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HEALING EVALUATION OF MANDIBULAR BONE DEFECT TREATED WITH PLATELET RICH FIBRIN SEEDED WITH DENTAL PULP STEM CELLS IN MALE ALBINO RATS

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ABSTRACT

Using tissue engineering-based therapies that utilize biomaterial scaffolds covered with osteogenic cells is the novel procedure of bone regeneration. Platelet rich fibrin (PRF) is a 2nd generation platelet concentrates used effectively in different applications in dentistry.

Aim: To investigate the effect PRF membrane, alone or in combination with human dental pulp stem cells (hDPSCs), on the healing of large mandibular bone defect in albino rats.

Methodology: Bone defects were created out in the mandibular angle of 36 male rats and divided into 3 groups: (I) Control gr. (II) PRF gr. and (III) PRF + hDPSCs gr.: the defect was filled with PRF membrane seeded with hDPSC. the animals were scarified after 15 and 30 days. Sections of 4 μm were processed for H&E and Masson's trichrome staining.

Results: measuring the surface area of bone defect at day 14 revealed a significant smaller defect size in control group than that of treated groups (II and III). At day 30, the bone defect of all groups markedly decreased in size. But, the quality of the regenerated bone in group III was superior than group II followed by group I which was confirmed by new mineralized bone revealed by Masson's trichrome staining.

Conclusion: using hDPSCs and PRF membrane had no effect on reduction of the bone defect size but it showed a high quality regenerative effect on the newly formed bone filling the defect which may represent a potential alternative for bone regeneration. But further investigations are needed.

KEYWORDS : Platelet rich fibrin, Platelet concentrates, human dental pulp stem cells, critical sized bone defect and natural biomaterial scaffolds.

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INTRODUCTION

The Bone in the maxillofacial region plays the most important role in keeping the facial appearance and function. So, one of the most important maxillofacial surgical challenges is reconstruction of large bone defect caused by severe trauma or tumor resection especially in the craniofacial region. The common procedure of bone regeneration is to use bone graft materials [1]. From a biologic point of view autogenous bone is the most suitable bone graft materials due to its shorter healing time, production of better bone quality in addition to there is no immune rejection may be present. But, its main limitation is difficulty of harvesting large amounts of bone. Furthermore, presence of many donor site complications and considerable resorption after grafting. Other complications also may be present such as wound dehiscence, infection that leads to implant failure [2,3]. Therefore, other bone alternatives including xenogeneic, allogenic and alloplastic materials have been used as possible treatments in spite of their restrictions as immune rejection possibilities, infection and pathogen transmission risk [4,5]

Nowadays, significant improvement has been developed in maxillofacial surgery by using tissue engineering based treatments which utilize scaffold materials loaded with osteogenic cells and/or osteo-inductive growth factors [6]

Growth factors and stem cells are the main factors of the healing potential of the tissue, therefore the researches have shifted to the blood derivatives, which are the most predisposed to the process for regenerating concentrate these molecules, as platelet derived growth factors (PDGF), platelets rich plasma (PRP), Platelets rich fibrin (PRF) and Platelets lysate (PL) [7].

Platelet concentrates have been used to improve healing of bone tissue in maxillofacial surgery. The main advantages of platelet-concentrates are their safety as an autologous source that help to improve early immovability of the graft. they stimulate bone formation with minimal inflammatory reaction and complications. They encircle high elements of growth factors, generated from platelets at the time of tissue damage, which are very essential for tissue regeneration. [8, 9].

PRF was first pronounced by Choukroun et al. 2006 [8]. It has been considered as second generation platelet-concentrate. It is a rich source of growth factors and cytokines. In addition, it considered as a biomaterial in clinical application [10]. PRF is a three dimensional polymerized fibrin meshwork which are biocompatible and biodegradable. This fibrin meshwork contains all factors that play an important role in wound healing as platelets, leukocytes, cytokines (interleukin-1b, IL-4 and tumor necrosis factor α), growth factors as transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), matrix glycoprotein and insulin like growth factor1 (IGF1). Also, it acts as a matrix for tissue forming cells migration as fibroblasts and endothelial cells that are responsible for angiogenesis and re-modeling [11,12].

