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TRANSMISSION ELECTRON MICROSCOPY AND IMMUNOHISTOCHEMICAL STUDIES ON THE EFFECT OF PLATELET RICH FIBRIN AT THE BONE- METALLIC INTERFACE IN THE MANDIBLE

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ABSTRACT

Background: A dental implant is an effective method for restoring edentulous dentition. Many biological materials were used to coat implant surfaces to enhance osseointegration. Growth factors like bone morphogenic proteins (BMP) were used for this purpose. Platelet rich fibrin (PRF) is a new approach on bone regeneration through the synergistic effect of many growth factors derived from platelets.

Purpose: The purpose of this study was the evaluation of the effect of platelet rich fibrin (PRF) and transforming growth factor (TGFβ) on the bone- metallic interface in dogs. Also, to determine the relationship between titanium implants and bone cells by the electron microscopy.

Methods & materials: Six healthy adult male dogs included in this study. The dogs were divided randomly into two groups. The dog's mandibles were used. The third premolar was extracted bilaterally and the right side used as a control while the left used as experimental side. PRF was prepared and the immediate implant was inserted. Ultrastructure and immunohistochemical examination of dogs were evaluated.

Results: The results demonstrated that the PRF and TGFβ enhance the osseointegration in the experimental group when compared with control group. During study of bone regeneration more advanced intervals were required for bone maturation.

Conclusions: The PRF accelerates early bone regeneration and there is a tight regulatory relationship between the TGFβ and the rapidity of bone formation.

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INTRODUCTION

Bone is a specialized supporting tissue characterized by its rigidity, hardness, regeneration ability and repair. It protects the vital organs, provides an environment for marrow and acts as a mineral reservoir for calcium homeostasis. In addition, it acts as reservoir of growth factors, cytokines. [1] Dentists have for a long time been researching materials and techniques for providing efficient and effective methods of restoring a depleted dentition. Amongst the most versatile of these are dental implants. [2]

Three terms may be used to describe individual aspects of the bone formation process that can occur at implants interface are osteoconduction, osteogenesis, and osteoinduction. In addition, a fourth term, osteopromotion, has been introduced to refer to the clinical promotion of osteogenesis beneath exclusionary membranes for guided tissue regeneration. [3] Simply, some authors have emphasized that bone integration is a healing process of damaged bone. [4]

It is essential that a sufficient number of bone forming cells congregate at the implant surface. The osteoblasts may be derived from mesenchymal stem cells, and the continuous layer of cells comprising the periosteum and endosteum. The number of osteoblastic cells reflects the amount of bone formation that occurs. [5] Osteocytes embedded in bone tissue are the longest-lived and most abundant cell type in adult bone, comprising 90–95% of all bone cells. They have multiple functions, influence bone remodeling by controlling the differentiation and activity of osteoblasts and osteoclasts. [6]

The growth factors are biological mediators that regulate the cellular events involved in tissue repair by binding to specific cell receptors. [7] Several growth factors produced by osteogenic cells, platelets and inflammatory cells participate in bone healing, including IGF, TGF-β , PDGF and FGF. The bone matrix serves as a reservoir for these growth factors. Additionally, the acidic environment that develops during the inflammatory process leads to activation of latent growth factors, which assist in the chemo-attraction, migration, proliferation and differentiation of mesenchymal cells into osteoblasts or chondroblasts.[8-10] The transforming growth factor beta (TGF-β) proteins play important roles in morphogenesis of many craniofacial tissues.[11] It is produced in the fracture site by platelets, inflammatory cells monocytes, macrophages, osteoblasts, osteoclasts, and chondrocytes. It is extracellularly present in the hematoma (fracture site and periosteum) during the immediate injury response. [12]

