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Eggshell-Derived Bone Graft Substitutes in Treatment of Acute Intra-Bony Periodontal Defect in Rabbits Histological Study

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ABSTRACT

Aim: This research investigated the probable efficiency of bone graft derived from eggshell in the treatment of intra-bony defect in rabbits.**Method**: 10 rabbits were enrolled in the study, for each rabbit two identical bilateral periodontal bony defects on the mesial aspect of lower first molars were surgically created, given total 20 defects. The rabbits were randomly divided into 3 groups. Group I,4 rabbits(8 defects): filed with hydroxyapatite bone graft. Group II: 4 rabbits, (8 defects): filled with eggshell granules. Group III the control group 2 rabbit(4 defects): left empty. The animals were sacrificed,and the specimens were collected for histological evaluationat 3& 6 weeks'post- surgery.A light microscope and immunohistochemical was used to assess the level of periodontal regeneration for expression of osteopontinbone regeneration marker.**Results**: group I & II, at three weeks, partial regeneration was observed with the formation of considerable novel woven bone.After 6 weeks, a reasonable amount of regenerated tissues was seen for hydroxyapatite treated specimens little bit more than eggshell bone granules. The final outcomes of regeneration showed that groups (I,&II) were comparable to each other and showed better regeneration than in control group (III). Intenseosteopontin expression at3 and 6 weeks' periods was observed for Group I & IIcompared to the control group. **Conclusion**: Eggshells powder is an inexpensive, easy obtained source of grafting material with reasonable biocompatibility and regenerative ability that can enhance bone formation and periodontal regeneration of intrabony periodontal defects in rabbit's model.

Keywords: Periodontitis, Three wall periodontal defect, eggshell bone substitute, Hydroxyapatite bone graft

Introduction:

Bone grafts, root biomodifications, soft tissue grafts, guided tissue rejuvenation and combinations of these operations are all considered periodontal regenerative procedures [1]. Bone graft materials are well known since long time to promote new bone formation and periodontal regeneration especially in intrabony defects[2].

Bone healing stimulation was attempted using numerous sources of bone grafts but are limited as they only provide osteoconduction and exhibiting no other properties of natural bone[3]. Autogenous bone grafts are regarded the gold standard graft material due to their exceptional biocompatibility and osteogenic qualities[4].

However, it shows many disadvantages such as second surgery, tooth ankyloses, donor site morbidity, root resorption, extended operation periods, and excessive expenses. As a result,

alternative options of bone graft supplies have been created and evaluated as substitutes for autogenous bone grafts[5].

On the other side, allografts produced from the same species donor, it requires the inactivation of proteins present in healthy bone normally and sterilization because of the possibility of disease transmission[6]. Xenogratfs are bone grafts from non-human animals, like bovine and are utilized as calcified matrices, it carries a riskypotential transmission of disease such as bovine encephalopathy and rejection of the graft [3].

Additionally, Synthetized bone replacements have been approved for clinical usage, however they are only osteoconductive and costly. They act only as inert defect filer relying on viable periosteal/bone for their success[7]. In recent years, a novel approach has been proposed using powdered chicken eggshell[8]. In regenerative surgery, the organic and mineral of the chicken eggshell's matrix has been proposed as a bone replacement option.It is comprised mostly of calcium carbonate (97.4%), magnesium phosphate (1.9%)and tricalcium phosphate(0.7%) hence the resemblance to the bone matrix[9].

Powdered eggshell is a non-toxic, substance that is very easy to work with., unlimited availability and lack of disease transfer risks. Eggshell-derived hydroxyapatite (EHA) was proven in various experimental animal studies to be an efficient biocompatible and osteoconductive biopolymer[10].

This study aimed to histologically and immunohistochemically assess the efficacy of the eggshell-bone substitute granules in treatment of infra-bony periodontal defects in rabbits.

Materials and Method:

Our study was developed in conformity with the standards for the responsible use of animals in research, which were recommended by the Ethical Committee of Tanta University's Faculty of Dentistry.

A sample of 10 adults, male healthy Neazland rabbits, weighting about 3.5-4.0 K g. were selected for the study. Separate cages with identical environmental circumstances were used to house the rabbits.Additionally, they were provided with a regular laboratory food twice a day and were allowed access to tap water.

for each rabbit two identical periodontal bony defects on the mesial aspect of lower first molar in both side of the mandible were surgically created bilaterally, given total 20 defects. The rabbits were randomly divided into 3 groups. G I study group (4 rabbits with 8 defects): The defects were filed with hydroxyapatite bone graft. G II study group (4 rabbits with 8 defects): The defects were filled with eggshell bone substitute. G III control group (2 rabbit with 4 defects): The defects were left empty.

