# THE ROLE OF PLATELET RICH FIBRIN IN BONE DEFECT REGENERATION

Mazen T Abou ElKhier*, Laila E Amin** *and* Nagla M Salama***

## *ABSTRACT*

**Background:** Natural tissue regeneration relies on a cocktail of signaling molecules and growth factors. During natural wound healing, activated platelets concentrate in the wound area and secrete factors that play a role in wound healing. Platelet rich fibrin (PRF) represents a revolutionary step in the platelet gel therapeutic concept. It needs only centrifugation of the natural blood without additives.

**Objectives:** This present study designed to evaluate the role of PRF on regeneration of bone defect.

**Design:** In this study 24New Zealand white male rabbits were used with bilateral cortical bony defects in the submental mandibular area, divided into two groups. The right bone defects were filled with PRF (experimental) and the left bone defects kept empty (control). Each group was equally subdivided into three subgroups; rabbits were sacrificed at 2nd, 4th and 6th weeks. Immunohistochemical analysis for collagen I and vascular endothelial growth factor (VEGF) were done.

**Results:** The histological findings illustrated that all experimental subgroups showed more deposition of osteoid tissue and well organized bone trabeculae. Immunohistochemical findings of collagen I revealed statistically to be the highest in PRF group. While VEGF illustrated a positive expression by bone marrow stromal cells and bone tissue cells in different value and the PRF group recorded the highest value.

**Conclusion:** It was concluded that PRF can accelerate bone regeneration and had effect on collagen I and VEGF expression.

**KEY WORDS:** Bone defect, Platelet rich fibrin, Collagen I, VEGF

* Associate Professor of Oral Biology Department, Faculty of Dentistry, Mansoura University.
** Lecturer of Oral Biology Department, Faculty of Dentistry, Mansoura University.
*** Associate professor of Oral Pathology Department, Faculty of Dentistry, Mansoura University.

## INTRODUCTION

The essential goal of dental treatment is the maintenance of the natural dentition in health and for optimum comfort, function, and esthetic. After surgical procedure healing usually occurs by regeneration. Regeneration has been defined as the reproduction or reconstitution of a lost or injured part to restore the architecture and function of the periodontium. It is possible to achieve bone regeneration by using auto grafts and biomaterials. Both have presented high rates of success. Regenerative surgery including the use of barrier membrane, graft material, can support the formation of tissue and allow regenerative rehabilitation and also functional reconstruction. Periapical surgery includes removal of diseased soft tissue and sometimes application of different graft material to enhance new bone formation at the defective site [1].

Enhancement of the regenerative process of human body by utilizing the patient's own blood is a unique concept in dentistry. Post-surgically, blood clots initiate the healing and regeneration of hard and soft tissues. Platelet rich fibrin (PRF) is coming up as a biological revolution in dental field. Using PRF is a way to accelerate and enhance the body's natural wound-healing mechanisms. Platelets primarily are involved in wound healing through clot formation and the release of growth factors that initiate and support wound healing[2].

The use of PRF, in turn, has proven to be more promising, because in its preparation protocol it is not necessary to add external compounds and this promotes natural fibrin polymerization and consequently the slow and gradual release of growth factors that get trapped in their meshes [3, 4].

The scientific rationale behind the use of these preparations lies in the fact that the platelet α-granules are a reservoir of many growth factors for hard and soft tissue repair [5, 6]. Growth factors released by α-granules include platelet-derived growth factors, transforming growth factor-β, vascular endothelial growth factor (VEGF), and epidermal growth factor[7].

The role of VEGF in the healing process involves regulation of the vascularity of the healing fracture as well as a direct effect of VEGF on osteoblastic differentiation and activity. As the fractured site is believed to be under hypoxic tension, the finding that osteoblastic cultures under hypoxic conditions produce VEGF suggests the possibility of an autocrine loop that regulates osteoblastic activity at the repair site. Another possible source of VEGF at the fracture site is the soft callus and fracture hematoma[8].

Among natural polymers, collagen is the most abundant protein in mammals. It provides structural and mechanical support to tissues and organs and fulfill biomechanical functions in bone, cartilage, skin, tendon, and ligament[9]. Collagen type I has been shown to support osteoblast, osteoclast, and chondrocyte attachment, proliferation, and differentiation in vitro as well as in vivo[10].

