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RESEARCH ARTICLE

Assessment of Peri-implant Bone Disorders Treatment by Bone Replacements: Histological and Immunhistochemical Analysis in the Rabbit Tibia

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Abstract

Aims: to assess the efficiency of bone replacements in peri-implant disorders formed in the rabbit tibia. Methods: Twenty-four rabbits used to receive 36 screws of implant in their tibia. A circular bone defect 6.1 mm in diameter/4 millimeter depth made in the rabbit tibia. Dental implant sgrew "4.1 mm × 8.5 mm" placed beyond formation of defect, offering a "2-mm space". These spaces were lauded either with blood clot (BC), particulate Bio-Oss(R) (PBO), or Bio-Oss(R) Collagen (BOC). Eight animals were euthanized after 30, 45, and 60 days. Histological and immunehistochemical analyses for protein expression of osteocalcin (OC), OPG, and RANKL, were estimated. Results: At 45 and 60 days, between the PBO and BOC categories and also in the PBO and BC categories, RANKL expression was significantly different. At 8weeks, there was higher significant relation of OPG expression in the PBO category than BC category. Conclusions: An indication of our data as compared to BC and PBO, BOC give good bone healing that was described by high OC, and OPG immunolabeling. So with PBO, BOC give hopeful biological characteristics supporting its plausible application in peri-implant spaces osteoconductive grafts filling.

Keywords: *Osseo integration, Bone replacements, Implant, RANKL, Osteocalcin.*

Introduction

Bone resorption in the first three months after extraction of a tooth is large, so the acceptable esthetic and functional effects will delay implants loading [1]. So for defeating determination, a study [2] reported implant placement to socket of newly removed tooth, categorized method as "immediate dental implant insertion". Although, when the tooth had been extracted, the diameter of implant is commonly narrower than the alveolar socket, so a space between the implant surface and bone wall is noticed, that cause dimensional changes in the height and thickness of alveolar walls [3, 4, 5].

In respect to the size of the space, achievement of osseointegration may occur with bone replacements or alone. 'The peri-implant marginal defect is usually big in its marginal part and need osseous healing from the implant surface to the walls of the defect'. A previous study [6] on dogs, peri-implant bone defects show bone regeneration in a 0.5-mm wide with a smooth surface implant. The conclusions show that in a 0.5-mm defect space, there was a regeneration of bone. Also, Botticelli *et al*. [7] said that beyond 4 months spontaneous bone fill occur in surrounding space defects higher with a "rough surface implant (SLA) and guided tissue regeneration (barrier membrane)". Although, a histological restoration for larger surroundings "peri-implant bone defect" not happen unless bone-regenerative methods and materials manipulation in different ways to promote growth of bone [8].

There were various materials to fill peri-implant spaces in a trial to raise the accuracy of the management of the case. So in this case, a marginal space loaded with bone replacements involving homogenous, heterogeneous, autogenous, or alloplastic bone. In latest time, regeneration physiologically enhanced by adding mineralized bone matrix and 10 % porcine collagen, BOC, which played as a bone replacement choice for bone , that give pro-

angiogenic features, rapid growth, endothelial cells reproduction & maturation, that [9]. Many researches [10, 11, 12] produced estimating BOC to occupy the alveolus of extraction and periimplant space. Presently, researches utilize BOC to laud peripheral space not produced good details concerning process of healing in bone [10, 11, 12]. Also, no record has addressed the processes of tissue repair of BOC assessed over various periods of assessment.

So, as the requirement for good conception about the repairment process of tissue utilizing BOC, we seek for rectifying space defects around implant made in rabbit which occupied by either clot of blood (BC), particulate Bio-Oss® (PBO), or Bio-Oss® Collagen (BOC). Principle of research is that PBO shows big effect of regeneration in bone, so intendance for differentiation with BOC to assess its effective ability for enhancement to formation of bone around implant placement beyond osteotomy produced around fabricated spaces.

The supposition was "that the healing process in circumferential space defects of 2 mm surrounding osseointegrated implants would favor BOC in comparison to PBO and BC". So to do this aim , analyses of histological, and immunohistochemical work to detect "osteocalcin (OC), osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B-ligand (RANKL)" proteins were assessed in research after operation at 4weeks, 6weeks, and 8weeks.

Material and Methods

The calculation of the sample size was derived from papers which were published previously [13, 14, 15, 16]. As Animal Research recommendations, the animal model was designed: The guidelines for fulfillment and subjection of researches in animals was reporting In Vivo Experiments (ARRIVE) [17]. Rabbits were housed in a" facilities at 25°C, in 12-hour light &dark intervals". Over interval of experiment, we put the rabbits individually, good diet gave normally.