Nowadays, PRF is used effectively in different applications in dentistry as treatment of different inflammatory bone lesions [13, 14], sinus elevation [15], gingival recession and socket preservation [16]

One of the most important strategies for bone regeneration is the use of stem cells. they are undifferentiated cells with high proliferative and regenerative potential so, they have the ability to differentiate into different types of tissues. Many niches of adult type human stem cells have been discovered as peripheral blood, bone marrow, skin, adipose tissue, dental pulp, of adult or exfoliating teeth, and periodontal ligament [17, 18]. Mesenchymal stem cells (MSCs) have been used successfully in scaffold based tissue engineering [19]. Dental stem cells display MSCs features and have the ability to differentiate into many cells as odontoblasts,

adipocytes, osteoblasts, chondrocytes and myocytes [20]. Dental stem cells can be harvested from many sources of dental tissues such as dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells, apical papilla stem cells and dental follicle stem cells. These cells can be attained more easily than bone marrow stem cells (BMSCs) [21]. But, large bone defect repair does not only need stem cells therapy, but also require bone grafts or scaffolds to encourage bone induction [22].

Many authors mixed platelet rich fibrin with different bone graft materials to accelerate bone defect healing as Karayürek et., al 2019 [23], Oliveira et., al 2015 [24] and Durmuşlar, et., al 2016 [25]

On the other hand, other authors used different types of bone grafting materials or scaffold loaded with stem cells to accelerate bone defect healing [26,27].

So, the aim of the current study is to investigate the effect PRF as a natural scaffold material, alone or in combination with human dental pulp stem cells (hDPSCs), on healing process of large mandibular bone defect in male albino rats.

MATERIAL AND METHODS

hDPSCs preparation and characterization:

Cryopreserved cell line of sub-cultured human dental pulp stem cells (hDPSCs) at 3rd passage were purchased from Nile Center for Experimental Researches, Mansoura city, Egypt. The recovery of the cells was performed under aseptic condition in a biosafety laminar flow hood. The frozen cells were thawed in a in a 37 °C water path for 60 mm. In a biosafety hood, cells were transferred into a sterile 15-mL tube containing a pre-warmed 5 mL growth media of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The hDPSCs were centrifuged for 3 min at 200/prm, the supernatant was discarted and the cell pellet was re-suspended with DMEM then transmitted into a T25 flask. The cells then incubated in a humidified incubator at 5% CO2 and 37 °C.

Characterization

To confirm that hDPSC maintain their phenotypic characteristics in vitro, the cells were expanded and characterized by Fluorescein Activated Cell Sorting (FACS) analysis through expression of the cell-surface markers that define its phenotype. $4X10^6$ hDPSC were trypsinated then re-suspended in phosphate-buffered saline (PBS) with 3% fetal bovine serum (FBS) and saturated concentration (1:100) of 4 fluorescein isothiocyanate-conjugated monoclonal antibodies; anti-CD105, anti-CD90, anti-CD34 and anti-CD45. The cells were incubated with isotype controls (human IgG peridinin chlorophyll protein complex) in a dark room for 30 minutes at room temperature to differentiate between the background signals, which is non-specific, and specific antibody signals.

PRF preparation

Blood samples were drained from 10 deeply anaesthetized rats directly by cardiac puncture. The samples were rapidly centrifuged for 10 min at 3000 rpm [7] till 3 layers were formed in the test tube: the cellular plasma on the surface, a PRF layer in the middle and red blood cells that accumulated at the bottom of the test tube. The PRF was easily separated from the tube by a sterile tweezer and squeezed gently between 2 sterile glass slabs to transform it into a membrane.

Seeding of PRF membranes with hDPSCs

In the laminar flow hood, PRF membrane pieces were placed in 24-well plates and they were sterilized by ultra violet light. hDPSCs were harvested on PRF pieces at a density of 5×10^4 cells/ well. Then they were incubated with 200 µL of DMEM in humidified atmosphere at 5% CO2 and 37 °C for 3

days. The cultures were microscopically observed every day for any contamination.

