Influence of Autologous Adipose Derived Stem Cells and PRP on Regeneration of Dehiscence-Type Defects in Alveolar Bone: A Comparative Histochemical and Histomorphometric Study in Dogs

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Background and Objectives: Autogenous bone grafts is considered to be the best choice for reconstructive surgery. Adipose Derived Stromal Cells (ASCs) represents a promising tool for new clinical concepts in supporting cellular therapy. The goal of our study was to investigate bone regeneration following application of autologous ASCs with or without Platelet-Rich Plasma (PRP) at dehiscence-type defects in alveolar bone in dogs.

Methods and Results: Standardized buccal dehiscence defects (4×3×3 mm) were surgically created in eighteen dogs, the defects were grafted with either ASCs -PRP, ASCs alone, or without grafting material. Three months later; a bone core was harvested from grafted and non grafted sites for histological, histochemical and histomorphometric assessment. There was no evidence of inflammation or adverse tissue reaction with either treatment. Defects grafted with ASCs-PRP showed a significantly higher result ($p \leq 0.05$), with a mean area % of spongy bone and compact bone of (64.96 ± 5.37 and 837.62 ± 24.95), compared to ASCs alone (47.65 ± 1.43 and 661.92 ± 12.65) and without grafting (33.55 ± 1.74 and 290.85 ± 7.27) respectively. The area % of lamellated bone increased significantly reaching its highest level in group A followed by group B. Also a significant increase in area % of neutral mucopolysaccharides and calcified reactivity of Masson's Trichrome stain in groups A and B compared to group C was obtained.

Conclusions: Our results suggest that, the addition of PRP to ASCs enhances bone formation after 3 months and may be clinically effective in accelerating postsurgical healing in both periodontal and maxillofacial surgical applications.

Keywords: ASCs, PRP, Dehiscence-type defects

Introduction

The loss of one or more dental elements not only harms

the patient psychologically, but also causes atrophy of the tooth supporting tissues. Alveolar bone deficiencies may be attributed to a variety of factors, such as pulpal pathology, traumatic tooth extraction, advanced periodontal disease, implant failure, tumor, or congenital anomalies (1).

A fenestration defect is an isolated bone thickness deficiency at the labial vestibule, while a dehiscence defect is heading in an apical direction. For ideal treatment, it is important to evaluate the extent of the dehiscence/fenestration defect (2).

Regeneration refers to the reconstitution of a lost or in-

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jured part by complete restoration of its architecture and function. Alveolar bone regeneration techniques have undergone many advances over a short period of time (3). Therefore, reconstruction of alveolar bony defects using minimally morbid techniques enhanced the success and patient acceptance (4).

Bone defects around dental implants are often seen when implants are placed in areas with inadequate alveolar bone, (dehiscence defects, fenestration defects, residual intraosseous defects) in extraction sockets, or around failing implants. Bone regeneration in these defects by means of bone grafts or substitutes may improve the long-term prognosis of dental implants (5, 6). These anatomic bone defects, which cause the exposure of parts of the implants, may impair bone integration within a short period, as well as cause related infection problems (7).

Successful regeneration of bone deficiencies in the craniofacial skeleton, whether arising from trauma, tumor resection, or congenital disorders, continues to be a major concern to reconstructive surgeons. The principles of osteogenesis, osteoconduction, and osteoinduction are used to optimize therapeutic approaches to bone regeneration (8).

The transfer of bone autograft remains the preferred reconstructive method but is inadequate for large defects (9, 10). There has been recent interest in the development of new grafting materials using allogeneic, xenogeneic and synthetic bioimplants for reconstructive bony procedures. Numerous studies have compared the effectiveness of these alternatives as potential replacements for autogenous bone grafts (11-13).

A viable alternative to these procedures could be a new discipline that combines cell therapy and tissue engineering to develop a more effective and safer therapeutic system for bone regeneration (14).