Twenty four male Albinus rabbits about 5 months old (New Zealand) with 3- 4 kgm body weight were entered in this research. The experiment was divided to three intervals: 30, 45, and 60 postoperative days (each interval contain 8 animals). For each interval, 4 rabbits gain 2 screws, one in each tibia and 4 rabbits had one screw "in any tibia right or left". Surgical part of experiment for implant placement was made as formerly displayed [18]. The anesthesias were given to the animals "intramuscular injection of ketamine hydrochloride 50 mg/kg and xylazine hydrochloride at 5 mg/kg". Then below the knee of the tibia 'an incision 3.0 cm in length was done. Dissection of the soft tissue was made with cautious and uses the periosteal elevator to lift it, to expose the bone tissue and insertion of the implant.

Then, a circular bone defect 6.1 mm in diameter/4 millimeter depth was made in each rabbit tibia. A screw of dental implant (4.1 mm × 8.5 mm) was placed after the formation of the defect, offering a 2-mm gap. These gaps were filled either with (BC), (PBO), or (BOC). One 4.1-mm diameter and 8.5-mm length cone-shaped screw, was placed (under 40 N of torque) in the hole of the tibia by "contra angle hand piece", the whole number was 36 screws. After that, we put the cover screws.

The bone defects space lauded by: BC, PBO, and BOC. With careful we reposition the soft tissue and by using a suture special type which is absorbable we sutured the incision, then we conduct antisepsis with PVPI. Immediately an intramuscular injection of "pentabiotic (0.1 mL/kg)" was given, also another dose after 5 days postoperatively. At 30, 45, and at 60 postoperatively days, 8 rabbits were euthanized of by (200 mg/kg) pentobarbital which is a lethal dose.

Histological Processing

By using reverse torque the screws were removed, then bone/implant samples were separated and dipped for 72 hours in 10% paraformaldehyde. After that, we decalcify the samples for three months in 5% ethylene-diaminetetra-acetic acid (EDTA). Then, in running water the samples were washed for 24 hours, dehydration, diaphonization and paraffin embedding, and then obtained "5-µm sections". Staining the sections for morphological tests of the repair of bone in the various groups and intervals by Hematoxylin and eosin stain (H&E).

Immunohistochemistry

The use of the primary antibodies was as following: receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and osteocalcin (OC).

After washing in tap water, with Harris hematoxylin specimens were briefly counterstained. Primary antibody as a negative control was neglected and with 1% PBS, the specimens were incubated in order to estimate staining the background while we use as a positive control, the lower cortex of the tibia in order to checking the antibodies affectivity. An examiner, who was blinded to groups of the experiment, in an optical microscopy we analyzed the digital images.

Protein expression quantification in the region of the threads of an implant was estimated by a quantitative analyses depend on scores (hyperpositive+++, super-positive ++, positive +, and negative-) depending on studies which are previously published [19, 20, 21]. The ratio of The RANKL: OPG was used in order to assess any formation or inhibition of osteoclast in the various categories [22].

Results

Structural Analysis

After 4 weeks, BC category showed in threads area of implant a reparation of bone tissue in its initial stages with connective tissue proliferation tendency with observation that CT get in touch with implant.

Around trabeculae of bone a cellularized CT was found and the cement line in the upper cortical region (Fig.1). In the PBO and BOC groups, the area of the threads of implant showed new bone formation and between the threads there were osteoblasts and CT (Fig.4, 7). At 45 days, in the BC group, we found CT at the threads of implant and upper cortical region but no indications of osseointegration (Fig.2). While, in the PBO and BOC categories, the area of the threads of implant showed new bone formation and at the bone/ implant contact there were osteoblasts and CT. In the upper cortical region, around the biomaterial there was new bone formation and small areas with CT were seen (Fig.5, 8). The results at 60 days of the BC category were the same of the prior 45-day period, and CT which is fibrous in the threads of implant also in the upper cortical area there were no indications of osseointegration (Fig.3).

In the PBO and BOC categories, at the thread area of implant there was a new bone formation and there was a CT around the biomaterial. Fibrous CT around the biomaterial was found in the PBO category at the upper cortical region (Fig.6). While, in the BOC category, bone trabeculae were found beside the biomaterial in upper cortical region (Fig.9).