Surgical procedures:

It has been reported that rat's immune system doesn't reject human DPSCs, as a result, immunosuppression tests were not performed[28]. Bone defects were created out in the right site according to Zhang et al., (2018) method [29]. The animals were anesthetized with 75 mg/kg ketamine and 25 mg/kg xylazine. Then, the surgical incision area was shaved and the skin was wiped with povidone-iodine. Using No. 15 Bard-Parker blade, 1-cm incision overlying and parallel to the inferior border of the mandible was made. Blunt dissection and retraction of muscles with forceps were performed to expose the lower border of the mandible. using a rounded surgical bur with copious irrigation, a full thickness rounded bone defect with 3ml diameter was created posterior to the molar area near the mandibular angle (Figure 1). This defect size was the maximum diameter available as only 1 mml was left intact on the upper and lower border of the mandible.

Animals used and study design:

46 male Sprague Dawley rats weighting from 200-250 gm were used in this study, 10 were used for PRF isolation and 36 were subjected to surgical procedure and divided into 3 groups. The experimental procedures were approved by the institutional animal care and use committee, Beni-Suef University. The animals were housed and cared in College of Veterinary Medicine, Beni-Suef University, Egypt. The rats were housed at a standard temperature and humidity and were fed a regular diet. After the acclimatization period, the rats were randomly divided into 3 groups (n=12) and the surgical procedures were carried out:

a) Group I: Control gr.; the bone defect was irrigated with saline and left empty

b) Group II: PRF gr. : the bone defect was packed with PRF membrane

c) Group III: PRF + hDPSCs gr.: the bone defect was packed with PRF membrane seeded with hDPSCs

According to the scarification time, each group

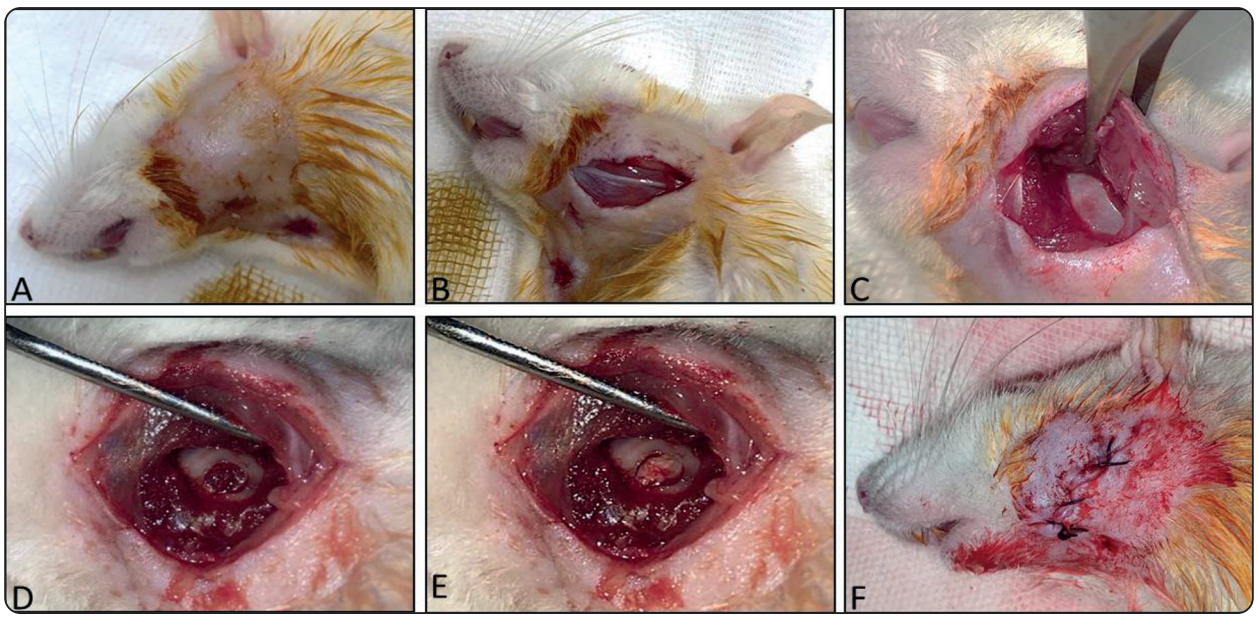

Fig. (1): A photograph demonstrates the surgical procedures of the study; (A) Shaving the surgical site, (B) Incision line at the lower border of the mandible, (C) Muscle reflection, (D) Bone defect, (E) PRF placement and (F) Suturing.

was then sub-divided into 2 subgroups;

- Subgroup A: the rats were scarified after 2 weeks.
- Subgroup B: the rats were scarified after 4 weeks

After 14 and 30 days, the rats were scarified by cervical dislocation. The mandibles were dissected, fixed in buffered formalin for 4 hours, decalcified with ethylene diamin etetra acetic acid (EDTA) for 2 weeks then embedded in paraffin blocks. Sections of 4 μm were processed for routine haematoxylin and eosin (H&E) and Masson's trichrome stains.

