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Freshly Isolated Versus Culture Expanded Bone Marrow Stem Cells in Healing of Bony Defects in Dogs

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ABSTRACT

Purpose: this study was conducted between 2011 and 2017 at the faculty of Veterinary Medicine Cairo University and aimed to compare the efficiency of freshly isolated bone marrow (F-BMMSC) versus culture expanded bone marrow (E-BMMSC) when added to the mixture of autologous platelet rich fibrin (PRF), collagen with nano-hydroxyapatite in healing of critical bony mandibular defects in mandibular site in dogs. **Material and methods**: 12 healthy adult Mongrel dogs were used in this study and bilateral mandibular critical bone defects (15X10 mm) were resected. Group A (The left side) contained: E-BMMSCs the expanded BMSC was seeded on collagen sponge with Nano-Hydroxyapatite and PRF membrane. Group B (The right side) contained: F-BMMSCs Gradient immediately separated BMSC seeded on the same scaffold. Radiographic follow up was done immediate, 4, 8, 12 weeks sacrificing was done 4, 8, 12 weeks postoperative. Local bone mineral densities (BMD) were measured on a DXA system. Histological evaluation was done using H&E. **Results** the results showed that group B right defects had better healing results than group A right defects all over the follow-up period, but the differences were not statically significant. **Conclusion** the study findings indicated that The F-BMMSCs that seeded in hydroxyapatite collagen type I HA-COL scaffolds combined with platelet rich fibrin PRF successfully repaired mandibular critical size defects, the same as the E-BMMSCs seeded in the same scaffold.

KEYWORDS

Bone, gradient, scaffold, expanded, stem.

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INTRODUCTION

Autogenously and synthetic bone substitutes were thought to have many limitations in treating critical size defects, so evolution of other treatment modality was needed. Improvement in the field of biotechnology and tissue engineering has offered new technique for repair of such defects, through the use of mesenchymal stem cells (MSCs), multipotent stem cells with osteogenic differentiation capabilities[1,2]. MSCs can be isolated from all connective tissues and bone marrow. The easiest location was found to be the tibial tuberosity via aspiration technique [3].

The bone marrow aspirate can be either cultured expanded or un-expanded. Processing of freshly (un-expanded) culture was done by Ficoll gradient separation of the aspirate, followed by (MNC) harvesting. It is rapid and easy process to increase concentration of MSCs that dramatically increases bone healing [4,5]. However, there is no consensus on the technique of bone marrow aspirate processing. So this study was carried out to compare between the use of Ficoll gradient separation and culture expanded bone marrow aspirate on repairing bone defect when added to collagen type I, non-hydroxyapatite and PRF membrane[6–8]

MATERIAL AND METHODS

Animal preparation:

This study included 12 healthy adult Mongrel dogs, with no gender predetermination, their weight ranged between (18 to 24 kg), their age ranged between 12 to 18 months years old. Were kept in the animal house under the veterinary observation at animal housing of the Surgery Department, Faculty of Veterinary Medicine, Cairo University.

Premedication and Anesthesia:

The anesthesia was done before bone marrow aspiration (15 days before the surgery). Atropine sulphate was injected subcutaneously 10-20 minutes before operation in a dose of 0.005mg/kg body weight. The anesthetic agent used was a mixture of Xylazine HCL 1mg/kg body weight and Ketamine HCL 5mg/kg via 20 gauge IV cannula through the cephalic vein

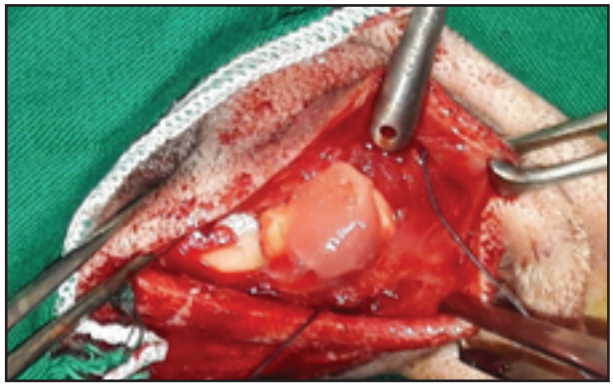
Surgical steps:

1. *Shaving and disinfection of surgical sites:* The dog skin was shaved; then washed twice with water and povidone iodine soap at both surgical and aspiration site.
2. *Bone marrow aspiration:* Using a sterile bone marrow trocar needle (18 gauge), bone marrow was aspirated into a plastic syringe containing 2 ml heparin.
3. *Expanded Bone marrow preparation E-BMMSCs: before 15 days of the surgery.* Aspirated 20 ml BM was then isolated with a density gradient at 2000 rpm/ min for 20 mins, washed with 15 mL saline (PBS), and re-suspended in (DMEM) then supplemented with 10% (FBS) serum and 1% penicillin/streptomycin. All cells were implanted at a concentration of 1.8×10^5 cells/cm3 in 50 cm^3 culture dish and incubated at 37°C.
4. *PRF protocol:*A blood sample was taken without anticoagulant in 10 ml tube which was immediately centrifuged at 3000 rpm 400 g for 10min.
5. *Immediate FICOLL gradient separation (F-BMMSCs preparation):* Another 20 ml bone marrow aspirate was used at the surgical field. Ficoll media (3.5 ml) was added to the 15 cm 4 falcon tubes, the 7.5 ml bone marrow sample were added on the Ficoll media solution.The samples were centrifuged for 30 min at 20°C. The upper layer containing plasma and platelets were drawn off leaving the MNC undisturbed at the edge. The upper layer was saved for later use. MNC layer was transferred to centrifuge tube by long cannula with syringe. The cells were suspended by gently drawing them in and

out of a pipette. Centrifuge was done at 400 g for removal of platelets for 10 min at 20°C.

6. *Preparation of the scaffold:* 2 Collagen cones were carefully put in 2 plastic dishes. 0.25 g of hydroxyapatite was mixed with each collagen scaffold. One of these cones was seeded by ten million E-BMMSCs through injection of the cells by 3ml syringe; the dish was labeled (L). The other cone was injected by 3ml syringe containing F-BMMSCs suspended on 2ml of Ringer's Lactate solution, and was labeled (R).

7. *Bone defects creation and augmentation:* Local anesthesia with vasoconstrictor was infiltrated into the periosteum and the local tissues. Midline 6 cm submandibular skin incision (extra oral approach). Blunt dissection of the mandible tissue. The periosteum incised. With low speed bur, bilateral mandibular border of the angle full thickness defects of 15 mm×10 mm were made between the angle of the mandible and the inferior alveolar canal surgically in each animal after determination of the size. (L) dish was inserted in the left gap. PRF membrane was inserted on the left scaffold. (Figure 1) Then, wound closure in layers. Right gap was performed as the left one. Right scaffold (R) was added.



Figure(1) The bone defect after filled with the scaffold with PRF.

8- *Radiographic X-ray analyses:* Mandibular radiograph was done under general anesthesia at 4, 8 and 12 weeks post-operative right and left. Comparisons were done between right and left in each case, and between groups each other's in order to determine the size of the healing defects.

9- *Sacrificing the animals:* The sacrifices were performed at the 4th, 8th, and 12th weeks post-operatively. The mandibles were placed in 10% Formalin solution for maximum 24 hours for DEXA then histological examinations.

10- *DEXA analysis:* (DEXA) analysis was done at sacrificed animal post-operation. The mandibles were harvested on groups A, B at 4, 8 and 12 weeks. The local bone mineral densities (BMD) were measured on a DXA system. To determine the BMD value of normal mandible and compare them with different constructs.

11- *Histological examination:* The specimens were stained by H&E for examination through the light electric microscope.

Statistical analysis

Special data files were developed in the computer conforming to the available data using SPSS (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) software program version 18.0. Data were analyzed and accessible in tables with means and standard deviation. Comparisons between the two methods used among studied cases regarding X-ray and DEXA results throughout times. Independent samples t test and Mann-Whitney test were finished for comparison between two samples and One-way ANOVA and Kruskal Wallis were done among more than two groups. p value was estimated for all previous statistics done. The relation was considered statistically significant when $p<0.05$. Tables were presented by using the same SPSS program.

RESULTS

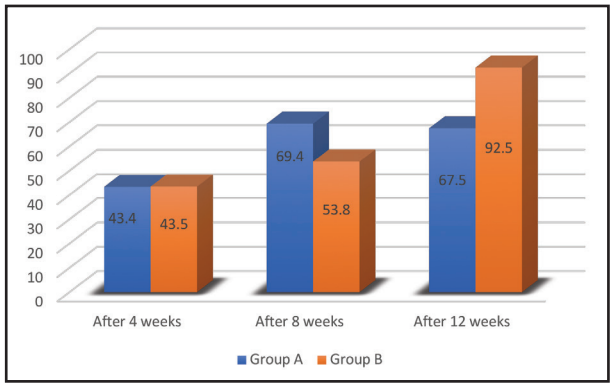
All animals survived the surgical procedure. Minor post-surgical edema was observed at the recipient site in each animal and resolved 4 to 6 days after the procedure. There was no sign of contamination.