Figure 1: View of threads in the BC category at 30 days

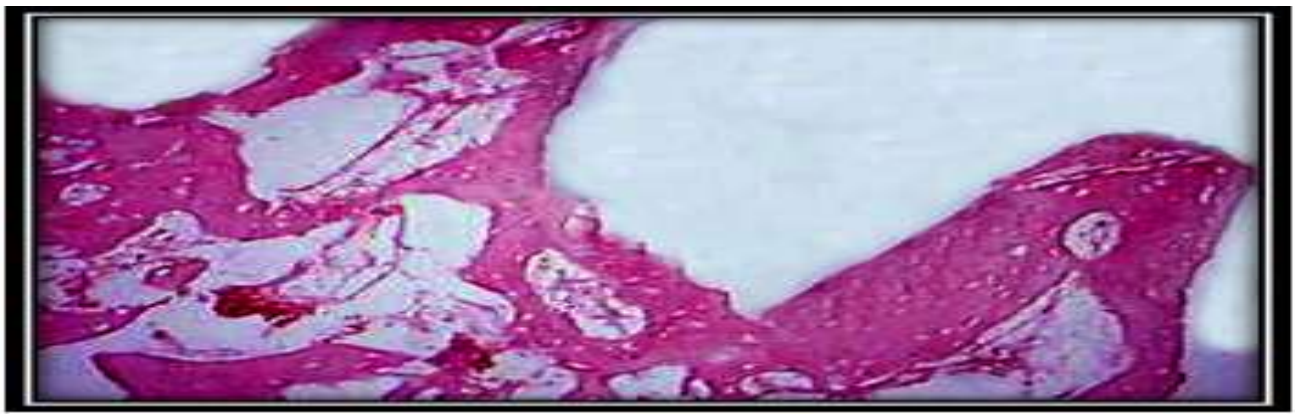

Figure 2: View of threads in the BC at 45 days

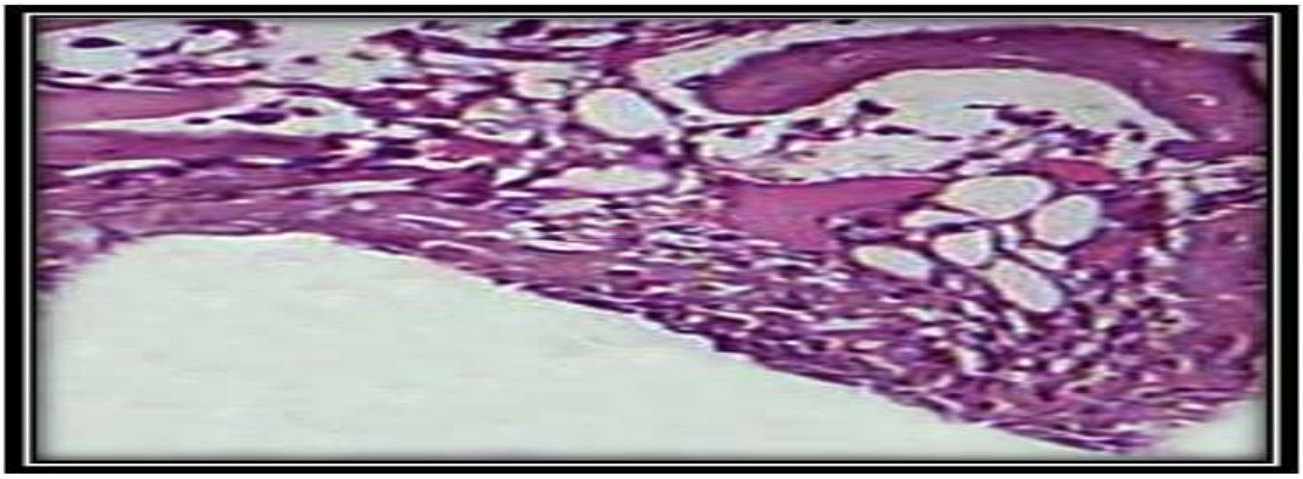

Figure 3: view of threads in the BC category at 60 days

Figure 4: view of threads in the PBO category at 30 days

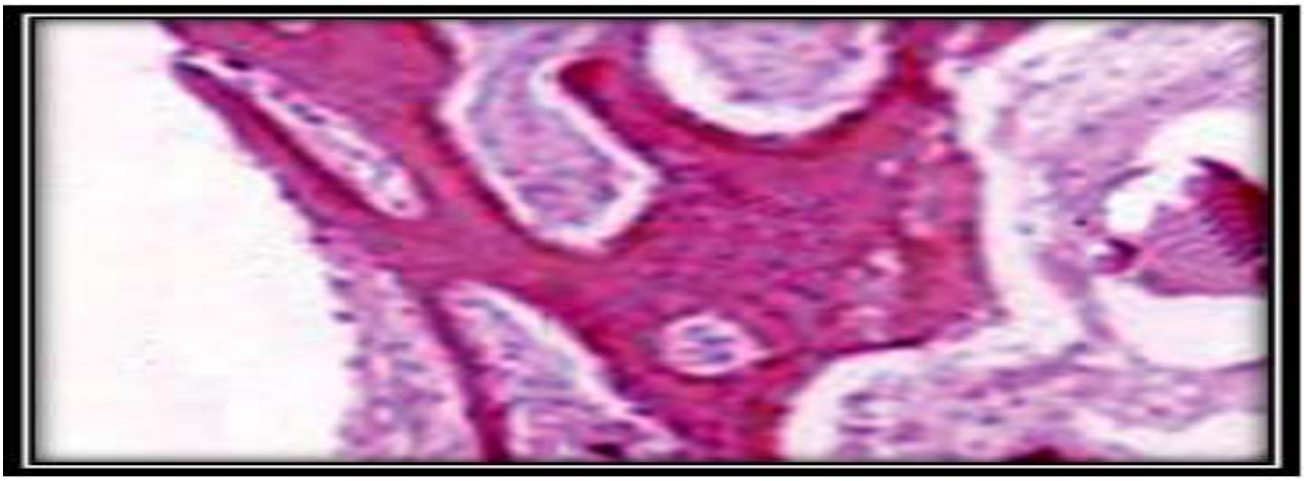

Figure 5: view of threads in the PBO at 45 days

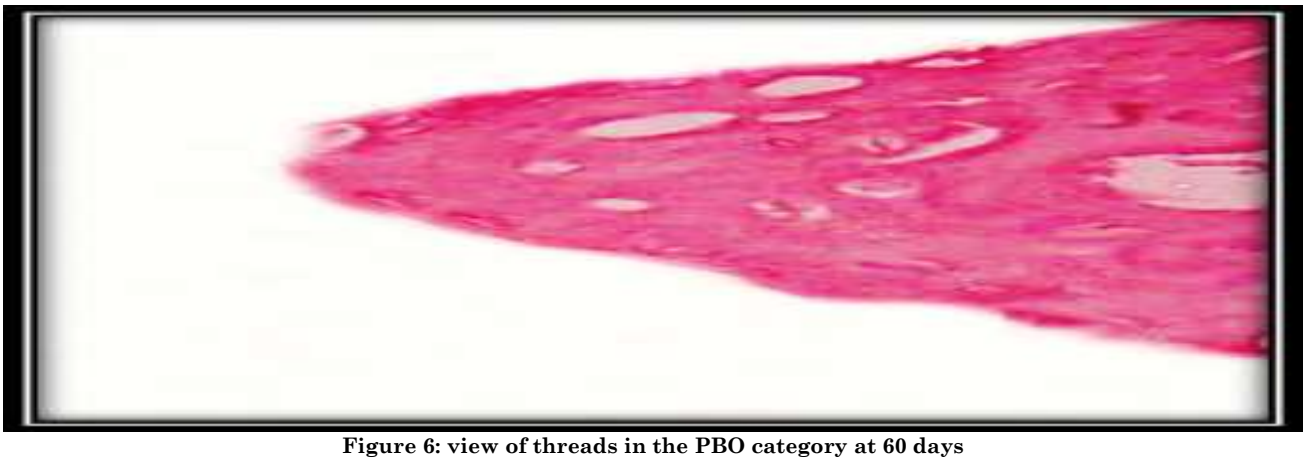