## MATERIALS AND METHODS

Twenty-Four New Zealand white male rabbits3 months of age, 2–2.5 kg body weights were used in this study. All animals were maintained in a 12h light and dark cycle and were fed standard diet with water. The rabbits were divided into 2 groups; control group (n=12), experimental group(n=12), and each group was equally subdivided into three subgroups.

### Surgical procedures:

All animals were anaesthetized by an intraperitoneal injection of xylazine (25mg/kg body weight) and ketamine (75mg/kg body weight). The submental region of all animals was shaved and the skin covering this region was scrubbed thoroughly with povidone iodine (Betadine). An incision was done in the submental region below the inferior border of the mandible using Bard-parker blade

No.15. Through this incision, the inner surface of symphyseal region of the mandible was exposed. Two equal holes surrounding the midline were created. No.3 rose head surgical bur was used to induce bony cavity of the same size (involving the cortex and spongiosa) under efficient saline irrigation. To ensure standardization of the bony defect, the same size of the bur was used making its head be contained in the bone defect, at the same speed of the micro motor device for all animals. The right induced cavity was filled with PRF(experimental group) while the left induced cavity was lifted empty (control group).The two edges of the skin were approximated and sutured using 3/0 silk mounted on half circle needle 3/0. After finishing the surgery, the animals received broad spectrum antibiotic covering (E-MOX) Vial Amoxicillin 500mg.

**Preparation of PRF:** 3 mL blood sample was collected from each rabbit from the marginal ear vein and PRF was prepared according to the protocol. Blood was centrifuged using a tabletop centrifuge (406G, Gyrozen, Daejeon, Korea) for 10 min at 3000 rpm. After centrifugation, the blood separated into three layers. The middle layer, which represents PRF was taken.

**Sacrificing of the animals:** Four animals from each group were sacrificed for histological assessment either at 2nd, 4th and 6th week.

**Histological analysis:** The mandible of the animal was resected and freed from soft tissue. The bone was fixed in buffered formalin fixative for 4h, decalcified in EDTA solution and then embedded in paraffin. Serial sections were made at $5\mu$ thickness.Sections of specimens will be prepared for Hematoxylin and Eosin stain (as routine stain) and for immunohistochemical stain for Collagen I (Polyclonal Antibody, Catalog#: PA1-36145, Thermo Fisher scientific) and VEGF (Polyclonal Antibody, Catalog#: PA5-16871, Thermo Fisher scientific).

**Computer Assisted digital image analysis:** Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 40 X objective. The result images were analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for slides with

- (H&E) for histomorphometric analysis measuring the percentage area of trabecular areas to the total surface area.

- IHC stain: for stain quantification, results were expressed as integrated density.

## Statistical Analysis:

Data was analyzed using Statistical Package for Social Science software computer program version 17 (SPSS, Inc., Chicago, IL, USA). Data were presented in mean and standard deviation. Student's t-test (unpaired) was used to compare between two different groups while one way Analysis of variance (ANOVA) and post-hoc Tukey test were used for comparing more than two groups. Pearson's correlation coefficient test was used to correlate different variables. *P* value less than 0.05 was considered statistically significant.

## RESULTS

## H&E results: (Fig.1)

### *Control group:*

At 2nd week; the bone cavity was filled with a granulation tissue, inflammatory cell infiltrate, blood vessels and small amount of newly formed woven bone (Fig. 1A).

At 4th week; the bone cavities filled with granulation tissues and some newly formed bone trabeculae starting to coalesce enclosing still large bone marrow spaces (Fig. 1B).

At 6th week; the bone cavities had more number of well-organized bone trabeculae, almost filling

the cavity enclosing relatively large bone marrow spaces with definite line separating them from the old bone (Fig. 1C).

**Experimental group:**

At 2nd weeks; the bone cavity filled by granulation tissue. The inductive osteogenic activity of the osteoblasts for healing occurred in all boundaries in bottom and all sides of the bone cavity and surrounded by granulation tissue. Small newly formed bone trabeculae were evident. (Fig. 1D).

At 4th weeks; the bone cavities filled with a network of trabeculae starting to coalesce enclosing still large bone marrow spaces (Fig. 1E).