Recently, it has been discovered that human adipose tissue has a higher concentration of adult stem cells than any other tissue in the body (15, 16). Adipose tissue might be a promising alternative source of stem cells that could have far reaching effects on several fields including bone engineering. Adipose Derived Mesenchymal Stem cells (AMSCs) studies demonstrate a diverse plasticity, including differentiation into adipo-, osteo-, chondro-, myo-, cardiomyo-, endothelial, hepato-, neuro-, epithelial and hematopoietic lineages, similar to that described for bone marrow derived MSC (17, 18).

The rationale for the local application of Platelet Rich Plasma (PRP) in bone surgery is the release of growth factors present in the platelet. They include platelet derived growth factor (PDGF), transforming growth factor- β

(TGF- β), insulin-like growth factor-I (IGF-I) which are involved in reparative processes including osteogenesis. There is local release of different growth factors which activate the osteoblasts, chondroblasts, fibroblasts and osteoprogenitor cells. They increase the extracellular matrix formation and haemopoiesis. The addition of (PRP) to the recipient site after decortication and perforation allows for growth factors to accelerate wound healing by stimulating angiogenesis and mitogenesis (19-21). However, their capacity to improve the osteogenicity of MSCs *in vivo* has not been well characterized. Recent studies have suggested positive, synergistic effects of MSCs and PRP mixtures in enhancing bone formation in the oral and maxillofacial regions (22, 23).

The purpose of this study was to evaluate the effect of the combination of Adipose Derived Stromal Cells (ASCs) and (PRP) on bone regeneration of dehiscence-type defects in alveolar bone.

Materials and Methods

Transplant preparation and placement

Experimental animals: Eighteen clinically healthy *Canis familiaris domesticus* (1- to 3-year-old, weighing 8 to 10.1 kg) were used in this study. These dogs were treated in accordance with the guidelines approved by the Animal Use Committee of Cairo University.

A) Adipose derived stem cell preparation: Isolation and culture of Adipose-Derived Stem Cells (ASCs); According to Tomiyama et al. (24) Adipose tissue was excised from both the omentum and the inguinal fat pad of dog under general anesthesia. The adipose tissue was resected and placed into a labeled sterile tube containing 15 ml of a phosphate buffered solution (PBS; Gibco/Invitrogen, Grand Island, New York, USA). Enzymatic digestion was performed using 0.075% collagenase II (Serva Electrophoresis GmbH, Mannheim) in Hank's Balanced Salt Solution for 60 minutes at 37°C with shaking. Digested tissue was filtered and centrifuged, and erythrocytes were removed by treatment with erythrocyte lysis buffer. The cells were transferred to tissue culture flasks with Dulbecco Modified Eagle Medium (DMEM, Gibco/BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco/BRL) and, after an attachment period of 24 hours, non-adherent cells were removed by a PBS wash. Attached cells were cultured in DMEM media supplemented with 10% fetal bovine serum FBS, 1% penicillin-streptomycin (Gibco/BRL), and 1.25 mg/L amphotericin B (Gibco/BRL), and expanded *in vitro*. When large colonies developed (80~90% confluence), cultures

were washed twice with PBS and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (Gibco/BRL) for 5 min at 37°C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50 cm² culture flask (Falcon). The resulting cultures were referred to as first-passage cultures and expanded in vitro until passage three.

Differentiation into neurocytes was achieved by adding beta-mercaptoethanol, dimethyl sulfoxide, and conditioned medium for neuron induction. Differentiation was confirmed by detection of nerve growth factor (NGF) gene expression in cell homogenate (25).

