EFFECTS OF THE COMBINATION OF PROPOLIS AND HYDROXYAPATITE ON BONE REGENERATION
(AN EXPERIMENTAL ANIMAL STUDY)
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ABSTRACT
INTRODUCTION: The surgeon is always working to replace lost bone and obtaining enough bone quantities to do his/her surgical operations. In the present study we examined propolis in order to determine the bone healing capacity of it.
OBJECTIVES: Comparing histologically & histomorphometrically the bone healing rate of the combination of hydroxyapatite plus propolis extract versus hydroxyapatite.
METHODOLOGY: Eighteen New Zealand white male rabbits were used. Bilateral designed critical-size bone defects were prepared in the right and left tibia of 12 Rabbits. Defect in the right tibia (positive control) & received hydroxyapatite, while defect in the left tibia (study) & received (hydroxyapatite + propolis). Bilateral critical-size bone defects were prepared in both tibia of the other 6 Rabbits (negative control) & left empty. Sacrificing were done at 3, 6 weeks postoperatively.
RESULTS: In the positive control group, at 3 weeks, specimens revealed formation of new bone covering the defect area. While, in the study group, bone consisted of thicker trabeculae with more regularly arranged osteocytes and relatively smaller bone marrow spaces. At 6 weeks, specimens revealed higher percentage of the formed bone in the defect area in both groups. However, in the negative control group, the regenerated bone was lower than in the other groups. Histomorphometrically, the mean percentage of bone surface area in study group was higher than positive control group through all experimental periods but the difference was statistically non-significant (P3 = 0.155, 0.136) respectively.
CONCLUSION: Study revealed that histologically HA plus propolis showed better convenient results.
KEYWORDS: Propolis, bone regeneration, hydroxyapatite, tibia.

INTRODUCTION
A common challenge facing dentists is large & delayed alveolar bone healing which happens after removal of any pathologic lesion targeting the alveolar bone or even after tooth extractions. Alveolar bone protects nerves, arteries, and glands, as well as supports facial expression & masticatory muscles (1). Because of this wide range of functions, alveolar bone loss can have a major effects on people's quality of life (1,2). Healing consists of wide processes including vascular alterations; inflammatory activation; migration, proliferation and differentiation of distinct cell populations; then, extracellular matrix production and maturation; bone formation, modelling and remodeling, finally restoration of the damaged tissues (3). Generally, the process of healing of a large bone defect requires a lot of time for bone regeneration (4). The fracture site is suffering from poor blood supply that reaches to the fracture area and also from a decreased quantity of phosphorus & calcium that are required for strengthening and hardening new bone surfaces (4). As a consequence, it is critical to repair bone defects using biomaterials designed as defect fillers that can accelerate bone regeneration (5).

Critical size defect (CSD) is an experimental approach which is used during in vivo assessment of tissue engineered constructs. CSD has been used as an experimental model in the preclinical field of orthopaedics and trauma surgery to measure the efficiency of newly developed biomaterials to stimulate bone regeneration before clinical application (5). Schmitz and Hollinger defined CSD in 1986 as "the smallest size intraosseous wound in a particular bone and species of
animal that will not spontaneously enter the healing processes during the animal's lifetime” (6).

Propolis is derived from Greek, where pro means "at the entrance to" and polis means "community" or "city," implying that this natural product is used in hive defense (7). Propolis is a material that is extremely rare as a pure material. Propolis or bee glue is a natural complex resinous mixture made by bees & obtained from beehives (7). Propolis comprises a waxy nature which is used by bees in building of the beehives & repairing them after the attack of outside invaders (8,9) . Also as a barrier against external invaders such as snakes, as well as rain and wind (7).

Propolis was prescribed for topical therapy as well as an antiseptic material & as a natural mouth disinfectant for wounds by Greek and Roman physicians (9). Propolis was widely used at the end of the nineteenth century due to its healing properties, and during the Second World War it was used in several Soviet clinics for tuberculosis treatment due to the observed decrease in lung problems and appetite recovery (10). Propolis was applied before to treat burns, stomach ulcers and sore throats in the Balkan countries (11). Also systemic use of propolis may decrease the duration time of bone regeneration after bone loss (12).