Figure 6: view of threads in the PBO category at 60 days

Figure7: View of threads in the BOC category at 30 days

Figure 8: View of threads in the BOC category at 45 days

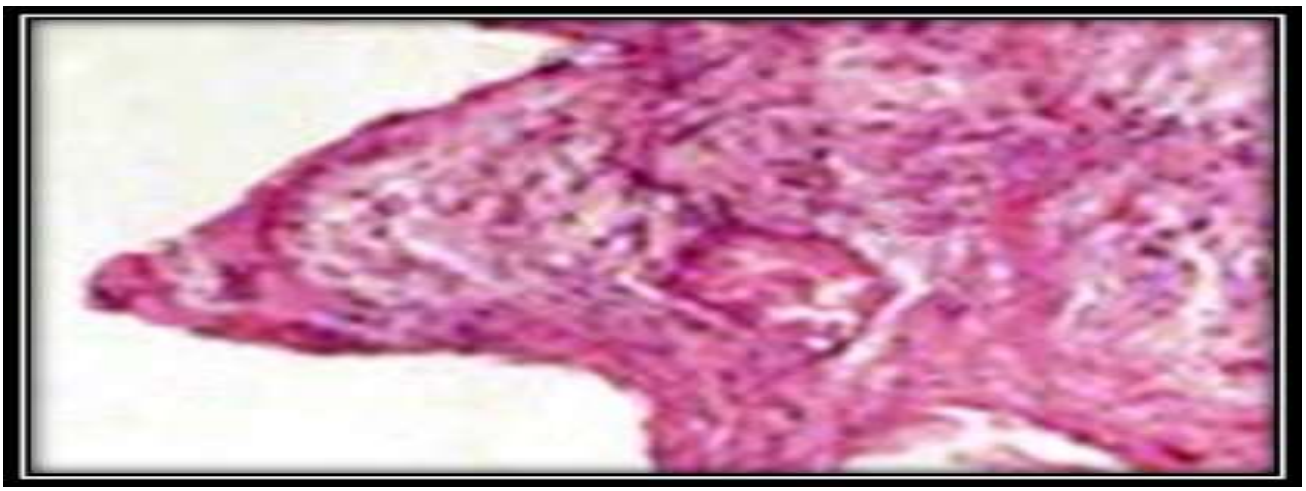

Figure 9: view of threads in the BOC category at 60 days

Immunohistochemical Analysis (IHC)

The using of primary antibodies -RANKL anti-OC and -OPG, conduct the IHC reactions. Analyzation of the reaction was done throughout the period of healing at the bone/implant contact.