Eggshell powder preparation

After pouring the egg contents, the adequate number of chicken eggs were rinsed using distilled sterile water. Using forceps, the external and internal shell membranes of the eggs were caustiously removed. The shells were ground into a fine powder then sieved to yield particles with a diameter of 1mm and a porosity of 75%. Three times in sterile distilled water with continuous agitation for one hour at room temperature, the powdered eggshells were washed. They were autoclaved for 18 minutes at 136° C[10].

Surgical procedures:

Anesthesia:

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The operation was carried out under general anaesthetic. The medications utilized were ketamine chlorhydrate (Rotexmedica, Trittau, Germany) at a concentration of 0.06 mg/L per kg body weight and xylazine hydrochloride at a concentration of 2%. (Xylaject, Adwea, Egyptian international pharmaceutical industries. 10th of Ramadan, city area, Egypt) 0.03 mg/L for every kilogram of body weight. Given intramuscularly for 12 hours following food and water deprivation.

Surgical steps:

Three walled intrabony defects bilaterally mesial to the mandibular first molars were formed in all rabbits. The animals were put in lateral recumbency, and the region was shaved and cleansed with betadine antiseptic solution (Povidone-iodine 10% w/v. El Nile company for pharmaceuticals and chemical industries, Cairo, A.R.E.).

On the alveolar ridge mesial to the mandibular first molar, A three-millimeter-long full-thickness incision was made. To reveal the related alveolar bone, minimal buccal and lingual mucoperiosteal flaps were raised. Under continuous water irrigation, a low-speed hand piece equipped with a 1 mm diameter round diamond bur was utilized to remove the mesial wall of the right mandibular first molar's alveolar bone socket.

The proportions of the defect were checked continuously using a clinical periodontal probe till the desired size and shape were achieved (W × L × D; 2 × 2 × 1, 5 mm). Root planting was used to remove remaining bone spicules and PDL fibers from the uncovered root surface using a hand periodontal curette. After properly rinsing the defective region with saline to eliminate all debris, the root surface was properly dried using sterile gauze.

The 20 defects that have been results were randomly divided into 3 groups G I (8 defects in 4 rabbits) filled with HA bone graft. G II (8 defects in 4 rabbits) filled with eggshell bone granules, and Group III (4 defects in 2 rabbits) the defects were left unfilled. To accomplish primary closure in all groups, the mucoperiosteal flap was adjusted and sutured with a resorbable interrupted suture (vicryl 5/0, International Sutures Manufacturing Co. Egypt).

Post-operative management:

To avoid surgical infection, the animals received ceftriaxone (Xoraxon, Medical Union Pharmaceuticals, Egypt) intramuscularly for three days. Three weeks later 4 rabbits from GI and 4 from G II and one from G III (Total 5 Rabbits) were sacrificed with an overdose of anesthesia. The other 5 rabbits with the same distribution were euthanized six weeks post-surgery. The bone samples were collected at each stage.

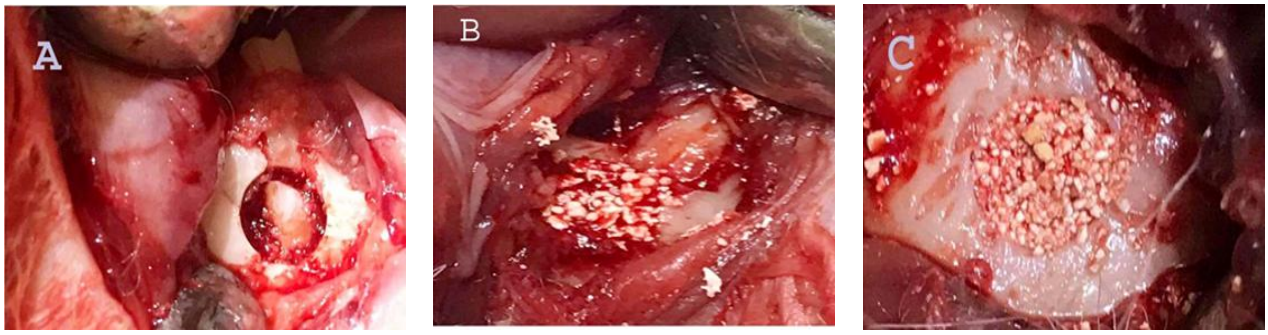

Figure (1)A: The mucoperiosteal flap raised and 3 wall defect created mesial to the root of the **B: HA bone graft in place fill the created defect** **C: Eggshell granules in place fill the created defect**

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Histological processing

Following euthanasia, the animal's jaw was removed, and the defective region dissected with a low speed hand piece equipped with a double-sided diamond disc. The samples were fixated in 10% buffered formalin for 48 hours and then decalcified in 10% EDTA.