There is a huge need for a biomaterial that accelerates bone regeneration in the medical field for restoring bony structure after trauma, bone infections & tumors (13). Hydroxyapatite (HA) could be prepared naturally or as a synthesized form. (HA) considered as a great artificial bone substitute material because of its biocompatibility & osteoconduction properties (14,15). Another advantage, (HA) increases the adhesion of bone cells and cell proliferation processes (16). So, the aim of the present study was to evaluate histologically and histomorphometrically the use of a combination of hydroxyapatite plus propolis extract versus hydroxyapatite in the acceleration of bone regeneration rate in the tibia CSD of rabbits.

**MATERIALS AND METHODS**

The present study was approved by the Institutional Ethics Committee for Animal Use of Alexandria University. Eighteen New Zealand white male rabbits of 5 months of age, with a weighing range about 3–3.5 kg were included in this study. Animals were acquired from the animal house of Medical Research Institute, Alexandria University. The rabbits were housed under the same normal environmental surroundings in the experimental animal house. The right tibia bone was served as the positive control side and the left tibia bone served as the study side in 12 rabbits. The positive control bone defects received hydroxyapatite while the study defects received hydroxyapatite plus propolis. Bilateral bone defects in both tibia were created & left vacant as negative control group in the other six rabbits.

The surgical operations were performed at the Institute of Medical Research, Alexandria University. The animals' water and feeding were stopped 12 hours before surgery. An intramuscular dose of (0.15-0.20mg)/Kg ketamine plus (1-2mg)/Kg lidocaine was used to anaesthetize the rabbits (17). The surgical area in both tibia of all rabbits were shaved before any procedure and the skin was rinsed and scrubbed with 2% povidone iodine to avoid contamination. An incision 4-5cm incision was made by surgical blade number 10 in the medial aspect of both rabbit thighs including skin and periosteum. The bone was then exposed after a flap was elevated. A critical size bone defect 6mm in diameter (18,19)& 5mm depth was created bilaterally using sterile trephine bur (6mm in diameter) with profuse saline irrigation to protect bone from heat generation (Fig. 1a). The dimensions of the (CSD) were checked with 6mm width and 5mm depth using the measures of trephine bur intraoperatively (Fig. 1b).

The positive control defects were filled by freeze-dried sterile synthetic hydroxyapatite (granules; manufactured by ACRO Biomedical Company, Taiwan) in the medial side of the right tibia of 12 rabbits (Fig.1e&f). The study defects were filled by hydroxyapatite & Propolis extract (MARTINEZ NIETO, S.A. Spain) in the medial side of the left tibia of the same previous 12 rabbits (Fig. 2a,b,c,d,e & f). The cover of propolis capsule were cutted with scissor and squeezed in order to obtain propolis extract (Fig.2a&b). Then, propolis extract were mixed with hydroxyapatite granules by stainless steel spatula in a dappen dish until having mixture (Fig. 2d). Mixture of hydroxyapatite and propolis extract was loaded using a blade part of periosteal elevator without any type of membranes from the dappen dish to the CSD until filling it (Fig.2e&f). The bilateral bone defects in the other six rabbits were created & left vacant as the negative control group. The wounds were sutured in layers, with non-absorbable Blue

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Monofilament polypropylene sutures. Finally, the rabbits were then returned to their cages separately, with no movement of their limbs. Following surgery, the rabbits were administered an antibiotic (Cefotaxime: Cefotax 1 g, Egyptian int. Pharmaceutical industries co. Eipico) for 5 days. The researcher and veterinary technician examined on the rabbits every day after surgery. At 3 and 6 weeks postoperatively, six rabbits from the positive control, study groups and three rabbits from the negative control group were euthanized with an overdose of ketamine (KET A-100) (20). The right and left sides of the tibia were collected. The defect were dissected out and processed for histomorphometric analysis and light microscopic examination.