At 6th week; the bone cavities had a well-organized bone trabeculae, with matured osteon and haversian system formation, and definite line separating old bone from new one (Fig. 1F).

**Immunohistochemical results:**

*Collagen I: (Fig. 2)*

At 2nd week, the control group showed week immunoreaction at the granulation tissue. At 4th week, increased positive reaction by the newly formed bone trabeculae, at 6th week, the section showed maximum immunoreaction by well-organized bone trabeculae. The experimental group showed more strong reaction.

*Vascular endothelial growth factor:(Fig. 3)*

At 2nd week, the control group express positive expression of VEGF by inflammatory cells and bone marrow stromal cells with strong intense identification of VEGF in the experimental goup. At 4th , 6th weeks, control group showed positivity expression of VEGF illustrated by osteoblasts, osteoclasts and bone marrow stromal cells, experimental group recorded a strong expression.

**Statistical analysis:**

*H&E (Table 1,2)*

ANOVA test revealed an overall significant difference between both groups in relation to the percent area of bone trabeculae. Moreover, Posthoctukey test for multiple comparisons showed significant difference between control group and experimental group at 2nd , 4th and 6th weeks.
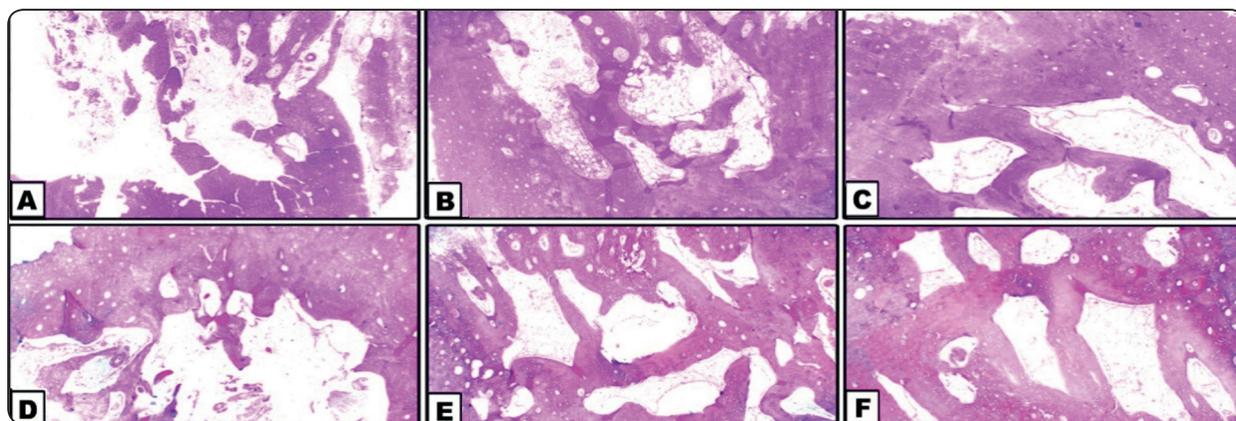