The samples were first decalcified, then dehydrated, paraffin infiltrated, and lastly lodged in a block of paraffin. Serial mesio-distal longitudinal segments (5 μm in thickness) from the central area of the defect were cut using a microtome (LEICA, RM 2245, Germany) for staining with Haematoxylin–Eosin (H&E), Masson's Trichrome, and immunohistochemistry.

Immunohistochemical analysis of the expression of Osteopontin:

The avidin–biotin–complex (ABC) technique was used for immunohistochemical labeling. Representative sections were deparaffinized in xylene and rehydrated in a declining sequence of ethanol strengths. The sections were washed with TBS (20 mM Tris- HCl,150 mM NaCl, pH 7.4).

After that they were incubated at room temperature for 30 minutes in 0.3% H_2O_2 in dH_2O to block endogenous peroxidase. Antigen retrieval was carried out as directed by the manufacturer. For 30 minutes at room temperature, slides were immersed in 100 l blocking solution (Abcam). At 4^oc overnight, a rabbit polyclonal primary antibody against OPN (Cat. No. ab8448, Abcam, Cambridge, UK) was used at indicated dilutions.

Samples were rinsed in 1X phosphate buffered saline (PBS) and then incubated at room temperature for 1 hour in a humidified chamber with HRPconjugated goat anti-rabbit IgG at a dilution of 1/10000 (in blocking buffer). To visualize peroxidase, samples were incubated in ABC solution at room temperature for an hour. The color reaction was then initiated by coating the segments with DAB solution (0.5 mg/ml DAB and 0.1% H_2O).

When the color response was sufficient, it was halted by washing with H2O for 5-10 minutes and then counterstained for 2 minutes with hematoxylin. Gradually dehydrated sections were placed on coverslips. Using a Leica light microscope, immunohistochemical staining was evaluated.

Results

The present study was conducted on 12 male albino New Zealand rabbits where all of the animals continued the duration of the study without any drop out or complications. The healing was clinically uneventful in all animals with no local or systemic reactions.

Group I (HA treated group):

At 3 weeks' period:

H&E sections showed:Newbone formation of woven pattern with accentuated reversal line. Bone spicules were surrounded with osteoclasts indicating bone remodeling into lamellar bone.Remnant of graft material, and minimal inflammatory cells infiltration were observed in connective tissue stroma between bone spicules, many blood vessels were also observed. Disorganized periodontal ligament fibers and A thin cementum layer was discovered in the defective region. Masson &trichrome section showed:A new immature bone formation.