Receptor Activator of Nuclear Factor Kappa-b ligand (RANKL)

BC category, there were various labeling of RANKL found, significant statistical difference "P=0.002" showed between 8 weeks which was 'negative' and the other intervals which was 'positive'. While in the PBO category, no significant statistical difference found in labeling of RANKL for all the intervals. Although, over time we noticed the decrease of labeling, varied from super-positive to positive. The labeling of RANKL varied "from negative to positive" in the BOC group. At 45 and 60 days, there was a 'significant statistical difference' (P=0.028) appeared and decreased labeling when the interval was end. When doing a Comparison between the groups, PBO showed higher RANKL labeling than BC and BOC at 30

days (P=0.003), while there was no difference noticed at 45 days. At 60 days, PBO there was higher RANKL labeling than in BC (P=0.006) (Fig.13). Although no significant statistical difference was showed among the categories about the RANKL/OPG ratio, in the BOC category found decrease amount than the other categories in all intervals of assessment (Fig.15). Osteocalcin (OC) in the BC group, osteocalcin labeling was noticed in the area of the threads of implant in all intervals, that assured cell viability. a statistically 'significant difference' (P=0.002) showed at 4weeks (2.00±0.00) "positive" while the rest intervalss (3.00±0.00) "super-positive". Positive OC labeling was noticed in the PBO category. In the BOC group, OC labeling give decreased expression through 30 days '2.70±0.54' with 45days '2.03±1.00', while equal among 45days and 60 days '3.00±0.00' (Fig.14).

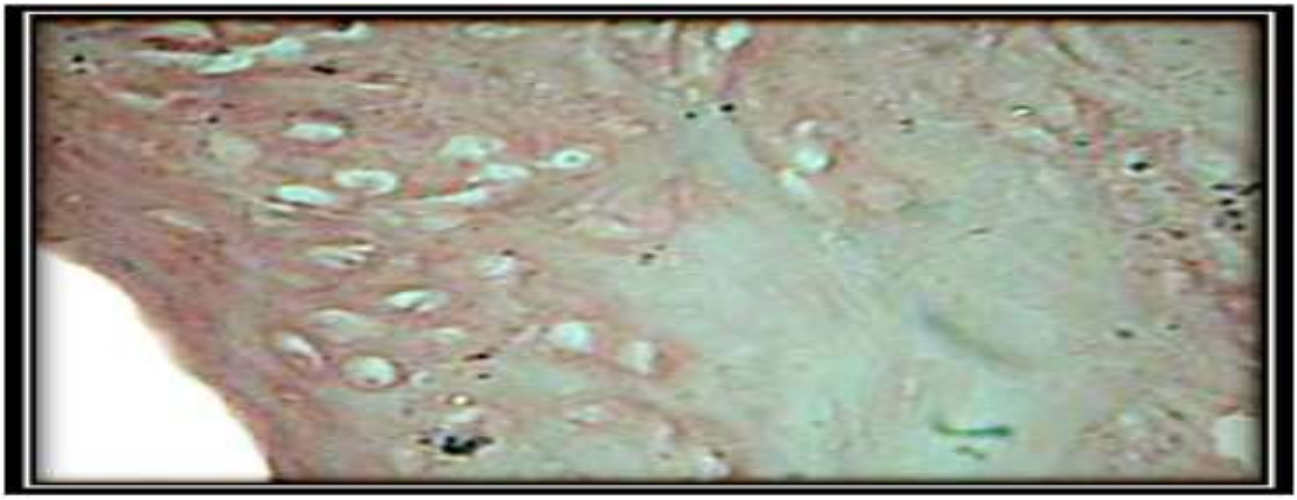

Figure 10: view of threads in the BOC category at 30 days for “osteocalcin" level