Histomorphometric analysis

The slides were examined with an Olympus microscope supplied with a 1/2 photo adaptor. Images were captured by a Toup View digital camera with an objective lens. The Digital images were introduced to Image-j software for digital analysis. In H&E stained slides, the surface area of bone defects was digitally measured by millimeter. While in Masson's Trichrome stained images, the area% of mineralized bone were calculated compared to the total area of newly formed tissue filling the bone defect.

Statistical analysis

All the data were tabulated and statistically analyzed with Statistical Package for the Social Sciences (IBM, SPSS, version 22). Paired Sample T-test was used to define significant differences between different groups. The difference between the groups was considered to be statistically significant at $P < 0.05$.

RESULTS

Characterization of stem cells

The flow cytometric analysis of hDPSCs revealed +ve expression of CD90 and CD105 (81.6% and 81.7% respectively). In contrast, it revealed –ve expression of CD34 and CD45; 5.8% and 3.9% respectively confirming the immunophenotypic profile of mesenchymal stem cells (fig 2).

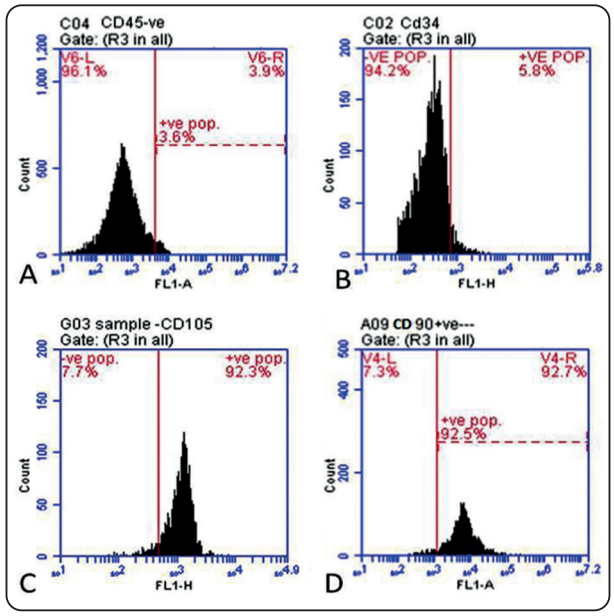

Fig . (2): Flow cytometric analysis of hDPSCs demonstrate –ve reaction to CD34, CD45 and + ve reaction to CD90, CD105.

Histological analysis

By evaluation the bone defect of all specimens under microscope, it was observed that the bone defect of all specimens were not completely healed or filled with bone either at day 14 or 30 (fig. 3). By measuring the surface area of the bone defect at day 14, it was found a smaller defect size in control group (3.1mm^2) than that of treated groups (3.9mm^2 in group II and 4mm^2 in group III) and these differences were statistically significant (0.002 and 0.00 respectively). At day 30, the bone defect of all groups markedly decreased in size (1.4, 1.68 and 1.62 mm^2 in group I, II and III) but the differences in defect size between control group and treated groups were not statistically significant (table 1,2).

The examination of the specimens at day 14, it was observed an increase of inflammatory cells in group I (which decreased markedly in group II and III) in addition to presence of necrotic tissue at the

TABLE (1): Demonstrate the means and Std. Deviations of bone defect surface area in each group.

	group1a	group2a	group3a	group1b	group2b	group3b
Mean	3.1855	3.9440	4.0070	1.4110	1.6845	1.6290
Std. Deviation	.09803	.06013	.05774	.12583	.18414	.24007

TABLE (2): Demonstrate the significant differences of bone defect surface area between all groups.