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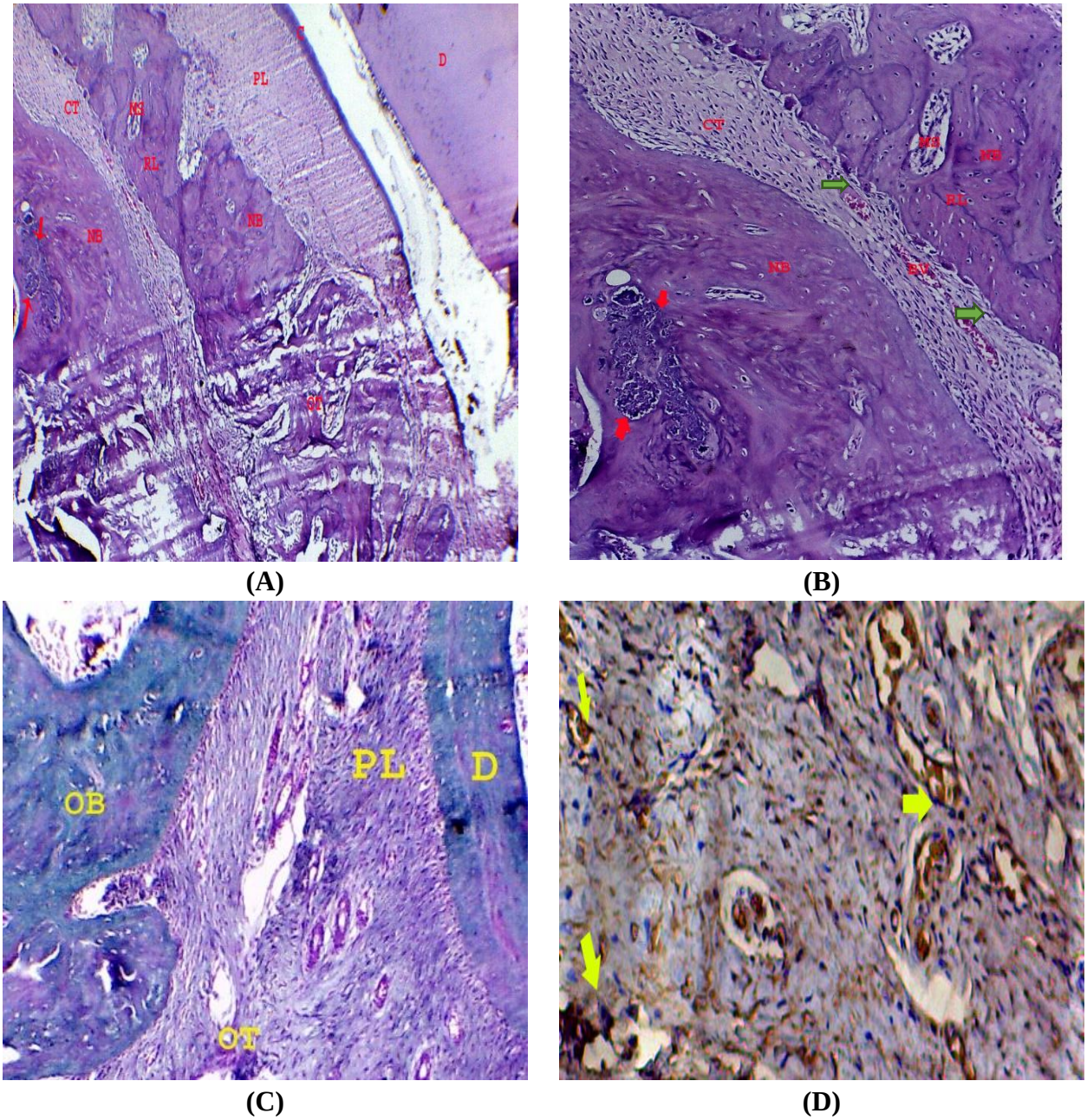

Figure (2)A: H&E section of HA treated specimen at 3 weeks showed area of highly cellular new woven bone formation NB, remnant of the graft material (red arrows),resting line RL and not well organized periodontal ligament fibers PL with thin layer of cementum B: Higher magnification showed osteoclast in their lacunae surrounded the bone (green arrows), remnant of graft materials (red arrows).C: Masson's &trichrome section showed newly formed bone formation where the bluish color indicates collagen fibrils in the bone matrix. D: Immunohistochemical staining of osteopontin, it showed high expression specially around blood vessels and in connective tissue (yellow arrows)

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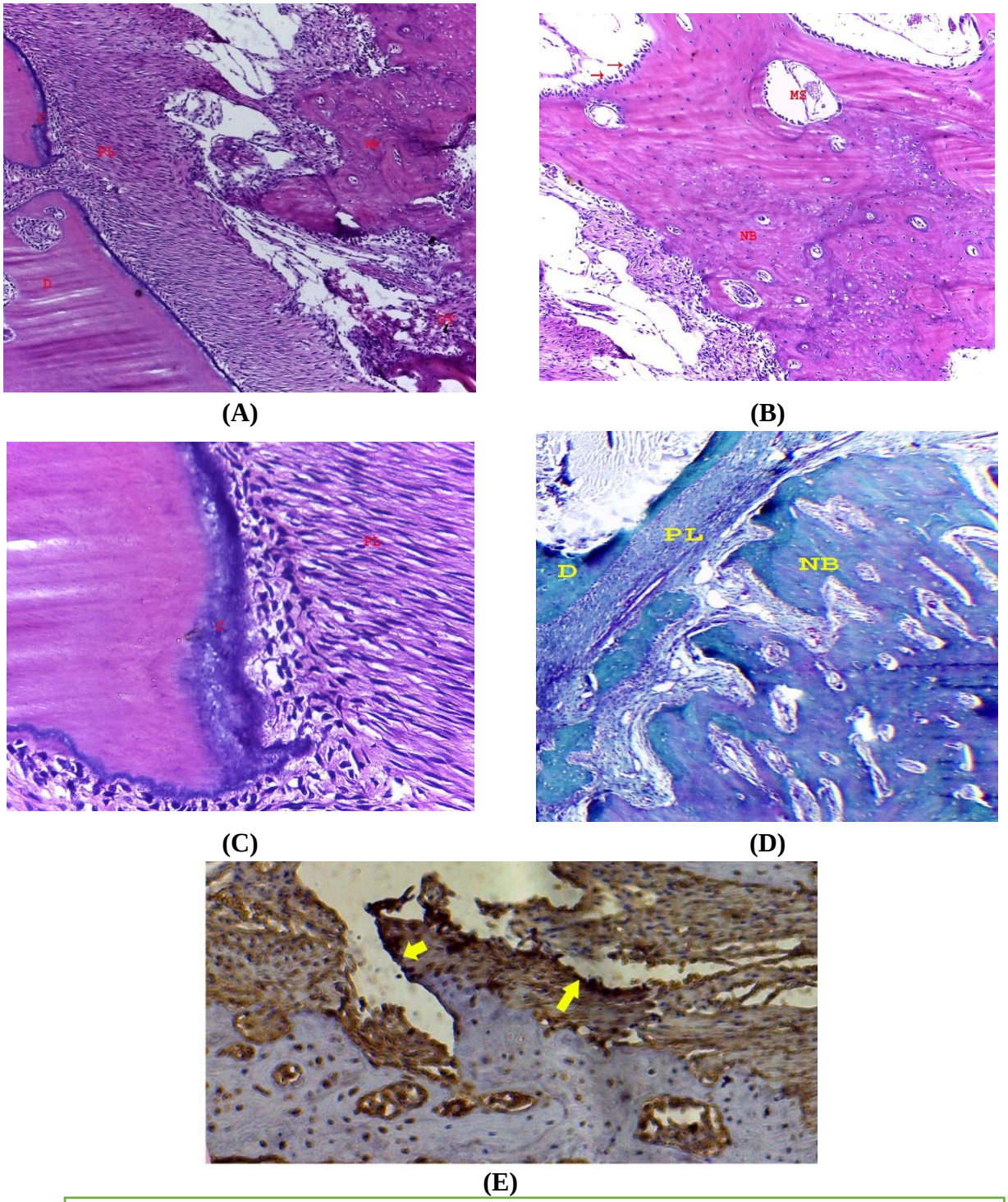
At 6 weeks' period