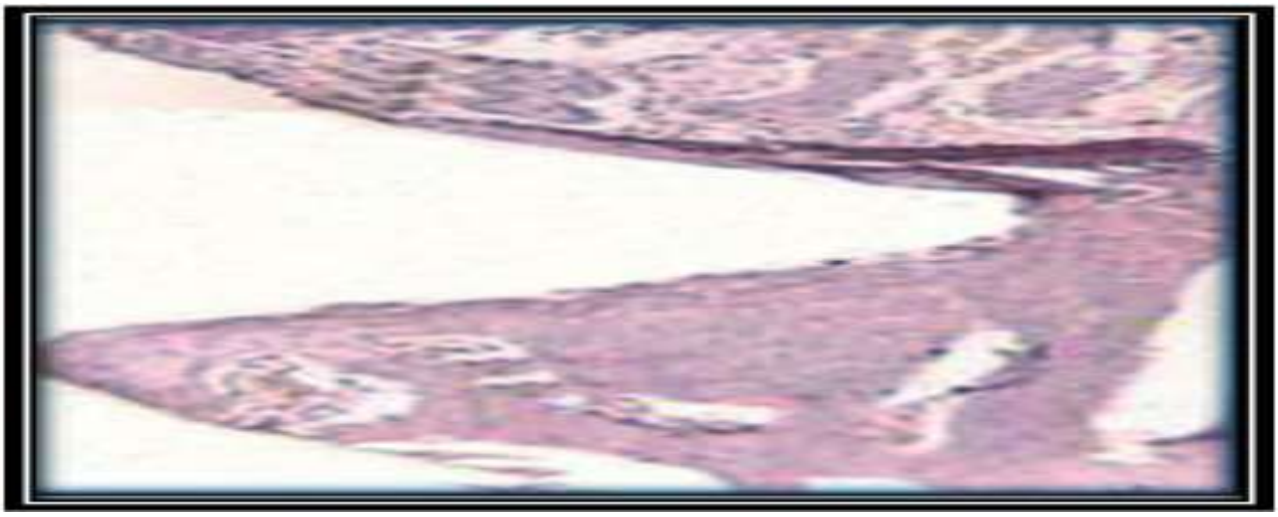

Figure 11: view of threads in the BOC category at 45 days for “osteocalcin" level

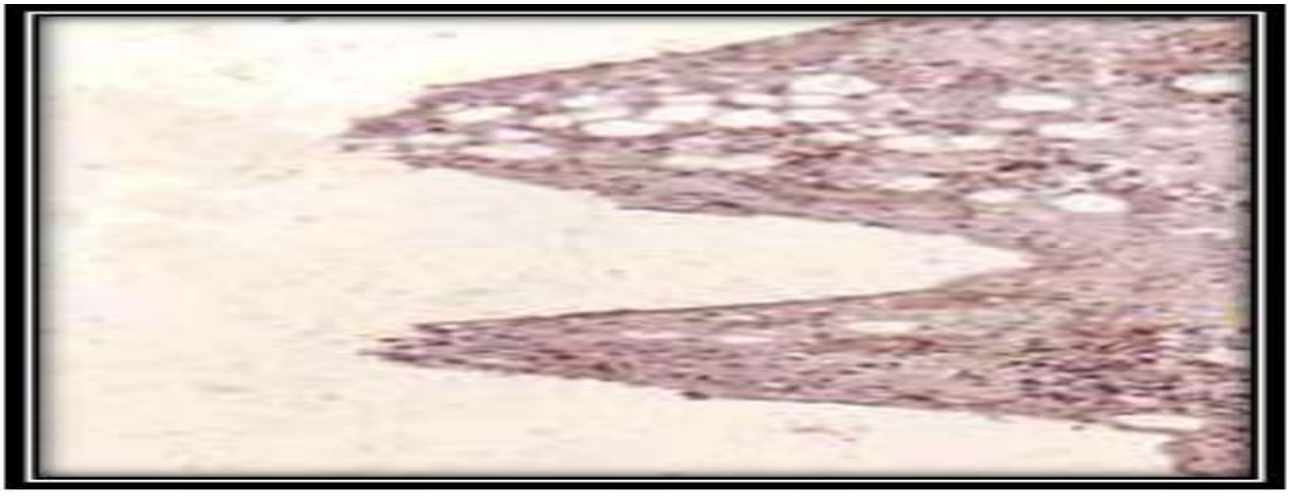

Figure 12: view of threads in the BOC category at 60 days for “osteocalcin" level