	Paired Differences							
				95% Confidence Interval of the Difference				
	Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	Sig. (2-tailed)
group1a - group2a	-.75850	.13833	.06916	-.97861	-.53839	-10.967	5	.002
group1a - group3a	-.82150	.08837	.04418	-.96211	-.68089	-18.593	5	.000
group2a - group3a	-.06300	.11782	.05891	-.25049	.12449	-1.069	5	.363
group1b - group2b	-.27350	.22220	.11110	-.62707	.08007	-2.462	5	.091
group1b - group3b	-.21800	.20380	.10190	-.54230	.10630	-2.139	5	.122
group2b - group3b	.05550	.12697	.06348	-.14653	.25753	.874	5	.446

border of the bone defect. A layer of newly formed bone matrix was observed in this group only. In group II and III, the granulation tissue increased with increased newly formed blood vessels with no bone matrix formation was observed.

At day 30, the bone defect size decreased markedly in all groups but the newly formed tissue was different. As group III revealed superior bone formation than group II followed by group I. The newly formed bone was cancellous bone with connected bone trabeculae and small sized bone marrow with numerous blood vessels. The granulation tissue in this group revealed numerous blood vessels with discreet areas of matrix formation. The newly formed bone in group II revealed cancellous bone with wide bone marrow and numerous blood vessels. The granulation tissue was still present in addition to appearance of muscle fibers instead of bone formation (muscular regeneration) in some areas. The least amount of bone formation was observed in control group with increase granulation tissue and muscular regeneration (fig. 3, 4).

Masson's trichrome staining analysis:

The cytoplasm and osteoid tissue in Masson's trichrome stained specimens appeared red in contrast to collagen fibers and mineralized bone that appeared blue, while the nuclei of the cells are colored dark brown to black. After 4 weeks, the defects of group I showed a small amount of loose irregular connective tissue with few collagen fibers (fig. 5D). packing the defects with PRF in group II stimulated formation of bon projections that form a thin layer inside the border of the bony defect. Also an integration was observed between The newly formed osteoid tissue and the proper lamellar bone (fig. 5E). New bone formation was markedly increased in group III, that was packed with PRF membrane seeded with hDPSCs. most of new tissue formed in these defects was trabecular bone (fig. 5F).

The statistical analysis of Masson Trichrome staining at day 30 revealed that, the mineralized bone represented 75% of the newly formed tissue in group III, followed by 55.7% in group II and finally 39.1% in group I. Paired Sample T-test revealed a statistical significant difference between all groups at day 14 and 30 (table3,4)