1. Light microscopic examination
For fixation, all specimens were immersed in 10% neutral buffered formalin and then rinsed. Then it was decalcified in 10% EDTA and dehydrated in increasing alcohol concentrations. Xylene was used to wash the specimens before they were embedded in paraffin wax. Using the standard procedure, 5 mm thick slices were cut transversely and stained with hematoxylin and eosin (H & E) (21).

2. Histomorphometric analysis
Measuring percentage of recently created bone in each specimen requires an accurate analysis like histomorphometric analysis which is the most reliable parameter for evaluating bone healing (22). The percentage of newly created bone surface area in the defect area was calculated morphometrically using the Image J 1.46r software. Three transverse sections were cut from each specimen at the center of the defect. An image was obtained from each section at the same magnification power. In each image, a rectangle with standardized dimensions was drawn to cover most regions of the defect. The rectangle's surface area was measured by selecting a region of interest (ROI) from tools and recording the results. The bone marrow gaps were chosen using a wand tracing tool within each rectangle, measured, and subtracted from the entire size of the rectangle to get the area occupied only by bone.

The outcomes were presented as percentages (the proportion of area occupied only by bone in relation to the total area of the standard rectangle). Then, for each rectangle in each section, the mean percentage of newly produced bone was computed. The approach was then performed in each of the three sections of each specimen. Each of the eighteen specimens in each group underwent the identical technique. After that all measurements of the fifty four specimens were organized in an Excel sheet. The terms used are those defined by the American Society for Bone and Mineral Research's Histomorphometry Nomenclature Committee (23).

Statistical analysis
Normality of variables were checked using Shapiro Wilk Test, descriptive and box plot. Data was found to be non-normally distributed. All variables were mainly presented by median, minimum, maximum and inter quartile range in addition to mean and standard deviation (SD). Comparison between groups were done using Kruskal Wallis followed by pairwise comparisons with Bonferroni adjustments. The differences between the independent 3 and 6 weeks values were assessed using the Mann Whitney U test. The significance level was chosen at 0.05 P value. All of the tests were two tailed. SPSS for Windows version 23 was used to analyse the data.
ointment in a dappen dish, e) Loading of the mixture of hydroxyapatite and propolis, f) CSD was filled with the mixture of hydroxyapatite & Propolis extract.

RESULTS
The results were evaluated through histological assessments and histomorphometrical analysis to assess the quality and quantity of newly produced bone.

Clinical observations
All rabbits survived through the entire study period.
No significant complications or clinical signs of infection or wound dehiscence that would impair osseous regeneration throughout the whole follow up period.

Histological results
After 3 weeks
Light microscopic examination of group 1 (negative control) specimens revealed the formation of woven bone in each side of the defect area and the central part of the defect was devoid of bone. The bone consisted of relatively thin spicules which contain osteocyte lacunae. Voluminous osteoblasts covered the bone surface which indicate active bone formation. Some blood vessels were seen in the bone marrow spaces (Fig. 3 A & B).

In group 2 (hydroxyapatite) specimens revealed the formation of woven bone covering the defect area. Plump osteoblasts covered the bone surface. Numerous blood vessels were seen in the relatively large bone marrow spaces (Fig. 3 C & D). In group 3 (hydroxyapatite + propolis) bone trabeculae covered the defect area with areas of active bone formation was seen (Fig. 3 E & F).

After 6 weeks
Histologic examination of 6 weeks specimens revealed the persistence of a space in the center of the defect. The newly established bone on each side of the defect contains numerous large osteocyte lacunae. The surface of the bone was filled with osteoblasts. (Fig. 4 A, B & C).

The bone became more mature with the formation of small osteons and thick cancellous bone trabeculae in comparison to 3 weeks groups. Reversal lines were also seen which indicate bone remodeling (Fig. 5 A, B & C) (Fig. 6 A, B & C).