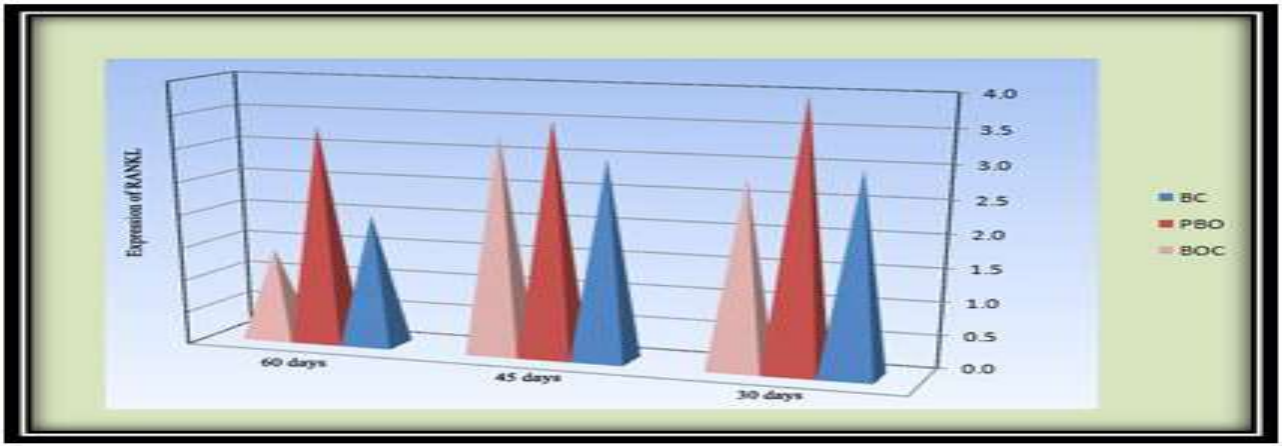

Figure13: Labeling of RANKL over time

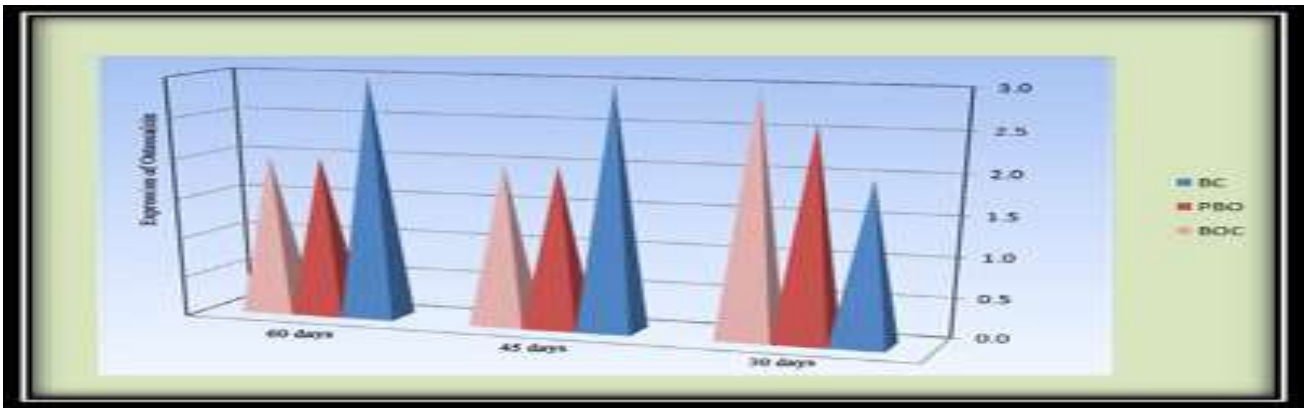

Figure 14: Labeling of Osteocalcin over time

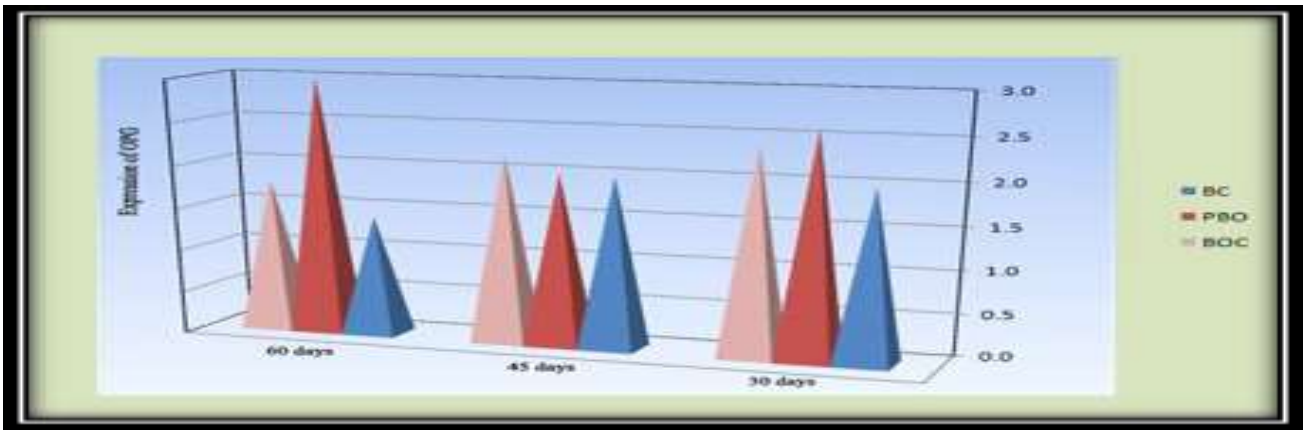

Figure15: labeling of OPG over time

Discussion

Conduction to new alveolar healing in a defect is by clotting the blood and maintenance of the clot at area of healing. Alveolar ridge volume preservation and healing promotion can be done by filling the alveolus with a type of biomaterial [23, 24]. Also, the insertion of biomaterial together with placement of implant can promote the restoration of function and esthetic. So, the purpose of this research was to assess the repair of bone around the dental implant in circumferential space defects loaded with BC, PBO, and BOC. The results showed that BOC gave good healing of bone when compared with BC and PBO categories, recognized by elevated OC, and OPG immunolabeling, particularly in the 45- day interval. BOC and PBO together have hopeful biological features assisting the feasible use of them as osteoconductive grafts to fill gaps around the implant.

Formation of bone as a consideration to immunohistochemical results was noticed in the BOC category, as proved by elevated OC, and OPG immunolabeling, particularly in the 45-day interval. The RANKL staining display the activity of osteoclast, which is constant all the time. This verity, related with decrease in the expression of OC in PBO & BOC categories through 8weeks interval,

ending to reduce the formation of bone, and as a result, the existence of elevated size of connective tissue. Primary stability of implant is achieved via physical attachment of bone with implant [18] that required to osseointegration [25], that biologic attachment of implant with bone was introduced. When an implant is not stable this may end in peri-implant fibrous connective tissue formation [26].

In histological analysis we find the identical results since we noticed a connective tissue around the biomaterial. A study regarding the Bio-Oss® [27] proved bad stability of implants in the created bone defects in the dog mandibles after 2 months. Bone repair in defects around the implant lauded with biomaterials have been estimated by many researches in the literature either using animal [6, 10, 12, 25] or human [3, 28] samples.

Construction of space around implant can be assessed by clinical, radiographic, histological and immunohistochemical analyses. The creation of a space between the implant threads and the surface of bone is due to the alveolus conical design which is commonly bigger than the dimensions of implant. Some studies [3, 10, 25] estimated this gap in regarding to the biomaterials insertion. Akimoto et al. [6] approved that the diameter of defecte in bone affects the rate of bone to implant attachment, which indicate a benefit of insertion of biomaterial to fill all defects.

In our study, a defect around the implant “diameter of 2 mm" larger than the 'implant diameter' was made to enhance the placement of a dental implant in a fresh extraction socket.