H&E sections showed thatthe defect was nearly filled with highly cellular newly formed bone most of the woven bone transformed into lamellar bone and reversal line was observed.Well organized periodontal ligament fibers inserted in the thin new cementum.Masson& trichrome showed: spicules of collagenous fibers formation within the lamellar bone formation



(A) **(B)**

(C) **(D)**

(E)

Figure (3)A:H&E section of HA treated specimen at 6 weeks showed area of highly cellular lamellar bone formation NB, resting line RL and not well organized periodontal ligament fibers PL with thin layer of cementum.B: higher magnification showed highly cellular lamellar bone with bone marrow spaces. C: showed well organized periodontal ligament fibers inserted in the thin new cementum. D: Masson's &trichrome section showed newly formed bone formation where the bluish color indicates collagen fibrils in the bone matrix. E: immunohistochemical

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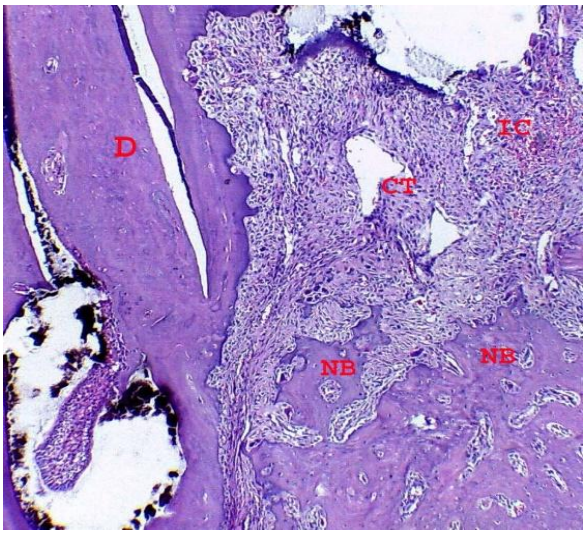
Group II (Eggshell treated group)

At 3 weeks' period

The defect was filled with vascular fibrous connective tissue, small new bone spicules were observed within the CT. A new woven bone formation was seen near the defect's perimeter, consisting of a network of bony trabeculae including blood vessels, bone marrow, osteoblast and osteocyte-like cells. Osteoclasts in their Howships lacunae were observed around the spicules of woven bone. Periodontal ligament fibers in a chaotic pattern and little bit of cementum were detected. A considerable amount of new immature bone was observed by Masson &trichrome section.