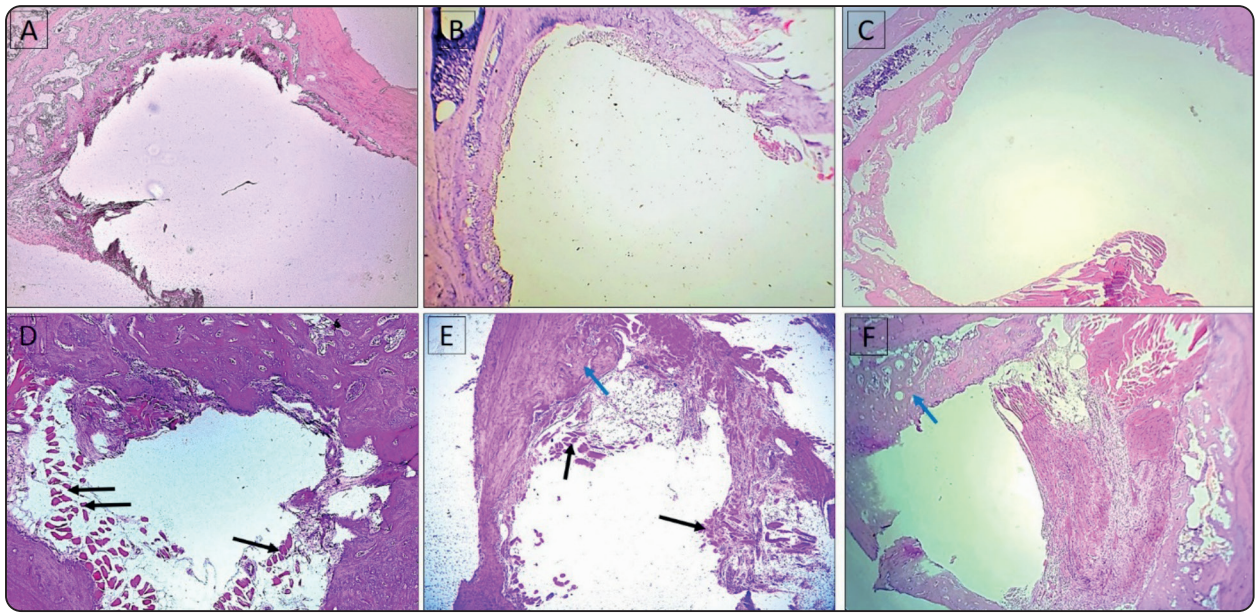

Fig. (3): A photomicrograph demonstrates the differences of defect size in each group; (A), (B) and (C) demonstrate groups I, II and III at day 15. (D), (E) and (F) demonstrate groups I, II and III at day 30. Muscular regeneration (black arrow), Bone regeneration (blue arrow). (H&E stain, x 40).

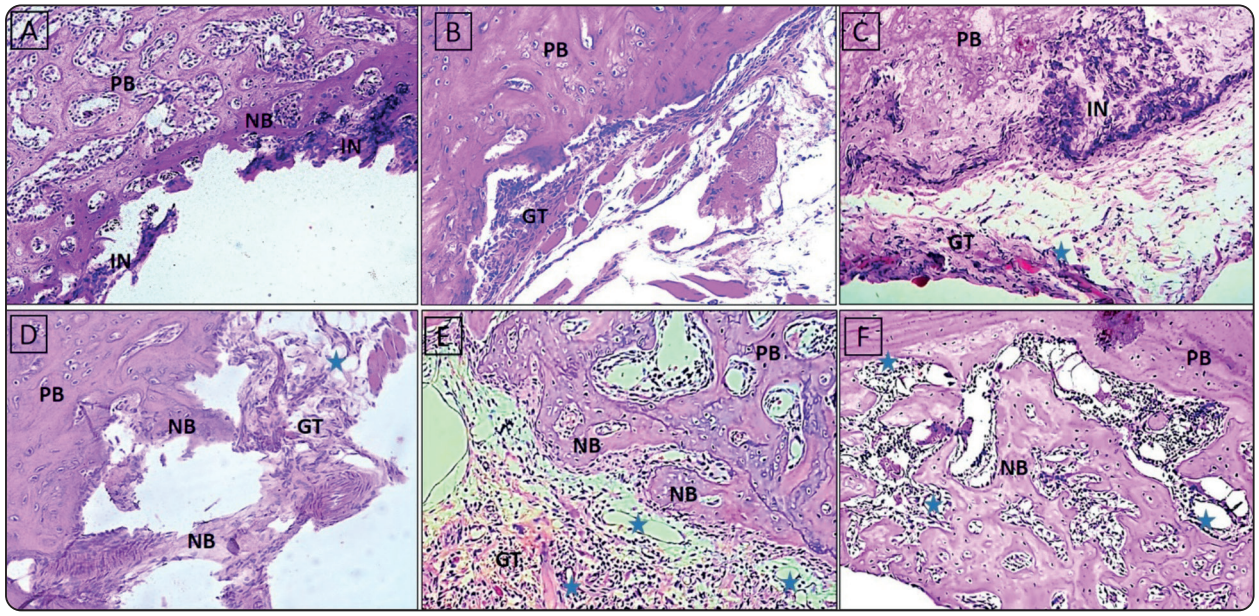

Fig. (4): A photomicrograph demonstrates the regenerated tissue in each group; (A), (B) and (C) demonstrate groups I, II and III at day 15. (D), (E) and (F) demonstrate groups I, II and III at day 30. (blue star) Blood Vessels, (PB): Bone Proper, (NB): New Formed Bone, (GT); Granulation Tissue and (IN) Inflammatory Cells, . (H&E stain, x 200).