X-Ray results:

At the experimental groups (A) and (B) little callus were observed in the defect post operation after 4 weeks reaching an average of 43.4 % and 43.5 % respectively. At 8 weeks post-operation, the quantity of newly formed bone highly increased in both groups reaching average of 69.4 % at group A and 53.8 % at group B. At 12 weeks group (B) shows nearly complete obliteration and higher opacification of the mandibular defects radiologically reaching an average of 92.5 % compared to an average of 67.5 % in group (A). On the other hand, x-ray analysis in each group revealed that the quantities of the radio-opacity also increased to reach the maximum level on 12 weeks reaching an average total value of 57.2% in group A compared to an average total value of 56.1% in group B.

Table 1: *Comparison between group A and group B according to X-ray percent improvement:*

	Group A Mean ±SD	Group B Mean ±SD	P
After 4 weeks	43.4±29.8	43.5±22.6	0.994
After 8 weeks	69.4±22.6	53.8±14.4	0.328
After 12 weeks	67.5±10.6	92.5±31.8	0.558
Total	57.2±26.3	56.1±26.7	0.907



Figure(1) Comparison between group A and group B according to X-ray percent improvement

Comparison between group A and group B according to X-ray percent improvement after four, eight and twelve weeks of plantation; the results revealed that the two groups insignificantly differed either after four weeks, eight weeks or twelve and in the total means of the two groups ($p<0.05$). (Table 1)(fig.2)

DEXA results:

The mean of total follow up periods in F-BMSCs group (B) the local BMD was 0.462 g/cm2, which was not significantly different from E- BMSCs group. After 4 weeks with better result among regular method E-BMMSCs scoring a mean of 0.589 g/cm2 compared to a mean of 0.498 g/cm2 in group B. At eight weeks F-BMSCs group (B) scored a mean of 0.5 g/cm2 compared to a mean of 0.488 g/cm2 in group (A). At twelve weeks. E-BMSCs group (A) Scored a mean of 0.401 g/cm2 compared to a mean of 0.388 g/cm2 in group (B).There was a significant difference between the two groups in 8 and 12 weeks of bone mass density.

DISCUSSION

Previous scientists studied the osteogenic and chondrogenic differentiation of BMMSCs when seeded in (COL/HA) scaffold. The study concluded that the COL/HA layer was the superior in osteogenic induction over either COL layer or pure HA. While COL layer or pure HA were better in chondrogenic induction [9].

The PRF was used to deliver GFs to the graft. It was proved to contain: transforming growth factor, (VEGF), (IGF-1),(PDGF-AB) , which help in the processes of stimulation of the BMMSC proliferation and differentiation in bone healing.[10,11]

Radiographic results at 4 weeks post-operative, in groups A and B were similar (43.4-43.5% respectively). It was reported that complete healing of the femur shaft was only observed at 4 weeks in 5 mm segmental defect model in rats when used of H-BMMSCs/COL/n-HA/TCP [12].

At 8 weeks in the current study, in groups A and B, the gap fill reached (69-53% respectively). Group A showed higher percentage of filling than group B but with no significant differences.

At 12 weeks, the radiographic results of our current study in groups A and B showed (67-92% respectively) healed bony defects. Group B recorded highly percentage of new bone than group A but with no significant differences. The healing was greater in both groups compared to the radiographic results of other studies that reported 41% new bone formation at 12 weeks when HA/b-TCP was loaded with BMMSCs for sinus augmentation in humans. The low percentage of their results may be due to the absence of the PRF growth factors present in our scaffold.[13].

CONCLUSION

The study findings concluded that bone marrow mesenchymal stem cells obtained from bone marrow can regenerate the critical bony defects; either freshly isolated F-BMMSCs or cultured expanded E-BMMSCs when seeded in collagen type I with a mixture of hydroxyapatite and PRF. The F-BMMSCs technique was easier and time saving procedure with low incidence of failure in preparation when compared to E-BMMSCs that necessitate laboratory equipment and additional operating sessions.

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