Platelet-rich fibrin (PRF), described by Choukroun et al.,[13] is a second-generation platelet concentrate which consist of fibrin membranes enriched with platelets and other growth factors that originate from anticoagulant-free blood harvest. [14,15] A fibrin network could provide more efficient cell proliferation and migration necessary for tissue regeneration. When fibrin is used with the autogenous bone graft can increase bone formation, and act as a scaffold for the restoration of bony defects. Fibrin is a recognized support matrix for bone morphogenic protein (BMP). [16] The PRF, as a natural and optimized blood clot, seemed the adequate adjuvant to improve the guided tissue regeneration. [17]

The aim of this study was to visualize the ultrastructural relationship between bone cells and the titanium implant surface during osseointegration. In addition, evaluate the effects of platelet-enriched fibrin and TGFβ on bone formation.

MATERIALS & METHODS

Animals: Six healthy adult male mongrel dogs were included in this study. Ethical committee, Faculty of Oral and Dental Medicine Al-Azhar University, approved the study. Animals were anesthetized and the dog's mandibles were used.

They were sacrificed 28 days after implantation. The third premolar was extracted bilaterally and the right side used as a control while the left used as experimental side.

Implant installation: After drilling of the socket using a Lindemann side-cutting bur, two cylindrical, threaded, endosseous titanium implants (3.8mm x 10mm), were placed in each animal one in left side and the other in the right side. The implants were capped by healing screws and the wounds were sutured.

PRF application: Blood samples were treated according to PRF protocol, PRF clot pressed gently into a membrane and slowly applied to the extraction site of the experimental group.

Sacrification of the animals: after a healing period of 4 weeks, the animals were euthanized. The regions of interest of the dog's mandible were dissected and prepared for subsequent investigations. Each group of the experimental and control groups were divided into two subgroups. The first was used for immuochemical investigation and other subgroup was used for electron microscope.

Immunohistochemistry: The implants and the surrounding bone were fixed for 72h in 10 % neutral buffered formalin, decalcified, dehydrated and embedded in paraffin wax. Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2. Slides were immersed in plastic containers (Coplin jars) filled with sufficient amounts of antigen retrieval solution, microwaved, allowed to cool, rinsed several times and dried out except for the tissue section. Then incubation with monoclonal antibodies against TGFβ was done for 30 min.

After blotting off excess buffer, a universal staining kit was used (Peroxidase LSAB 2 System, Dako, code No K 0672) according to manufacture instructions. Tissue sections were treated with biotin for 10 minutes, then rinsed and washed with PBS as before. Streptavidin was added for 10 minutes then rinsed and washed with PBS for 5 minutes as before. The slides were incubated with diaminobenzidine for 5- 10 minutes, then washed in distilled water and counterstained using Mayer's Hematoxylin. The tissue sections were washed, dehydrated, cleared and mounted ***with DPX***, and a cover is slipped over. The immune-histochemical examination was performed by evaluating the concentration of antigen within tissue for estimating the relative intensity of a chromogen label of TGFβ.

Electron microscopic preparation: The tissue specimens were cut into cubes of 1mm3, fixed in 2.5% glutraldehyde in phosphate buffer at pH 7.4 for 24 hours in refrigerator at 4ºC. Dehydration of the tissue specimens was performed by their immersion in ascending grades of ethanol (50-90%) for 15 minutes each was followed with absolute alcohol for 15 minutes and then embedded in the araldite resin. The blocks were trimmed and the semithin sections were cut 1 μm thick. The tissue sections were stained with toluidine blue stain for semithin sections. The ultrathin section (60-90 nm) were cut from the trimmed tissue block a preparation to be stained and examined with Philips transmission electron microscope at 60 or 80 K.V by loading the ultrathin stained tissue sections into the electron microscope.