TABLE (3): Demonstrate the means and Std. Deviations of new bone formation areas %, revealed by Masson's Trichrome stain, in each group

	group1a	group2a	group3a	group1b	group2b	group3b
Mean	26.8660	22.8850	47.4610	39.1000	55.7370	75.0920
Std. Deviation	1.05214	1.11001	.92736	1.00000	1.20083	1.80887

TABLE (4): Demonstrate the significant differences of area percentage of new bone formation between all groups

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
group1a - group2a	3.98100	.08672	.03878	3.87333	4.08867	102.652	5	.000
group1a - group3a	-20.59500	.32711	.14629	-21.00116	-20.18884	-140.785	5	.000
group2a - group3a	-24.57600	.31003	.13865	-24.96096	-24.19104	-177.251	5	.000
group1b - group2b	-16.63700	.20494	.09165	-16.89147	-16.38253	-181.525	5	.000
group1b - group3b	-35.99200	.81976	.36661	-37.00986	-34.97414	-98.176	5	.000
group2b - group3b	-19.35500	.64031	.28636	-20.15005	-18.55995	-67.591	5	.000

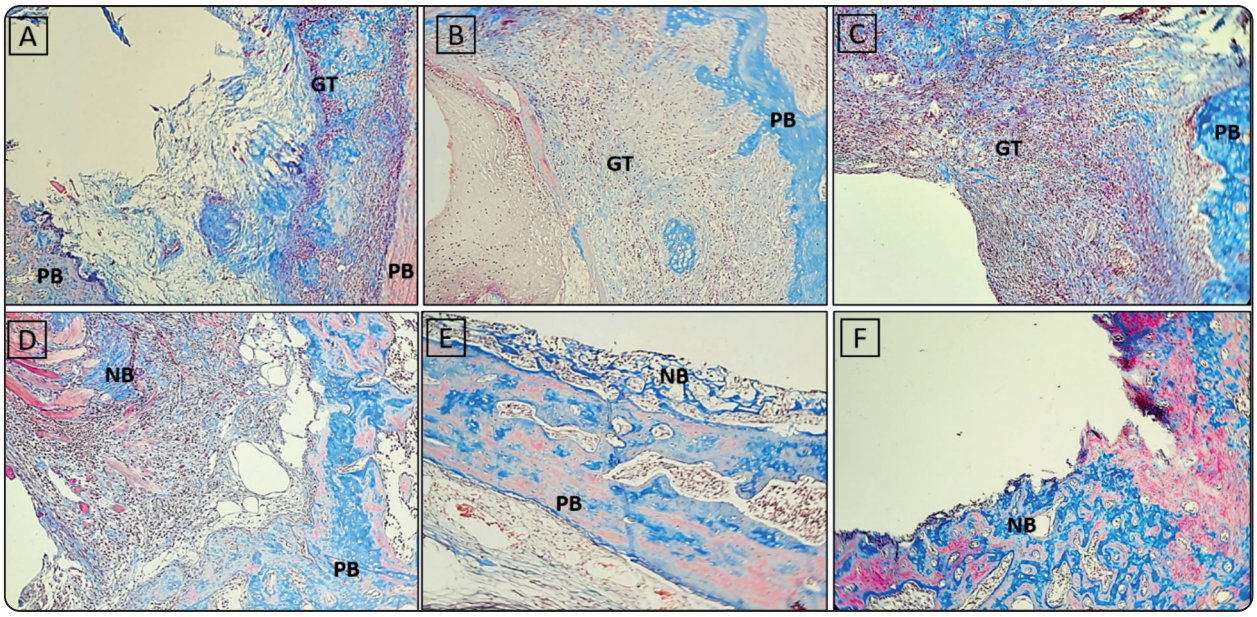

Fig. (5): A photomicrograph demonstrates the newly formed bone in each group; (A), (B) and (C) demonstrate groups I, II and III at day 15. (D), (E) and (F) demonstrate groups I, II and III at day 30. (PB): Bone proper, (NB): New Formed Bone and (GT) Granulation Tissue. (M.T stain, x 100).

DISCUSSION

Proper restoration of large bone defects is considered one of the major challenges for craniofacial surgeons as it influences the quality of patient's life. Different types of bone grafts have been used as autografts, allografts and xenografts which provide osteogenesis or/and osteoinduction properties [22].

It has been approved that, loading of stem cells on scaffolds has significantly accelerated the bone regeneration process [17,26,27,30].