Fig. (1) Photomicrograph of control group showing (A) 2nd week, bone cavity filled with granulation tissue and dilated blood vessels. (B) 4th week, a network of newly formed bone trabeculae filling the bone cavity. (C) 6th week, more organized bone trabeculae were formed with large marrow spaces. Experimental group showing: (D) 2nd week, the bone cavity filled with granulation tissue and small newly formed bone trabeculae. (E) 4th week, the bone cavities filled with a network of trabeculae starting to coalesce enclosing still large bone marrow spaces. (F) 6th week, well-organized bone trabeculae were formed, with matured osteon and definite line separating old bone from new one (H&E, x100).
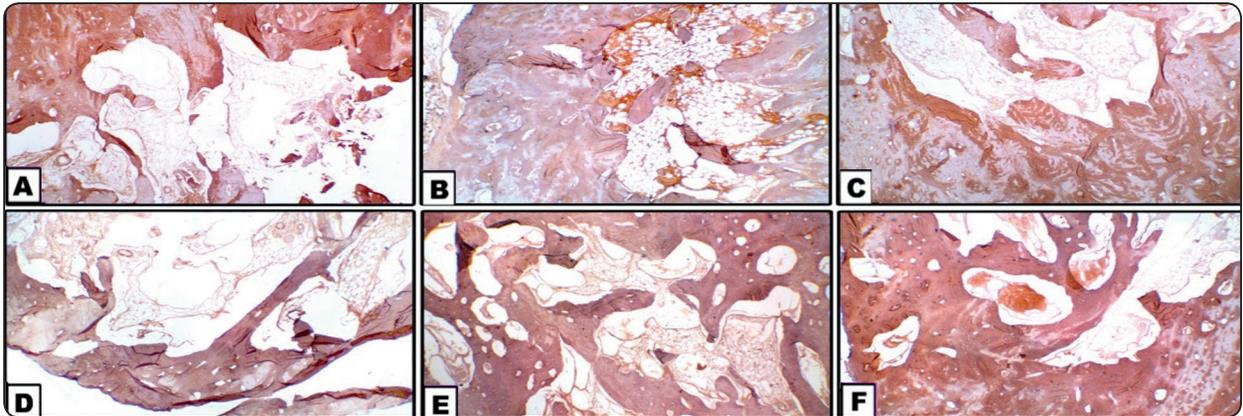
Fig. (2) photomicrograph of collagen I. Control group (A) 2nd week, (B)4th week, (C) 6th week. Experimental group (D) 2nd week, (E)4th week, (F) 6th wee (IHC, DAB chromogen, X100).
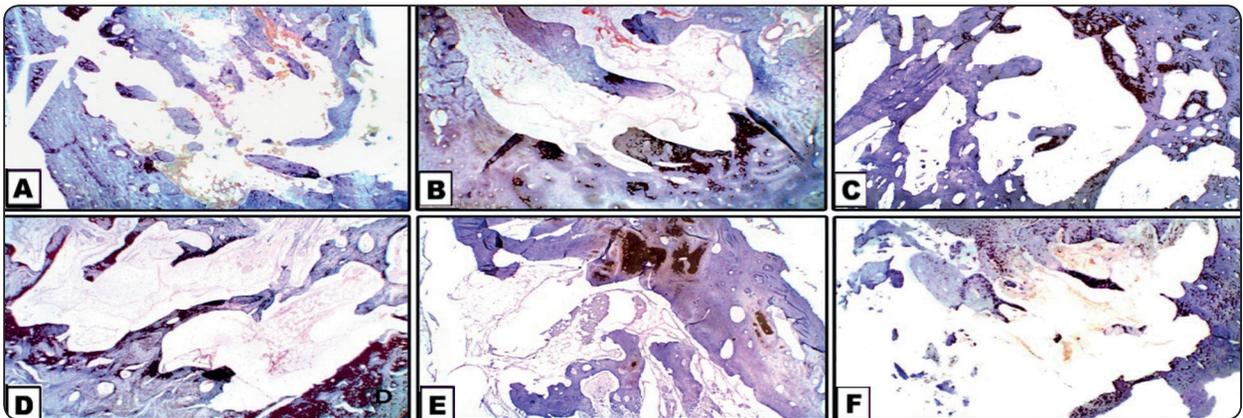


Fig. (3) photomicrograph of VEGF. Control group (A) 2nd week, (B)4th week, (C) 6th week. Experimental group group (D) 2nd week, (E)4th week, (F) 6th week (IHC, DAB chromogen, X100).

TABLE (1): Means± STD for the percentage area of bone trabeculae of control and experimental group at three examination periods.

| Percent area | | Cont. group | Exp. group | P |
|---|---|---|---|---|
| 2weeks | Mean | 29.8 | 40.30 | 0.019* |
| | ±SD | 8.600 | 9.600 | |
| 4weeks | Mean | 37.20 | 49.60 | 0.035* |
| | ±SD | 11.20 | 13.10 | |
| 6weeks | Mean | 67.00 | 78.60 | 0.026* |
| | ±SD | 11.20 | 10.30 | |

*SD: standard deviation*      *P:Probability*      *\*: significance <0.05*      *Test used: Student's t-test*

TABLE (2): Multiple comparisons between three examination periods in both groups.