RESULTS

The immunostaining reaction of TGFβ was evaluated according to the intensity and pattern of distribution. The positive immunohistochemical staining for TGFβ appeared as nuclear and cytoplasmic brownish reaction. The immunohistochemical staining showed different immunostaining Fig. (1). In the control group, the whole tissue expressed TGFβ (brown), with more prominent staining in immunostaining of the experimental group revealed

brown staining in all cell types and interstitial cells. TGF-β was weakly expressed at the bone- metallic interface. It is strongly expressed in the walls of blood vessels, vascular hemopoietic buds and in the centers of ossification. In addition, strong immunoreactions in lining surfaces of the bone marrow cavities were observed**.**

The transmission electron microscopy observations of the tissue sections at the control side, containing a 4 week implant revealed typical cellular activity taking place at and near the interface. Near the implant, a tissue with a characteristic morphology typical of a woven bone formed. A thin amorphous zone was interposed between the bone and implant. Random arrangement and orientation of the collagen fibrils was also noticeable **(Fig.2A)**. At the experimental side, mature bone was found in areas transformed at the earlier stages after implantation. The mature bone contained well organized collagen fibers **(Fig.2B)**, indicates the bone area related to the woven bone and also the mature lamellar bone with the aligned collagen fibers of well resolved banding. Active osteoblasts, cement line at the junction between old and new bone, collagen fibrils and matrix vesicles were also observed **(Fig.2C)**.

In the control group, the pre-osteoblast and osteoblastic cells were found to continue at 4 weeks of implantation, as shown in **(Fig.2D)**. But, the osteocytes and osteocyte canaliculi in close proximity to the implant were observed at the experimental side. The cell in lacunae displayed in the micrograph developed numerous processes which spread equally well in the direction of the implant and also in all other directions, contributing