B) Platelet-rich plasma preparation; A 10 ml volume of autologous blood was drawn from each animal. Briefly, the PRP preparation was performed according to a previously reported protocol (26). One monovette was filled with 8.5 ml solution (7 ml blood and 1 ml citrate-phosphate-dextrose-adenine [CPDA] solution for anticoagulation). The first spin was performed at 2400 r.p.m. for 10 min dividing the blood into three basic components; red blood cells (RBC), Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP). PRP and PPP were collected in a second monovette. Then, a second spin was performed at 3600 r.p.m. for 15 min. The PPP was removed so that only the PRP pellet remained in the monovette. Accordingly, 0.4 ml volume of PRP was produced containing a mean platelet count value of $2520 \pm 834 \times 10^3 / \mu\text{l}$ and high mean concentration values of growth factors (i.e. 295 ng/ μl PDGF-AB and 500 ng/ μl TGF- β 1) (27). At the time of application, constitution of ASCs/PRP admixture was performed by mixing ASC pellet of (10^7) with 1 mL of PRP before implantation. The ASCs/PRP admixture was then activated with a 10% calcium chloride solution and 100 U/ml sterile bovine thrombin. Platelets released their granular content and platelet gel was obtained. Within a few seconds, the PRP displayed a sticky consistency that is relatively easy to apply to the surgical defects.

C) Animal model and surgical procedure; All surgical procedures were carried out under aseptic conditions and general anesthesia. The animals were premedicated with ketamine and xylazine and anesthetized with halothane. The perioral tissues and gingiva were disinfected with povidone iodine (Betadine, Purdue Pharma, Norwalk, CT). Surgery was conducted with due attention to aseptic technique. Standardized buccal dehiscence defects (4×3×3 mm) were surgically created in each dog (Fig. 1A). Eighteen dogs were divided into three groups. In six dogs the defect was grafted with Adipose Tissue-Derived Stem Cells (ASCs) and PRP (group A), and in other six dogs

the defect was grafted with Adipose Tissue-Derived Stromal Cells (ASCs) alone (group B), while the defect in the last group was left unfilled as a negative control (group C). The flaps were replaced and sutured using resorbable sutures (Dexon 3-0, Davis and Geck, Monofil Inc, Manati, Italy). Antibiotic and analgesic therapies were applied for 7 and 3 days, respectively (cephalosporin 1 g/day and ketoprofen 500 mg/day). During the first postoperative week, the animals were checked daily for signs of infection.

D) Specimens preparation for histological and histochemical examination; Three months later, a bone core was harvested from grafted and non grafted sites using a 3-mm trephine drill (Cizeta Surgical, Bologna, Italy), after scraping away the overlying soft tissues. The fixed specimens were decalcified in 20% ethylenediamine-tetra-acetic acid (EDTA) (Quality Biological, Inc., Gaithersburg, MD, (<http://www.qualitybiological.com>) PH 7 with a change per week for ten weeks until decalcification was completed. Neutral EDTA decalcifying solution consisted of: EDTA, (di-sodium salt) 26 gm, distilled water 1750 cm³ and 26 gm sodium hydroxide was added gradually to adjust the PH of the solution.

I. Histological examination: The specimens were dehydrated in ascending grades of ethanol, infiltrated in xylene, embedded in paraffin. Paraffin blocks were sectioned at a 5 μm thickness and stained with hematoxylin and eosin stain (H & E) by the standard technique (28).

II. Histochemical examination:

1. Masson Trichrome technique: (29) for detection of collagen and lamellar bone formation.
2. Alcian Blue-Periodic Acid Schiff (AB-PAS) (pH 2.5) technique (30):

Results Acidic mucopolysaccharides (AMP) red

Neutral mucopolysaccharides (NMP) ... blue

III. Histomorphometrical examination: All the stained sections were examined by image analyzer computer system using the software SIS, Germany, which comprises a light microscope (Olympus Bx60 Japan), capable of performing high speed digital image processing for the purpose of cell measurement. It is calibrated automatically to convert the measurement units (pixels) produced by the image analyzing program into actual micrometer units.

For histomorphometrical analysis of H&E stained sections, the surface area of spongy bone and the width of compact bone were measured. The area % of spongy bone was measured in each case, where five fields was captured using a magnification (×200). For each field of spongy bone the surface area of bone was measured in a measuring frame of area 192000 pixel² and then the area % was calculated. The color of bone trabeculae was automatically

selected, converted into gray color, then masked by a red color, and finally the image was converted into black and white to allow automatic measurement by the computer system. For the compact bone, five other fields were selected using a magnification ($\times 100$). The width of compact bone selected was measured at three different points. The mean value was calculated for each field.