| Percent area | 2 weeks | | 4 weeks | | 6 weeks | | P | P1 | P2 | P3 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | ±SD | Mean | ±SD | Mean | ±SD | | | | |
| Cont group | 29.8 | 8.6 | 37.2 | 11.2 | 67 | 11.2 | <0.001* | 0.26 | <0.001* | <0.001* |
| Exp group | 40.3 | 9.6 | 49.6 | 13.1 | 78.6 | 10.3 | <0.001* | 0.16 | <0.001* | <0.001* |

*SD: standard deviation      P: Probability   *: significance <0.05 Test used: one way ANOVA followed by post-hoc tukey*

*P1: significance between 2weeks and 4weeks time groups        P2: significance between 2weeks and 6weeks time groups*

*P3: significance between 4weeks and 6weeks time groups*

## Collagen I (Table 3, 4)

ANOVA test revealed an overall significant difference between both groups in relation to the integrated density of positive immunohistochemical reaction for collagen I. Moreover, Posthoctukey test for multiple comparisons showed significant difference between control group and experimental groups at 2nd , 4th and 6th weeks.

TABLE (3): Showed means± STD for the collagen I integrated density of control and experimental group at three examination periods.

| Int.Density(*10$^7$) | | Cont. group | Exp. group | P |
|---|---|---|---|---|
| 2weeks | Mean | 92.6 | 107.6 | 0.02* |
| | ±SD | 13.3 | 12.90 | |
| 4weeks | Mean | 97.30 | 116.1 | 0.036* |
| | ±SD | 17.40 | 19.66 | |
| 6weeks | Mean | 122.5 | 136.5 | 0.039* |
| | ±SD | 13.80 | 14.40 | |

*SD: standard deviation   P:Probability        *: significance <0.05        Test used: Student's t-test*

TABLE (4): multiple comparisons between three examination periods in both groups.

| Int.Density(*10$^7$) | 2weeks | | 4weeks | | 6weeks | | P | P1 | P2 | P3 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | ±SD | Mean | ±SD | Mean | ±SD | | | | |
| Group I | 92.6 | 13.3 | 97.3 | 17.4 | 122.5 | 13.8 | <0.001* | 0.7 | <0.001* | 0.002* |
| Group II | 107.6 | 12.9 | 116.1 | 19.66 | 136.5 | 14.4 | 0.001* | 0.46 | 0.001* | 0.02* |

*SD: standard deviation      P: Probability   *: significance <0.05 Test used: one way ANOVA followed by post-hoc tukey*

*P1: significance between 2weeks and 4weeks time groups        P2: significance between 2weeks and 6weeks time groups*

*P3: significance between 4weeks and 6weeks time groups*

**Vascular endothelial growth factor (Table 5,6)**

ANOVA test revealed an overall significant difference between both groups in relation to the integrated density of positive immunohistochemical reaction for vascular endothelial growth factor. Moreover, Posthoctukey test for multiple comparisons showed significant difference between control group and experimental groups at 2nd , 4th and 6th weeks.

TABLE (5): Showed means± STD for the integrated density VEGF of control and experimental group at three examination periods.

| Int.Density(*10⁷) | | Cont. group | Exp. group II | P |
|---|---|---|---|---|
| 2weeks | Mean | 6.422 | 3.937 | <0.001* |
| | ±SD | 1.6 | 1.15 | |
| 4weeks | Mean | 1.145 | 2.917 | <0.001* |
| | ±SD | 0.2800 | 0.8400 | |
| 6weeks | Mean | 2.999 | 1.335 | 0.026* |
| | ±SD | 0.6800 | 0.4200 | |

*SD: standard deviation*          *P:Probability*          *\*: significance <0.05*          *Test used: Student's t-test*

TABLE (6): Multiple comparisons between three examination periods in both groups.

| Int.Density(*10⁷) | 2weeks | | 4weeks | | 6weeks | | P | P1 | P2 | P3 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | ±SD | Mean | ±SD | Mean | ±SD | | | | |
| Group I | 6.422 | 1.6 | 1.145 | 0.28 | 2.999 | 0.68 | <0.001** | <0.001** | <0.001** | 0.001* |
| Group II | 3.937 | 1.15 | 2.917 | 0.84 | 1.335 | 0.42 | <0.001** | 0.03* | <0.001** | <0.001** |

*SD: standard deviation      P: Probability   \*:significance <0.05  Test used: one way ANOVA followed by post-hoc tukey*

*P1: significance between 2weeks and 4weeks time groups*

*P2: significance between 2weeks and 6weeks time groups*

*P3: significance between 4weeks and 6weeks time groups*

**Pearson's correlation test** (to detect the correlation between different variables) revealed

1. Significant negative correlation between VEGF and Collagen I   in experimental group while non significant correlation   in control group (Fig.4).