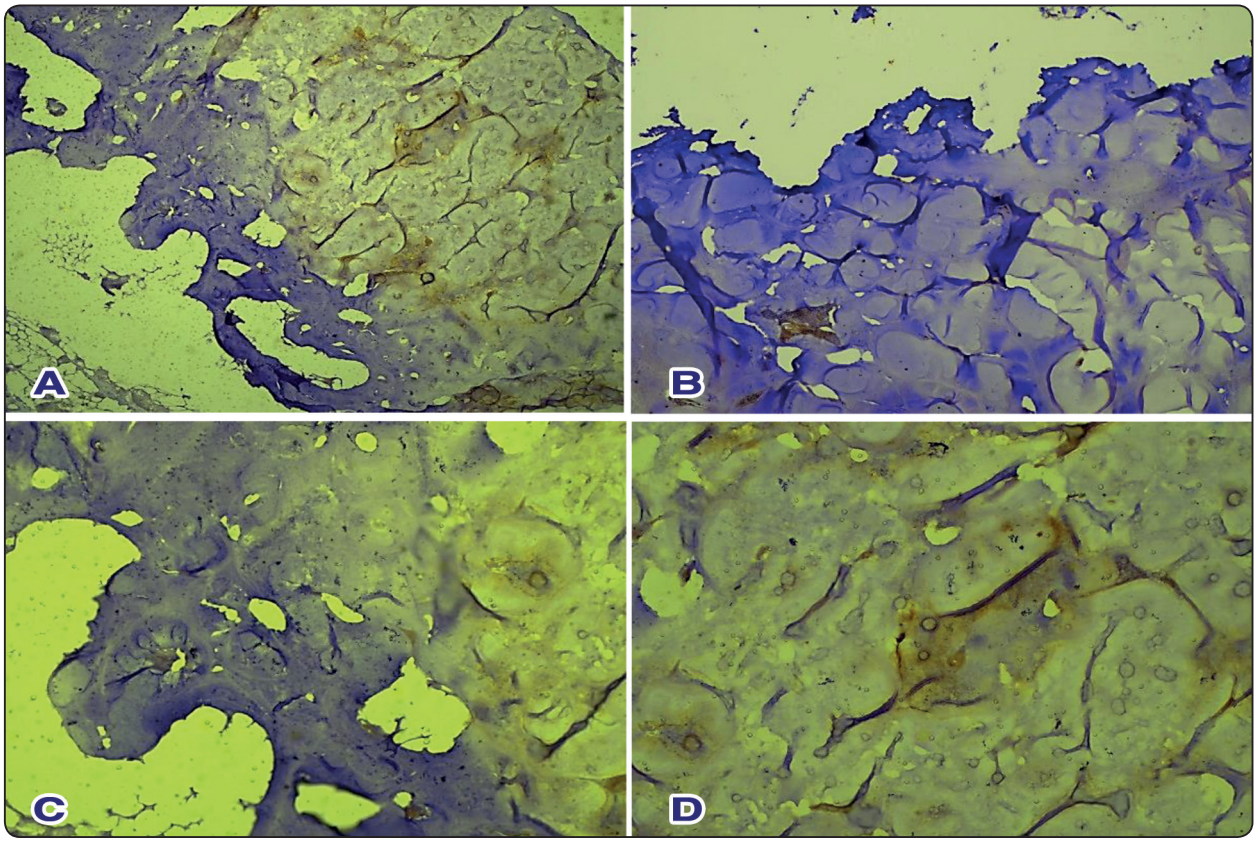

Fig. (1): Immunohistochemical photomicrographs of the control group (A) and experimental groups (B) showing, positive immunoreactions in blood vessel walls and in ossification center. (C&D) shows higher magnification of (A).