The area containing the most uniformly positive histochemically stained tissues were selected for evaluation us-

ing magnification ($\times 200$) and followed the same previous steps.

Statistical analysis

Statistical evaluation of data was carried out using the SPSS/PC_ Statistics software package (version 15; SPSS Inc., Chicago, IL). Group means $\pm$ standard deviations (SD) were calculated for the quantitative variables. Comparison between groups was done using non-para-

Fig. 1. (A) A buccal dehiscence defects ($4 \times 3 \times 3$ mm) were surgically created. (B) Group A: a photomicrograph showing increased number of marrow spaces (MS) with osteoblast lining. (C) Group B: a photomicrograph showing decreased lamellar maturation, note the deposition of thick osteoid tissue with well observed osteocytes (O). (D) Group C: a photomicrograph showing woven bone threads (W) with minimal amount of lamellar bone ($\surd$) and increased marrow spaces (MS).

metrical Kruskal- Wallis and Mann-Whitney tests. $p \leq 0.05$ was considered statistically significant.

Results

The healing process was uneventful in all the animals over the 3-month

Histological results

After 3 months all bone defects were filled with newly formed bone with variable amounts, thickness and density in each group. Both group A and B had parallel fibred and lamellar structures. As regard group A, a newly formed trabecular width with a recognizable amount of osteoblast was observed. Moreover, the bone architecture revealed an increased number of active marrow spaces with osteoblast lining (Fig. 1B). In group B an observable decrease in bone maturation was noticed with deposition of a thick and uniformly distributed osteoid bone concomitant with a relative increase in osteocytes. The cellularity of woven bone was highly detected (Fig. 1C). In group C the bone morphology was mainly consisted of woven bone threads with minimal amount of lamellar bone (Fig. 1D).

Histomorphometrical results

Masson trichrome histochemical stain applied to mature normal bone exhibited two main reactions: a blue reaction mainly localized to the osteoid tissue and collagen fibers distribution, and a red reaction for lamellar bone formation. As regard group A histochemical results of all specimens in this group revealed an increase in calcified bone as evident by red reaction, while in group B the blue reactivity was more pronounced than the red one. Similar results with weaker reactivity of red calcified tissue were detected in group C indicating thick and organized blue collagenous fibers, bundles of highly formed cellular woven bone (Fig. 2).

Alcian blue (pH 2.5) PAS stain: Normally, the completely formed bone matrix exhibited a high reaction for NMP represented by a faint blue color i.e positive to Alcian blue stain. However, As regard to group A, a highly positive reaction for NMP represented by obvious blue color and a decrease amount of AMP in bone matrix were noticed as a result of proceeding complete bone matrix formation. Also in group A, since normal bone formation was nearly completed Alcian blue staining gave highly positive reaction due to the increase in minerals deposition in bone matrix, While distribution of PAS positive reactivity was weakly noticed due to the decrease acidophilic reactivity. In group B, decreased amount of NMP

Fig. 2. Masson trichrome histochemical stain: (A) A photomicrograph of group A showing lamellar bone with marked accumulation of connective tissue and osteoid tissue (Masson trichrome $\times 40$). (B) A photomicrograph of group B showing lamellar bone with increased distribution of connective tissue in association with osteoid tissue deposition (Masson trichrome $\times 40$). (C) A photomicrograph of group C showing woven bone with multiple bone marrow spaces, entrapped osteocytes and moderate connective tissue (CT) (blue color) (Masson trichrome $\times 40$).

Fig. 3. Alcian blue (pH 2.5) PAS stain: (A) A photomicrograph of normal bone (Alcian-PAS $\times 100$). (B) A photomicrograph of group A showing increased distribution of NMP (blue color) in the formed bone (Alcian-PAS $\times 100$). (C) A photomicrograph of group B showing equalized distribution of NMP and AMP (Alcian-PAS $\times 200$). (D) A photomicrograph of group C showing increased distribution of AMP (red color) (Alcian-PAS $\times 200$).

will give rise to poor reactivity of Alcian blue stain than that of group A, and almost increase of PAS reactivity. Similar results to group B were noted with group C but with weaker reactivity to NMP (Fig. 3).