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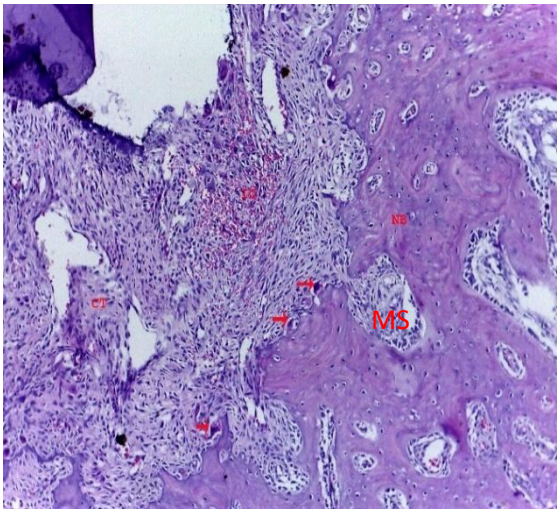

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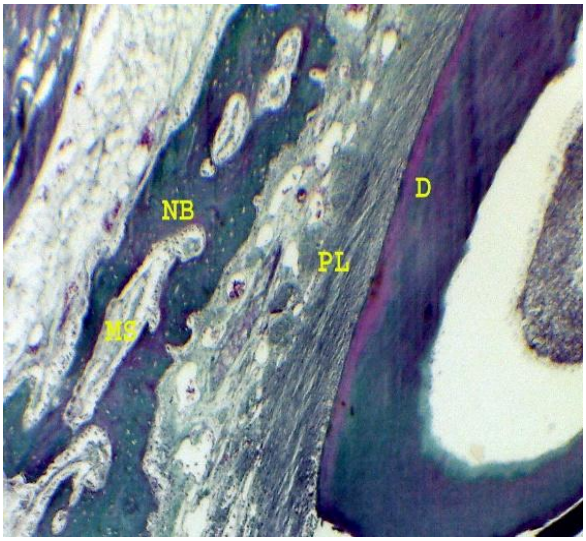

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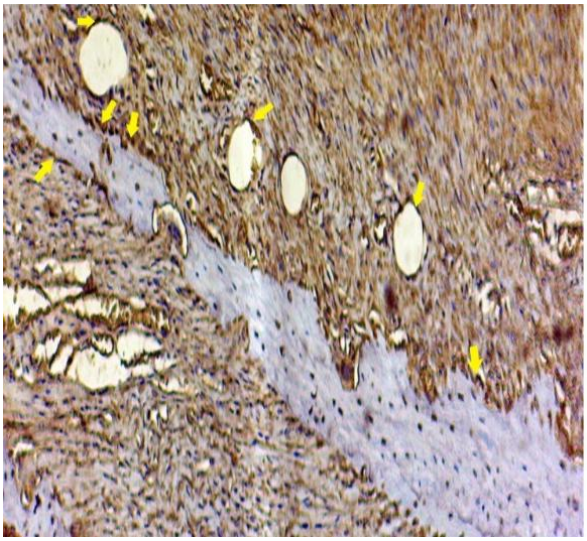

(D)

Figure(4)A: H&E section of eggshell treated specimen at 3 weeks the defect is filled with vascular fibrous connective tissue, small new woven bone spicules at the periphery of the defect NB within the CTwith marrow spaces MS, blood vessels. B: higher magnification showed Osteoclasts in their Howships lacunae were observed around the spicules of woven bone (red arrows).C:Disorganized periodontal ligaments PL. Masson& trichrome showed: dispersed of collagenous fibers formation around and within the new bone (the greenishbluish color).D:immunohistochemical staining of osteopontin, it showed high expression specially

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At 6 weeks' period

H&E sections showed: New bone formation of woven pattern and fibro-cartilaginous bone formation, isolated freshly created bony island was discovered that was mostly composed of woven bone with some mature lamellar bone. Accentuated reversal lines were also observed. Mild inflammatory cells infiltration was present. Masson& trichrome showed: increased new osteoid bone formation within the CT.