Histomorphometric results
Table (1) showed comparison between negative control, positive control and study groups according to the percentage of surface area of the newly formed bone at 3 & 6 weeks by means and standard deviation (SD).

The mean percentage of new bone surface area in group 1 (negative control) was 14.99 after 3 weeks, 26.57 after 6 weeks, and that increase was statistically non-significant as P value was P=0.249. The mean percentage of bone surface area in group 2 (positive control) was 49.63 after 3 weeks, 60.03 after 6 weeks, and that increase was statistically significant as P value was P=0.046 (P≤0.05). The mean percentage of bone surface area in group 3 (study group) was 75.76 after 3 weeks, 83.50 after 6 weeks, and that increase was statistically significant as P value =0.028 (P≤0.05).

It was observed also that all values of the mean percentage of surface area of newly formed bone of group 3 (study group) showed higher results than positive control and negative control groups.

After 3 weeks, there was a significant increase in the mean percentage of newly formed bone in study group in comparison to the negative control group where P<0.0001. However the increase was not significant with group 2 (positive control) (P<0.155).

After 6 weeks, there was a significant increase in the mean percentage of newly formed bone in study group in comparison to the negative control group where P<0.0001. However the increase was not significant with group 2 (positive control) (P<0.136).

Figure 3. Light micrograph (LM) of the defect area of group 1 (negative control) at 3 weeks. (A): shows the newly formed bone spicules (short arrows) surrounding the defect (stars). (B): Inset of the preceding micrograph at a higher magnification showing osteocytes lacunae at its center (short arrows) and plump osteoblasts (long arrows) lining the endosteal surface of the newly formed bone. Blood vessels are seen (arrow heads). Light micrograph (LM) of the defect area of group 2 (hydroxyapatite) at 3 weeks. (C): shows woven bone spicules (arrows) surrounding large bone marrow spaces (stars). (D): Inset of the preceding micrograph at a higher magnification showing the structure of the newly created bone which contains irregularly arranged large osteocytes lacunae at its center (short black arrows) and orderly arranged...
osteocyte lacunae (long black arrows) at the periphery of the bone spicules. Plump osteoblasts (arrow heads) line the endosteal surface of the newly formed bone. Well vascularized (red arrows) bone marrow spaces are seen. LM of the defect area of group 3 (hydroxyapatite + propolis) at 3 weeks. (E): showing bone formation (arrows) at the defect area. (F): Inset of the previous micrograph at a higher magnification showing the structure of the bone trabeculae which consist of areas of immature bone at the center with large irregularly arranged osteocytes (short arrows) surrounded by areas of mature bone with regularly arranged osteocytes (long arrows). Areas of intercommunicating collagen fibers mapping the shape of the future trabeculae (arrow heads). (H&E, x40 in A,C,E x200 in B,D,F)

**Figure 4.** LM of the defect area of group 1 (negative control) at 6 weeks. (A): shows the newly created bone (arrows) with the presence of a gap (stars) at the center of the defect. (B): Inset of the previous micrograph at a higher magnification showing the structure of bone at the periphery of the defect. It consists of thick cancellous bone trabeculae (short arrows) surrounding vascularized bone marrow spaces (long arrows). (C): Higher magnification of the previous micrograph inset showing some large irregularly arranged osteocytes (short arrows) and osteoblasts (long arrows) lining the surface of bone. (H&E, x40 in A, x100 in B, x400 in C)

**Figure 5.** LM of the defect area of group 2 (hydroxyapatite) at 6 weeks. (A): Shows mature bone development (arrows) at the defect area. (B): Higher magnification of the previous micrograph inset showing small osteons (short black arrows) and cancellous bone trabeculae (long black arrows) surrounding relatively smaller bone marrow spaces (stars). Blood vessels (arrow heads) and reversal lines (red arrows) are also seen. (C): Inset of the previous micrograph at a higher magnification showing areas in the center of the bone trabeculae with large haphazardly arranged osteocyte lacunae (short arrows). The endosteal surface of bone trabeculae is lined with flattened osteoblasts (long arrows). (H&E, x40 in A, x100 in B, x400 in C)