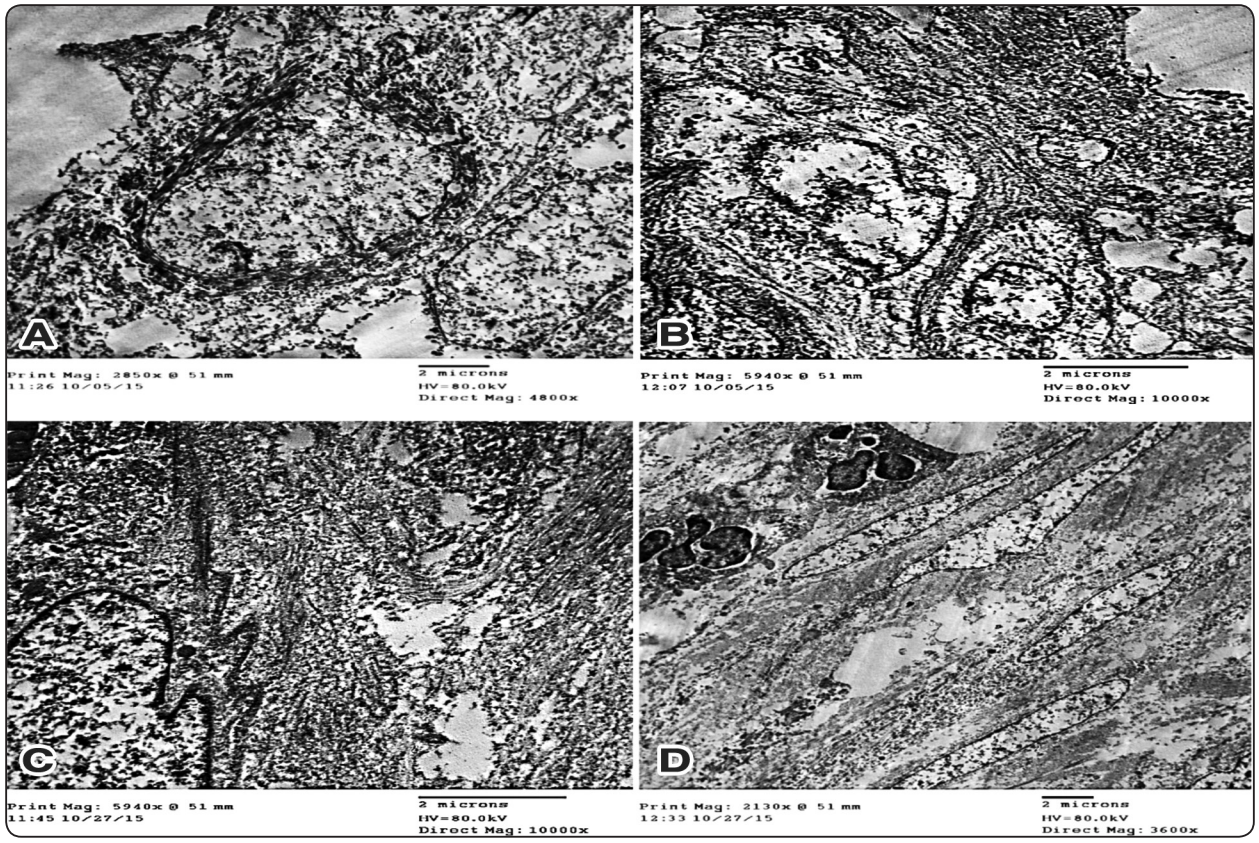

Fig.2: Electron micrograph showing random arrangement and orientation of the collagen fibrils (A), well-organized collagen fibers with a characteristic banding pattern (B). Active osteoblasts, cement line at the junction between native and new bone, collagen fibrils and matrix vesicles (C). Preosteoblasts and osteoblasts surrounded by bone matrix (D).

to bone tissue formation **(Figs. 3A)**. Additionally, calcifying globules are observed on the collagen fibers, and deposition of crystals similar to those in bone is evident indicate the banding pattern of collagen fibers of the bone.

The osteoclasts appeared and they increased in amount. These cells are multinucleated, with numerous mitochondria, and vacuoles. Some possessed numerous microvilli, and some kept away from bone tissues, while still others were located in the immediate vicinity of capillaries with morphologically similar mononuclear cells **(Fig. 3B)**. Attachment occurs via the sealing zone and resorptive activity along the ruffled border. An electron dense interfacial matrix layer lamina limitans is observed between the sealing zone and calcified tissue surface.

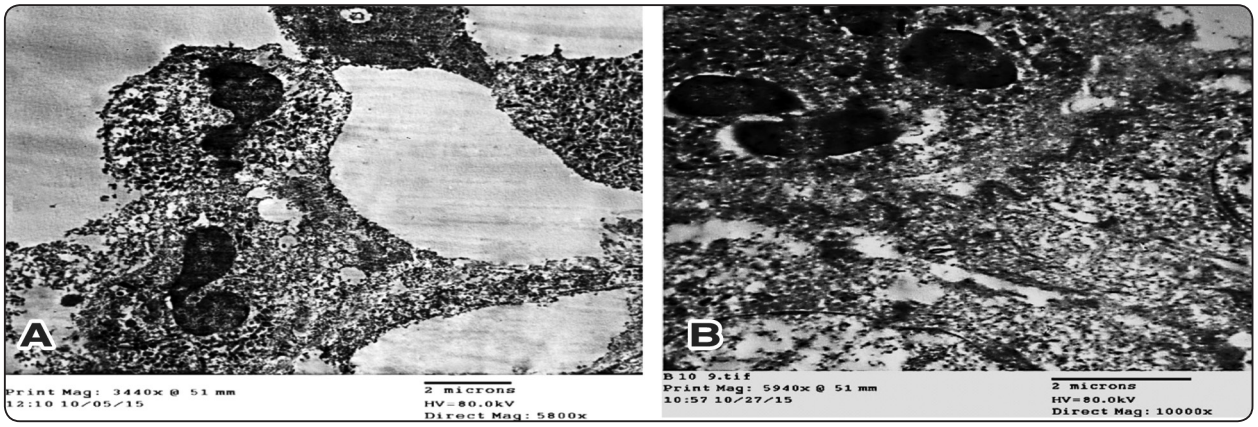

Fig. (3) Electron micrograph of an osteocyte. The cell, housed in its lacuna (L), emits a cytoplasmic process (CP), which exits the lacuna through a canaliculus (A). The osteoclasts attached to bone. Osteoclasts are multinucleated large cell. Attachment occurs via the sealing zone and resorptive activity along the ruffled border. An electron dense interfacial matrix layer lamina limitans is observed between the sealing zone and calcified tissue surface (B).