2. Significant negative correlation  between H&E and VEGF in experimental group  while non significant correlation  in control group (Fig.5) .

3. Significant positive correlation between H&E and collagen I in experimental and control group (Fig.6)
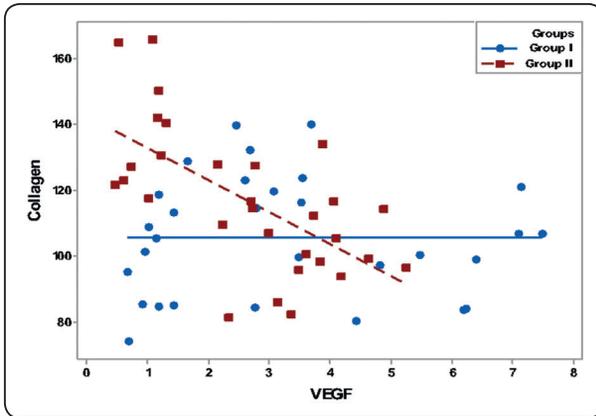
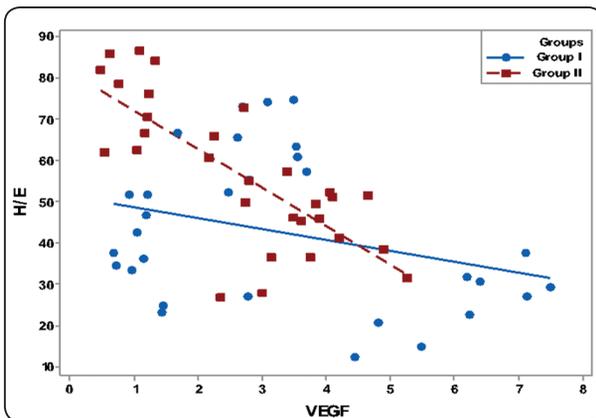Fig. (4) Correlation between collagen and VEGF.
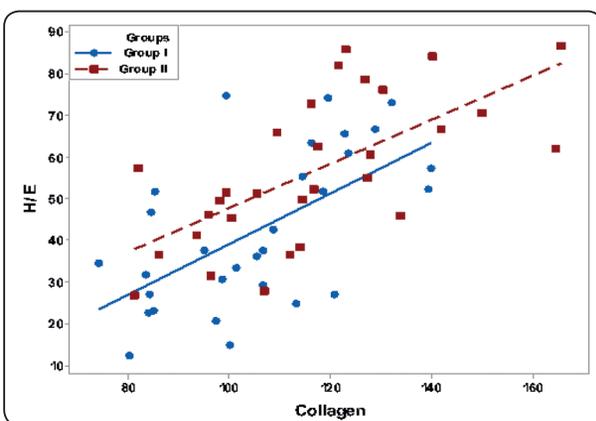


Fig. (5) Correlation between H&E and VEGF.



Fig. (6) Correlation between H&E and collagen I.

## DISCUSSION

PRF can be used to promote wound healing, bone regeneration, graft stabilization, wound sealing, and hemostasis. Because the fibrin matrix is better organized, it is able to more efficiently direct stem cell migration and the healing process. Release of growth factors from PRF through *in vitro* studies and good results from *in vivo* studies led to optimize the clinical application of PRF[11]. Dohan *et al.*, proved a slower release of growth factors from PRF and better healing properties. It was observed that the cells were able to migrate from fibrin scaffold; while some authors demonstrated the PRF as a supportive matrix for bone morphogenetic protein as well[12].

In the present study, the experimental group showed faster new bone formation and more well organized bone trabeculae, these come in acceptance with He L, et al., who reported that PRF released autologous growth factors gradually and expressed stronger and more durable effect on proliferation and differentiation of rat osteoblasts. The use of PRF seems to be one of the most promising methods to enhance bone healing in a controllable, and relatively long-term effect, way [13].