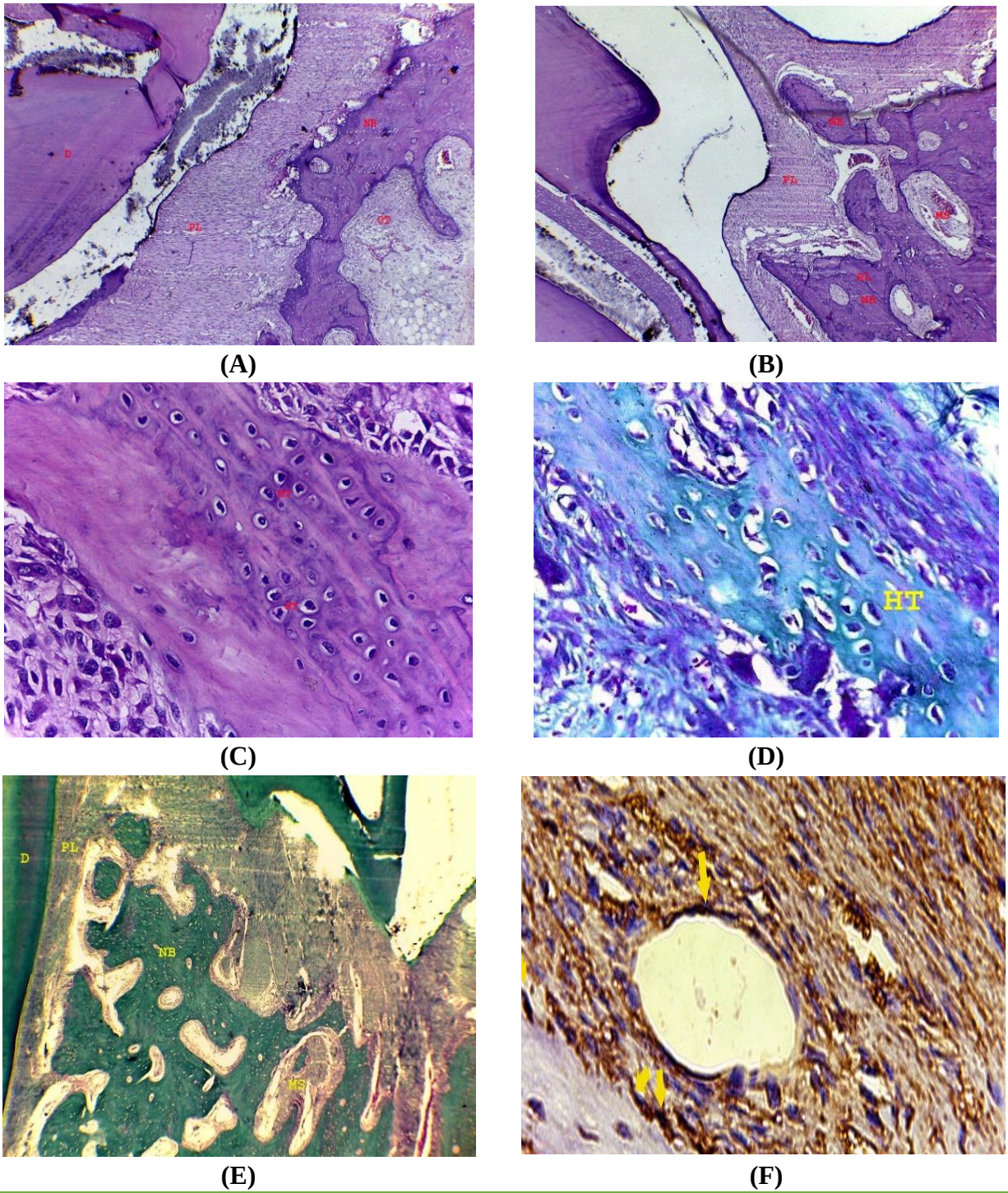

Figure (5)A & B: H&E section of eggshell treated specimen at 6 weeks isolated newly formed bony island consisted mainly of woven bone with some mature lamellar bone NB within the CT with marrow spaces MS, accentuated reversal lines RL and blood vessels BV. Disorganized periodontal ligaments PL.C:Higher magnification showed fibro-cartilaginous bone formation. D & E:Masson& trichrome showed: interconnected bone trabeculae with collagenous fibers formation (the greenish bluish color). F:Immunohistochemical staining of osteopontin, showed high expression specially around blood vessels and in connective tissue