DISCUSSION

The bone has a hierarchical structure at both the microscopic and macroscopic levels, which allows it to perform its primary function of providing support and distributing biomechanical load. Osseointegration is a dynamic process; this study was to examine the changes in bone cells and their relationship with the implant surface.

PRF is an immune and platelet concentrate that contains all the constituents of a blood sample favorable to healing and immunity. It was considered by **Choukroun et al. 2000** [13] as a natural fibrin-based biomaterial favorable to the development of micro-vascularization and able to guide cell migration. **Dohan et al. 2006** [15] evidenced that; such a membrane can accelerate wound healing.

The findings of the current work revealed the appearance of a thin amorphous material interposed between the bone and implant in the control group. Beneath this layer woven bone formation with different cellular activity and random arrangement of collagen fibers were observed. The experimental group showed a greater cellular activity with well-organized collagen fibrils. Mature bone appeared at the bone-implant interface. These results may be interpreted by the direct effect of PRF on the proliferation and differentiation of bone cells.

TEM showed morphological remodeling taking place, through the process of implant surface transformation and deposition of the bone tissue. Studies of **Jung et al. 2003**[18] **and Nevins et al. 2005**[19] have showed that bone regeneration procedures may be enhanced by the addition of specific growth factors. Platelets content a variety of autologous growth factors, including platelet-derived growth factor (PDGF), transforming growth factors β1 and β2. **Wing et al. 2011** [20] reported that these growth factors have important role in the anabolic action of osteoblasts differentiation as, regeneration of alveolar bone and the adhesion of platelet to the roughened titanium surface of the implant as fibronectin.

Our results coincide with **Jiing-Huei et al. 2011**[21] who reported that, PRF stimulate osteoblast differentiation; and **David et al. 2010**[22] who investigated that, PRF can stimulate proliferation of the bone mesenchymal stem cells. This also may agree with **Yamada et al. 2004** [23] and **Ito et al.** 2005 [24] reported that bone formation could

be achieved using platelet-rich plasma (PRP) as a scaffold.

In the present research, the specimens of the mandible examined for the presence of TGFβ. The results showed different immunoreactions. In the control group, the whole tissue expressed TGFβ. On the hand, the immunostaining was more prominent in the experimental group revealed brown staining in all cell types and interstitial cells. This is in accordance with several studies. [25-29] **Hämmerle et al. 2004** [25] and **Funato et al. 2007** [27] attributed that TGF-β has many roles, including promoting angiogenesis, which is essential for orderly fracture repair, stimulating bone formation by inducing differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts. As match as regulating cartilage matrix calcification, stimulating osteoblast activity and intraosseous wound regeneration. Other actions recorded by **Penarrocha et al. 2004** [26] and **Vasconsellos et al. 2006** [29] include inhibiting osteoblast differentiation and mineralization and the formation of osteoclasts, also increasing the production of other bone and cartilage components such as collagen, fibronectin, osteopontin, osteonectin, thrombospondin, proteoglycans, and alkaline phosphatase.

CONCLUSIONS

From the results of the present study we can conclude that:-

- At 4 weeks, there is marked enhancement of the bone formation after the application of the PRF.
- The activity of bone cells is in proportional to the TGF β growth factor intensity.
- Bone formation occurred from native bone site to the implant bone surface and contrariwise in the PRF-treated group. But in the control group, bone formation occurred from native bone site to the implant bone surface only.

RECOMMENDATIONS

Further comparative studies of multiple and longer periods for investigation is recommended because the use of single time point (4 weeks) making it difficult to predict various temporal changes in the bone cells numbers, activity or morphology. Use of larger doses of PRF is also recommended because the response may be dose-dependent, increasing with increase the amount of PRF.

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