isolated from the bone marrow of the dogs, gaining the advantages of osteogenesis and osteoinduction of autografts. Osteogenic differentiation in-vitro was induced by subculturing cells in osteogenic medium supplemented with l- ascorbic acid, b-glycerophosphate and dexamethasone. This medium is a routine for osteogenic differentiation of bone marrow stromal cells as mentioned in several studies. [28] In vivo, osteogenic ability of the two groups of scaffolds; seeded and unseeded scaffolds was evidenced by radiographic, histological and histomorphometric analysis. The results of the present study showed higher bone regeneration capability of group I_2 where the defects were treated with BMSCs seeded scaffolds. Radiographic examination of the mandible was used to evaluate the bone formation at different time intervals. There was a significant increase in bone density at the defect site by increasing the time period up to 16 weeks in subgroup I_1C. This result might be an indication for benefits when implanting a scaffold in vivo where it can induce the differentiation of stem cells normally found within our bodies into osteoblasts inducing bone formation. This finding could be explained by a study carried by Boulila et al [19] where their results showed a series of physicochemical reactions in the periphery of the material leads to a gradual degradation of scaffolds and its transformation into an apatite layer. The

composition of bioactive glass makes the surface of the implant very reactive when exposed to an aqueous environment, leading to in vitro and in vivo biological activities. This mechanism was explained by Hench [29] who stated that the osteoinduction property of the bioactive glasses is attributed to the release of its dissolved elemental constituent such as silicon, calcium, sodium and phosphate species as it degrades into the physiological environment. The authors reported that certain combinations of some of these ions (such as silicon and calcium) trigger the osteoprogenitor cells to differentiate and produce new bone. Molecular biology studies have shown that seven families of genes involved in osteogenesis are stimulated by bioactive glass dissolution products, including insulin growth factor II (IGF-II), IGF binding proteins, and various proteases that cleave IGF-II from their binding proteins. IGFs are involved in the synthesis of collagen by osteoblasts, an essential component of bone [30-32]. The bone bonding is attributed to the formation of an HA layer, which cooperates with the collagen fibrils of the damaged bone to form a bond causing the proliferation and attachment of progenitor cells of the bone cell differentiation and excretion of the bone extracellular matrix, followed by its mineralization. Moreover, osteogenesis was confirmed by its positive red and green reaction to the Goldner's Masson Trichome stain, showing the production of newly formed immature bone and mature bone respectively. The results showed a significantly higher amount of mature bone formation in group I_2 than I_1 group. This was coincidence with the results of Wang P et al [33] where the new bone area fraction at 12 weeks for MSC seeded constructs was about 2.8-fold the that of unseeded constructs. In addition, Liao et al [34] found that the time of bone matrix development in a defect site was shortened from 2 weeks to 1 week when nanocomposite scaffolds were enriched with hMSCs. A study by Wang P et al [35] reported that the cell-encapsulated scaffold groups generated significantly more new bone than the counterpart without cell encapsulation at 3 months. In another study [36], nHA/CS/PLGA scaffolds combined with pre-osteogenic MSCs achieved the most bone regeneration than unseeded scaffolds. The high osteogenic activity of group I_2 scaffolds might be attributed to the effect of concentration of soluble ions of bioactive glass on osteoblasts differentiation and bone formation. It is accepted that the rate and type of dissolution ions released from bioactive glasses determine gene expression; however, the intracellular signaling pathways remain uncertain. The high bioactivity of nano bioglass could be related to the faster dissolution rate and the deposition process of hydroxyapatite layer as a result of the smaller the filler size and the larger the surface area. Finally, the significant difference between I_2 and I_1 groups pointed to the role of stem cells in enhancing the osteogenic activity of the scaffolds, indicating that seeding the cells on Chitosan/Bioglass composite scaffold yield a higher tendency for bone formation as demonstrated in the present study.

CONCLUSION

Under the circumstances of these investigations, it can be concluded that:

1. Chitosan and bioglass ceramic composite scaffold could be a good candidate to be used in the regeneration of large bony defect.
2. The use of osteogenically differentiated BMSCs accelerates and enhances bone tissue engineering.

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