The natural and slow polymerization occurring during centrifugation process of PRF leads to formation of a homogenous 3-dimensional organization of the fibrin network. The absence of anticoagulant in the test tube leads to massive platelet activation, bolstered by the presence of a mineral phase on the tube walls (residual glass particles).A progressive polymerization mode signifies increased incorporation of the circulating cytokines in the fibrin meshes (intrinsic cytokines). This configuration increases the lifespan of these cytokines, as they are released and used only at the time of initial cicatricle remodeling [12].

By statistical evaluation, the current study revealed that, there was an increase in the percentage of bone trabeculae in both study and control groups, and there were significant differences in the experimental group compared to the control

group from the second to the sixth post-operative weeks. That comes in agreement with Cypher TJ& Grossman JP, that the maximal promoting effect of PRF occurred at day 14. the bone graft healing is a sequential process involving inflammation, revascularization, osteogenesis, remodeling, and incorporation into the host skeleton to form a mechanically efficient structure, the in growth and proliferation and differentiation of osteoblasts occurred during the initial 14 days[14].

Regarding our result in the collagen fibers formation and organization, by immunohistochemical detection of collagen type I, statistical analysis revealed significant difference between the control and experimental group along the examination periods. These finding agree with the study evaluated the effect of platelet-rich fibrin (PRF) on the osteogenesis and angiogenesis of rabbit calvarial defects. They analyzed not only the area of newly formed tissue relative to the total defect, but also the quality of regenerated tissue by measuring the percentage of the osteoid to the newly formed tissue. They found a significant linear increase of newly formed tissue (NB) and newly formed bone relative to newly formed tissue(NBR), including osteoid formations(OS) value in the PRF group[7].

The current study showed positive expression of VEGF by bone marrow stromal cells, adipocytes, mesenchymal stem cells, endothelial cells, and bone cells include osteoblasts and osteocytes in different periods in both groups but in different value. Therefore, our primarily data provide evidence that VEGF activity is essential for appropriate bone formation and mineralization in response to injury. Several growth factors are expressed in distinct temporal and spatial patterns during bone repair. Of these, vascular endothelial growth factor, VEGF, is of particular interest because of its ability to induce neovascularization (angiogenesis), VEGF also acts to recruit and activate osteoclasts as well as stimulate osteoblasts chemotaxis, differentiation, and matrix mineralization. Suggesting a functional role for this growth factor in bone formation and remodeling[15].

The statistic evaluation of overall periods showed that PRF group records the highest value in the mean of positive VEGF; As VEGF itself has no osteo-inductive capacity, bone-forming cells were probably recruited from pericytes, circulating cytokines and cells, the blood clot and the fractured bone ends. VEGF potentiates the actions of several cytokines, and it mediates the angiogenic actions of most growth factors. It could therefore complement other cytokines such as basic fibroblast growth factor or bone morphogenetic proteins in enhancing bone healing[16].

PRF can release high quantities of multiple growth factors including TGFβ-1, PDGF, and VEGF. In PRF, the fibrin network gradually builds up during centrifugation and in the absence of anti-coagulant agents. This results in a dense fibrin structure, in which platelets and leukocytes are entrapped during centrifugation. This reservoir property of the fibrin network enhances the gradual release of growth factors and other mediators,resulting in prolonged maintenance and stimulation of stem cells by PRF[17].

Dohan Ehrenfest DM et al., showed that this dense fibrin membrane releases high quantities of three main growth factors (Transforming Growth Factor b-1 (TGFβ-1), platelet derived growth factor AB (PDGF-AB); VEGF and an important coagulation matricellular glycoprotein (thrombospondin-1, TSP-1) during 7 days. Moreover, the comparison between the final released amounts and the initial content of the membrane (after forcible extraction) allows us to consider that the leucocytes trapped in the fibrin matrix continue to produce high quantities of TGFβ-1 and VEGF during the whole experimental time[18].

In conclusion, the results of this study suggested that PRF may improve the bone regeneration and enhance bone quality. Further large scale and long-term *in vivo* studies are needed to strengthen our results and to improve adequate application of PRF for bone-healing procedures.

## ACKNOWLEDGEMENTS

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