The area % of lamellated bone increased significantly throughout the experimental groups reaching its highest level in group A followed by group B. Similar and more confirming results were obtained with histochemical results with a significant increase in area % of neutral mucopolysaccharides and calcified reactivity of Masson's Trichrome stain in the therapeutic groups A and B compared to the control group C (Table 1).

Discussion

Atrophy of the alveolar bone always presents difficulties for carrying out procedures which involve the placement of implants, and this problem has prompted the development of a number of techniques in order to obtain bone in sufficient quantity and quality. Buccal dehiscence defects are one of the most commonly encountered problems in implant dentistry.

Since bone and cartilage tissue engineering requires large amounts of osteogenic/chondrogenic precursor cells, new sources of progenitor cells are needed. Compared with Bone Marrow Mesenchymal Stem Cells (BMSCs), ASCs have the same ability for osteogenic differentiation, and this ability is maintained with increasing donor age

Table 1. The histomorphometric analysis of study groups after 3-months healing period (values are mean $\pm$ SD)

	Control	Group A	Group B
Area% of spongy bone	33.55 $\pm$ 1.74	64.96 $\pm$ 5.37	47.65 $\pm$ 1.43
P1		0.002	
P2			0.002
P3			0.002
Area % of compact bone	290.85 $\pm$ 7.27	837.62 $\pm$ 24.95	661.92 $\pm$ 12.65
P1		0.002	
P2			0.002
P3			0.002
Masson trichrome	16.36 $\pm$ 0.91	8.10 $\pm$ 2.65	11.15 $\pm$ 0.95
P1		0.002	
P2			0.002
P3			0.041
Alcian-PAS	13.26 $\pm$ 2.14	31.58 $\pm$ 7.94	22.00 $\pm$ 9.95
P1		0.002	
P2			0.394
P3			0.240

P1=control vs. Group A, P2=control vs. Group B, P3=group A vs. Group B

*p is significant at the ≤ 0.05 .

(31). The simple surgical procedure, the easy and repeatable access to the subcutaneous adipose tissue, and the uncomplicated enzyme-based isolation procedures make this tissue source for MSCs most attractive for researchers and clinicians of nearly all medicinal subspecializations. Therefore, ASCs was chosen in this study as they represent an alternative source of autologous adult stem cells that can be obtained repeatedly in large quantities under local anesthesia with a minimum of patient discomfort. Most importantly, a comparative analysis of MSCs obtained from bone marrow, adipose tissue, and umbilical cord clearly showed that ASCs were not different regarding morphology, immune phenotype, and success rate of isolating MSCs, colony frequency, and differentiation capacity (32, 33).

Collagen is a member of major importance in the extracellular matrix bone tissue. It also changes according to the physiologic state of bone, as any alteration in its distribution can be indicative for the different changes in bone tissue during the study (34). In the present study, the decreased amount of osteoid matrix in the control group (C) indicates a delayed sign of bone repair. while increased amount of calcified tissue in the therapeutic groups (A and B) reflects accelerated bone remodeling and effective therapy. Moreover, the presence of thin osteoid layer covering most of the peripheries of bone trabeculae

in groups A and B is a sign for continuous remodeling this was in accordance with Loveridge, (1999) (35).

In correlation with the histological findings in the present study, the histochemical results of this work revealed that the control group showed rich acido mucopolysaccharides reaction. Torricelli et al. (2002) (36), during their study of bone healing, stated that mucopolysaccharides macromolecules increased at the site of new bone formation. Furthermore, histological and histochemical description of the first secreted matrix resembling a cement line introduced in bone remodeling by mono nuclear mesenchymal cells. Moreover, histochemical findings of the present study revealed presence of both neutral and acidic mucopolysaccharides in collagenase matrix. The PAS positive reactions were related to immature collagen that had high hexose percentage, which predominate in early stage of bone healing (37).