In BC group there was still spaces because of no new bone formation in the cervical and medial (medullary) region. At 30 and 45 days, the PBO and BOC categories gave significant new bone formation with the existence of biomaterial at the interface between bone and implant. While, at 60 days, we found a connective tissue around the biomaterial in the PBO category. Carmagnola et al. [9] used Bio-Oss® and evaluated reactions of tissue around implant and manifested that after 4 months there was no integration between the host tissue and the demineralized bovine bone, which gave a suggestion that the defect of bone was large. A study [10] was done in dogs to estimate bone restoration at defects around implant lauded by BOC. After 6 months, the biopsies collection was done to do histological analysis. The authors estimate that BOC change hard tissue healing, assessing the bone-to-implant attachment. These findings were the same as results of a study in dogs done by Han et al. [12] which approved that after 8 weeks, the integration of the PBO and BOC was firmly to bone surface.

Important details detected by the analyses of immunohistochemistry regarding the attitude of cell according to the expression of proteins about bone metabolism during the osseointegration. Osteocalcin expression by osteoblasts and bone matrix hydroxyapatite binding gave us the importance of its study. Also for 'bone mineral content' maturation importance, which just appear next to finishing active osteoblast proliferation, acting as a stage of differentiation and maturation of osteoblast cells. Osteocalcin activated during the initial steps of bone reparation [31].

The findings of our study found that the BOC category gave elevated OC labeling more than the BC category at 30 days. At 60 days, there was an elevated OC labeling in BC category more than the PBO and BOC categories. Formation and activation of osteoclast is regulated by Osteoprotegerin protein, which is found when the bone resorption process was inhibited. In spite of the same labeling of OPG in all intervals, higher labeling in PBO category at 60 days. While, RANKL labeling is associated with stimulation of bone resorption.

The findings of this research manifested that the PBO category explained high labeling of RANKL than BC category on 4weeks and 8weeks. OPG and RANKL have reverse actions, the analysis of ratio detects their relative influence on bone remodeling [22]. BC category manifested decreased amounts to other categories over all intervals; BOC shows higher inclination to inhibition of bone resorption. So, depend on our results, as a conclusion the BOC, also PBO, manifests good biological properties supporting its possibility for using in osteoconductive grafts for lauding peri-implant spaces and may be considered a safe and expected biomaterial for pre-clinical and clinical use.

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