DPSCc compromises more advantages than other human stem cells as their availability, accessibility and multipotency that turning them into a superior chosen source for using in clinical applications [31,32]. In addition, their high differentiation rate rendering

them to be considered as an actual valuable source for bone healing [31] as their osteoinduction ability has been proven invitro [33] and invivo [27, 34] .

So, hDPSCs have been used in the current study as an alternative osteoinductive source instead of human BMSCs due to their technical difficulties in collection and firm restrictions for donor patients.

Bone defect size in the present study hasn't been completely healed by any type of tissue (except some granulation tissue at the border of the defect) at day 14 and partial decrease in defect size was observed at day 30. These results coincide with Zhang et al., 2018 [29] who found incomplete healing of bone defect size of aged rats up to 12 weeks either in control group or stem cells group.

It was surprising that, the PRF groups (II and III) showed significant larger defect size than control group at day 14. This result may be due to filling the bone defect with PRF prevent bone clotting formation inside it which is a very important factor in bone healing process. Haematoma formation is the first step in the inflammatory phase which is the initial phase of bone healing process [35]. By haematoma formation, regeneration begins by migration of immune cells and regenerative cells, MSCs and endothelial cells, towards the bone defect region [35] [36] [37]. That was confirmed by Schell et al., 2017 who proved that intentional removal of the haematoma during surgery adversely influenced the healing process of the fractured bone [38].

Although PRF have important factors essential for bone healing process as the factors present in thrombus [11, 12], but it seems to be the delaying factor of bone defect restoration in PRF groups was the resorption time of PRF. It is biodegradable, three dimensional polymerized fibrin meshwork. This fibrin meshwork starts to be resorbed after 10 days of placement. Then it is completely resorbed up to 28 days [39]. So, using PRF in guided bone regeneration as a barrier membrane have been developed to prevent fast growing soft tissues from entering the slowly growing compartment containing bone exposure [40]. It have been also used in sinus lifting [41] and over-top of titanium meshes to reduce their chance of exposure [42].

At day 30, muscular tissue was observed in some areas, of newly formed tissue, in group I followed by group II. The same result was observed by Zhang et al., (2018) in control group and the group treated with normal BMSCs. This may be due to entrapment of surrounding tissue inside the bone defect especially in control group and in PRF group after its resorption.

On the other hand, the quality of formed bone in PRF group (II) had a superior quality than group I after 30 days as more bone formation was observed at the border of the defect, the areas where resorption of PRF takes place, which is coincided with Awadeen et., al [30] who found that the new bone formed in PRF group was better than the control group. This result is due continuous and gradual release of growth factors from PRF during their resorption period [43]. also the structural arrangement of PRF fibers and progressive polymerization significantly increases the integration of circulating cytokines into the fibrin mesh work which increase the lifespan of these cytokines [44].

Presence of PRF leads to leukocytes attraction which are not only shown to secrete a wide array of growth factors responsible for bone wound healing and regeneration, but also act to fight incoming pathogens from infection [45].

In the current study, group III (treated with hDPSCs and PRF) revealed more new bone formation than group II and muscular regeneration was less observed also unlike group I and II. This is exactly what was reported by Awadeen et al., (2020) as he found statistical significant in new bone formation in the bone defect that was filled with PRF seeded with BMSCs than that filled with PRF alone especially at day 30 [30]. This result also was in agreement with that of Knapen et al., (2015) who

found that using of PRF, as a fragments or a thin membrane, has no negative effect on the process of bone regeneration and they attributed that to early engraftment of the cells to PRF membrane at surgical time, when no osteoblast progenitor cells or connective tissue with blood vessels are available in the defect [46].

These results were supported by Chen et al., (2012) who reported that using human platelets lysate (a derivative of PRF) in growth media, in vitro, stimulates DPSCs proliferation and increases their osteogenic capacity when they are transplanted in vivo more than DPSCs when they were transplanted alone [47].

CONCLUSION

Based on the present study, the combined treatment of PRF membrane loaded with hDPSCs had no effect on the reduction of bone defect size but it showed a high quality regenerative effect on the newly formed bone filling the defect which may represent a possible alternative treatment for bone regeneration. But further researches are needed to accelerate the reduction of defect size.

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