Some researchers suggested that the ASCs in the grafted fat repairing the local tissue either directly or through the promotion of angiogenesis or neovascularization, by the secretion of cytokines, such as hepatic growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PGF), transforming growth factor ($TGF\beta$), fibroblast growth factor (FGF-2), and angiopoietin (38). Others emphasize the relation between preadipocytes and macrophages, and suggest that the healing effect may be related to an enhanced immune response leading to tissue remodeling and the removal of damaged cells. Lastly, the release of hormones, cytokines, or growth factors by the ASCs and local tissue may direct integration or differentiation of the fat-grafted material (39).

PRP is a new application of tissue engineering and a developing area for clinicians and researchers. Recently, a combination of PRP with different types of grafting materials and barrier membranes has also been used in intrabony defects and regenerative periodontal therapy (40, 41). The results of the present study showed that PRP with ASCs induced alveolar bone regeneration, we did not find epithelial invasion or ingrowth, which is characteristic of the inadequate healing process that marks periodontal disease, especially in ASCs/PRP and ASCs groups at all time points. We speculated that this phenomenon may be due to the strong connection between the defect and the apical root side so that epithelial ingrowth could be completely prevented. Furthermore, the gel property of PRP allows ASCs to be injectable and to stay in the implanted site. Most important, this autologous product eliminates concerns about immunogenic reactions and disease transmission (42).

Histomorphometry of cancellous bone is a useful tool

to estimate changes in bone remodeling because approximately 25% of cancellous bone is resorbed and replaced every year in adults compared to only 3% reduction in cortical bone and this explains why in most of papers focused more on cancellous bone (43). The histomorphometric results in the present study revealed that defects grafted with ASCs-PRP showed a significantly higher result ($p \leq 0.05$), with a mean area % of spongy bone and compact bone of (64.96 ± 5.37 and 837.62 ± 24.95), compared to ASCs alone (47.65 ± 1.43 and 661.92 ± 12.65) and without grafting (33.55 ± 1.74 and 290.85 ± 7.27) respectively. These results implicated that ASCs can produce synergic effect with PRP. Our findings confirm previous reports in which the application of the PRP in dentistry may have successful results and beneficial outcomes in the tissue regeneration, bone remodeling and general wound healing.

Platelet-derived growth factor is considered one of the primary healing hormones in any wound and is found in great abundance within platelets. These growth factors enhance bone formation by increasing the rate of stem cell proliferation and inhibiting osteoclast formation, which decreases bone resorption (21). It is proved that growth factors added to bone grafts and synthetic bone substitutes lead to better results in bone regeneration and shortening the healing period. Although the growth factors and the mechanisms involved are still poorly understood, the ease of applying PRP in the dental clinic and its beneficial outcomes, including reduction of bleeding and rapid healing, holds promise for further procedures. However, the efficacy of PRP in bone regeneration remains unclear, in particular because PRP does not contain bone morphogenetic protein (BMP). BMP is the most potent osteoinductive protein that promotes stem cell differentiation into the osteoblastic lineage and the only known growth factor that can induce ectopic bone formation (44). On the other hand, many other studies have reported that PRP has a positive influence on bone regeneration with or without any stem cells (45). The positive effect of PRP on bone remodeling could be attributed to the angiogenetic, proliferative and differentiating effects on osteoblasts of the high concentrations of TGF- β and PDGF contained in PRP (44). The improved results observed with the ASCs-PRP group may in fact be due to the bone forming capacity of ASCs, with synergistic effect from PRP which served as a cytokine cocktail or a cell delivery carrier.

Within the limit of this study, it can be concluded that after a 3-month healing period the addition of ASCs in combination with PRP enhanced the amount of newly formed bone in the alveolar bone of dogs compared with

ASCs alone, as PRP may become a routine treatment modality for periodontal regeneration and improve surgical results in a variety of procedures in the field of oral and maxillofacial surgery in future. In addition the use of ASCs in the future is an exciting concept and one that will surely continue to be explored further in the laboratory and clinically as it can be easily harvested and proliferated.

Potential conflict of interest

The authors have no conflicting financial interest.

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