**Figure 6.** LM of the defect area of group 3 (hydroxyapatite + propolis) at 6 weeks. (A): shows mature bone formation (arrows) at the defect area. (B): The previous micrograph inset has been magnified to show thick cancellous bone trabeculae (short arrows) surrounding vascularized small bone marrow spaces (long arrows). Small osteons (arrow heads) can also be seen. (C): Inset of the previous micrograph at a higher magnification showing some large irregularly arranged osteocyte lacunae (short arrows). Osteoblasts (long arrows) line the endosteal surface of bone trabeculae. Several reversal lines are also seen (arrow heads). (H&E, x40 in A, x100 in B, x400 in C)

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<th>Table 1: Comparison between the study groups regarding percentage of surface area of newly formed bone.</th>
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DISCUSSION

Large bony defects which result from infections, accidents and invasive tumors considered as serious complications. Usually these deficiencies didn’t heal adequately, resulting in unfavorable treatment outcomes. In these circumstances, selecting a suitable graft material to induce new bone development will be essential (24). Propolis is reported by Pileggi et. al. (25) to affect both osteoclasts and osteoblasts. Hydroxyapatite (HA) has been considered as the gold standard studied material for various load-bearing biomedical applications (26). Confirmed reports on hydroxyapatite in combination with propolis are limited. So, the objective of this study was to compare the use of hydroxyapatite combined with propolis extract versus hydroxyapatite alone in acceleration of bone healing rate in the tibia bone defects of the rabbits.

In the present study, the combination of hydroxyapatite plus propolis resulted in an increase in new bone formation in comparison to hydroxyapatite alone. However, the increase was not significant.

The animal model which we used in our research were rabbits. Rabbits are the first choice of animal models in musculoskeletal research, according to Neyt et al. (27). Another reason is that rabbits are inexpensive and easy to keep. Furthermore, Castaneda S et al. reported that rabbits have a faster rate of bone regeneration than primates and other rodents (28).

In the present research, we have chosen the tibia because of its size, which is ideal for creating a bone defect; and its accessibility for surgical procedures. One of the major disadvantages of smaller animal models is the limited tissue harvest but tibia of the rabbit give adequate volumes of tissue required for histological assessment. A study was done by Laverty M in 2010 agreed that rabbit’s bone and cartilage is adequate enough to enable harvesting (29).

In this study the grafting materials were applied in two surgically created standardized bone defect area with size (6mm diameter* 5mm depth) in the tibia of each rabbits including negative control group. This standardization gives more reliable data as it allows equal healing conditions.

In the current experimental work, study periods (3&6 weeks) were chosen to cover expected phases in bone healing. Rabbits are characterized by a rapid healing response when compared to that in human, so the histological assessment was done after 3 weeks postoperatively to evaluate the early tissue reactions induced by the applied materials. Also the choice of the period (6 weeks) was done according to a research done by Terjesen T. who assessed healing of rabbit tibial fractures using external fixation, and determined that the maximum period for spontaneous bone fracture healing is roughly 6 weeks (30).

No clinical signs of adverse reactions, infection, delayed healing or dehiscence were noted in any of the studied defects. This means that, the application of propolis is safe, no additives, which prevents possibility of cross contamination and allergic reactions.

In our study the newly formed bone was assessed histologically and histomorphometrically in accordance with Reddy M.S. (31) in 2000 who said that the most accurate method to examine the true extent of osseous regeneration is histological evaluation, neither clinical nor radiographical examinations, as they cannot provide any accurate information about the type or degree of bone regeneration.

Histomorphometric analysis of the current study showed an increase in percentage of surface area of newly formed bone in comparison to group 2 (positive control) but the increase was not significant.

One of propolis effects is promotion of wound healing and actually this is the function of the flavonoids which increase the formation of both growth factors fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor A (VEGFA). (FGF-2) acts as a pleiotropic growth factor capable of stimulating progenitor osteoblasts and fibroblast cells (32). Propolis stimulates osteoblast proliferation, differentiation, and maturation, according to Lim YK et al. (33). Propolis loaded implants has been reported for increasing the expression of bone morphogenetic proteins (BMP) 2 and 7 at the surrounding tissues (34). Increasing expression of BMP-2 and 7 leads to increase new bone production around the implant structure, as well as improved mandibular and implant adhesion (34).

Histological results of our study showed that propolis plus hydroxyapatite resulted in bone formation improvement. After 6 weeks thick
cancellous bone trabeculae was formed and also small osteons were seen. Our findings match those of a study by Altan et al. (12), they found that rats with rapid maxillary expansion and propolis treatment (100 mg/kg/day for 12 days) resulted in improved bone production, as evidenced by a larger number of osteoblasts and new maxillary bone. A research was done by Kresnoadi et al.(35), who did orthodontic tooth movement (OTM) in male guinea pigs propolis treatment (2% propolis in polyethylene glycol; 0.1 mL propolis extract) for 3 and 7 days resulted in improved alveolar bone development, as shown by increased osteoblast number and protein expression of osteocalcin. Another study confirmed our results, Wiwekowati, W. (36) concluded that propolis addition (5% propolis gel for 17 days) in the alveolar bone of rats with OTM can increase osteoblast cells number.

Light microscopic examination of the current work of group 3 (hydroxyapatite plus propolis) at 6 weeks revealed the formation of new bone with well vascularized bone marrow. This supports the results of the study of Altan BA et al.(12) who reported that the group of rats which has expanded premaxillary suture plus propolis showed new formed capillaries. These results demonstrated that propolis administration may stimulate new blood vessels formation.

In addition, propolis have direct effects on osteoclasts resulting in osteoclastogenesis inhibition. Between many osteoblast markers, there is osteoprotegerin (OPG) expression which was up regulated by propolis. OPG prevents the binding of Receptor activator of NF-κB ligand (RANKL) to receptor activator of NF-κB (RANK), thereby halting RANKL signaling and osteoclastogenesis (37). A study was done by Wimolsantirungsri et al.(38) revealed decreased RANKL and RANK expression in osteoclast precursor cells following propolis treatment, which lead to inhibition of RANKL-RANK signaling pathway, resulting in a reduced osteoclast differentiation. Similarly, Yuanita et al. (39) discovered that propolis decreased osteoclast number while increasing osteoprotegerin (OPG) expression in the periapical region of alveolar bone in rats with Enterococcus faecalis-induced chronic apical periodontitis. Our findings are in accordance with those of Meimandi-Parizi et al. (40), who found that male Wistar rats with critical bone defects in the radius bone treated with demineralised bone matrix and propolis had enhanced development of new bone tissue, woven bone, and cartilage tissue.

The current study are also supported by a study by Atlan BA et al.(12) that revealed that propolis have a role in increasing osteoblast proliferation through it’s increased expression of osteoblast markers. Also propolis may also help bone regeneration by lowering the expression of inflammatory cytokines that are essential in osteoclast differentiation (38). These qualities are thought to be beneficial in the treatment of a variety of medical issues, including bone loss and fractures as propolis promote bone healing and enhance bone regeneration.

CONCLUSION
At the end of this study, we concluded that the use of hydroxyapatite combined with propolis shows a better histological evidence of bone formation compared to the use of hydroxyapatite (HA) bone graft alone. Also, this was confirmed by the histomorphometrical results. Furthermore, the osteoinductive potential of propolis was almost confirmed, makes propolis the first choice in the field of bone regeneration. We recommended a larger sample and shorter time periods between scarifications of the animals to know the exact time at which bone formation started. Future studies are needed with longer periods of follow up on the potential role of propolis and its related ingredients either alone or as a complementary therapy in dentistry. Further clinical studies are recommended to enlighten the use of propolis in the enhancement of bone healing among various bone defects in the oral and maxillofacial regions.

CONFLICT OF INTEREST
“The authors declare that they have no conflict of interest”.

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