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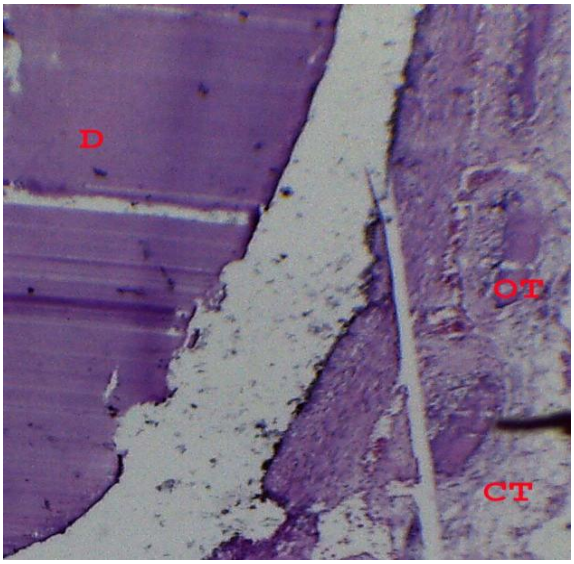
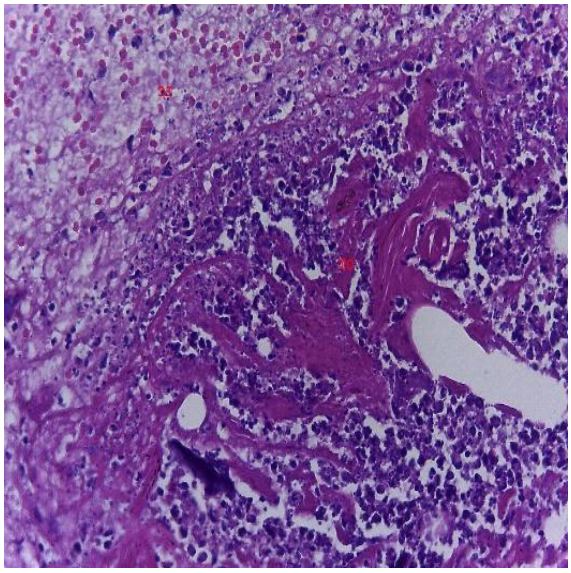
Group III (control group):

At 3 weeks' period

H &E stained sections showed that the defect was filled with a highly vascular fibrous tissue and extravagated RBCs with mild to moderate inflammatory cells infiltration. Masson and trichrome section showed layersof collagen fibers with minimal bone regeneration activity was observed



(A)



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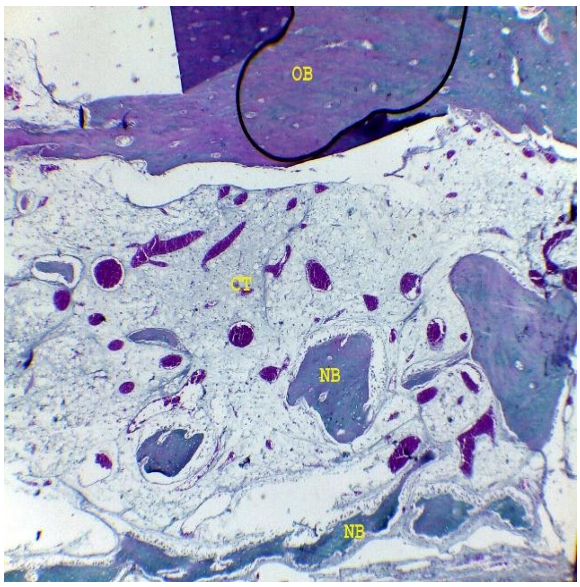

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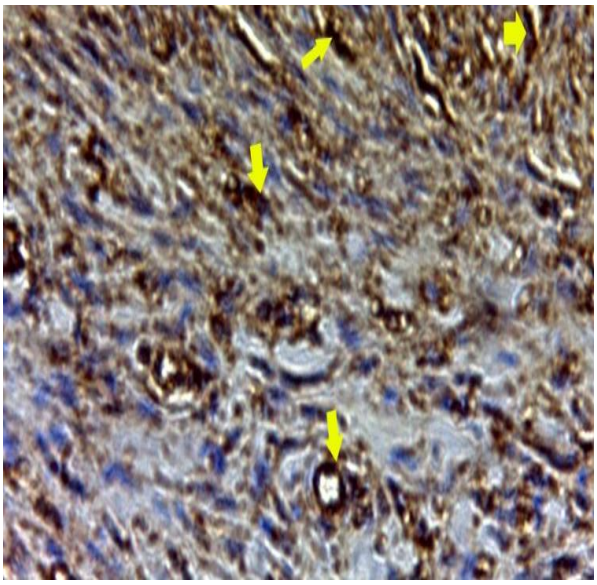

(D)

Figure (6)A & B:H&E section of control specimen at 3 weeks the defect was filled with a highly vascular fibrous tissue and extravagated RBCs with mild to moderate inflammatory cells infiltration IC small part of osteoid tissue OT. C: Masson and trichrome section showed layer of collagen fibers with minimal bone regeneration activity D:Immunohistochemical staining of osteopontin, showed low level of expression (yellow arrows)

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At 6 weeks' period:

At 6 weeks' control treated specimens showed newly bone spicules scattered in the defect center and new bone formation at the periphery. On a higher magnification, the trabeculae of this bone lined by apparent osteoblastic activity. Non functionally oriented collagen fibers of the Periodontal ligament were seen, thin layer of cementum was noticed. Inflammatory cells infiltration was also still observed.

Masson &trichrome sections showed osteoid bone formation within the collagenous fibers

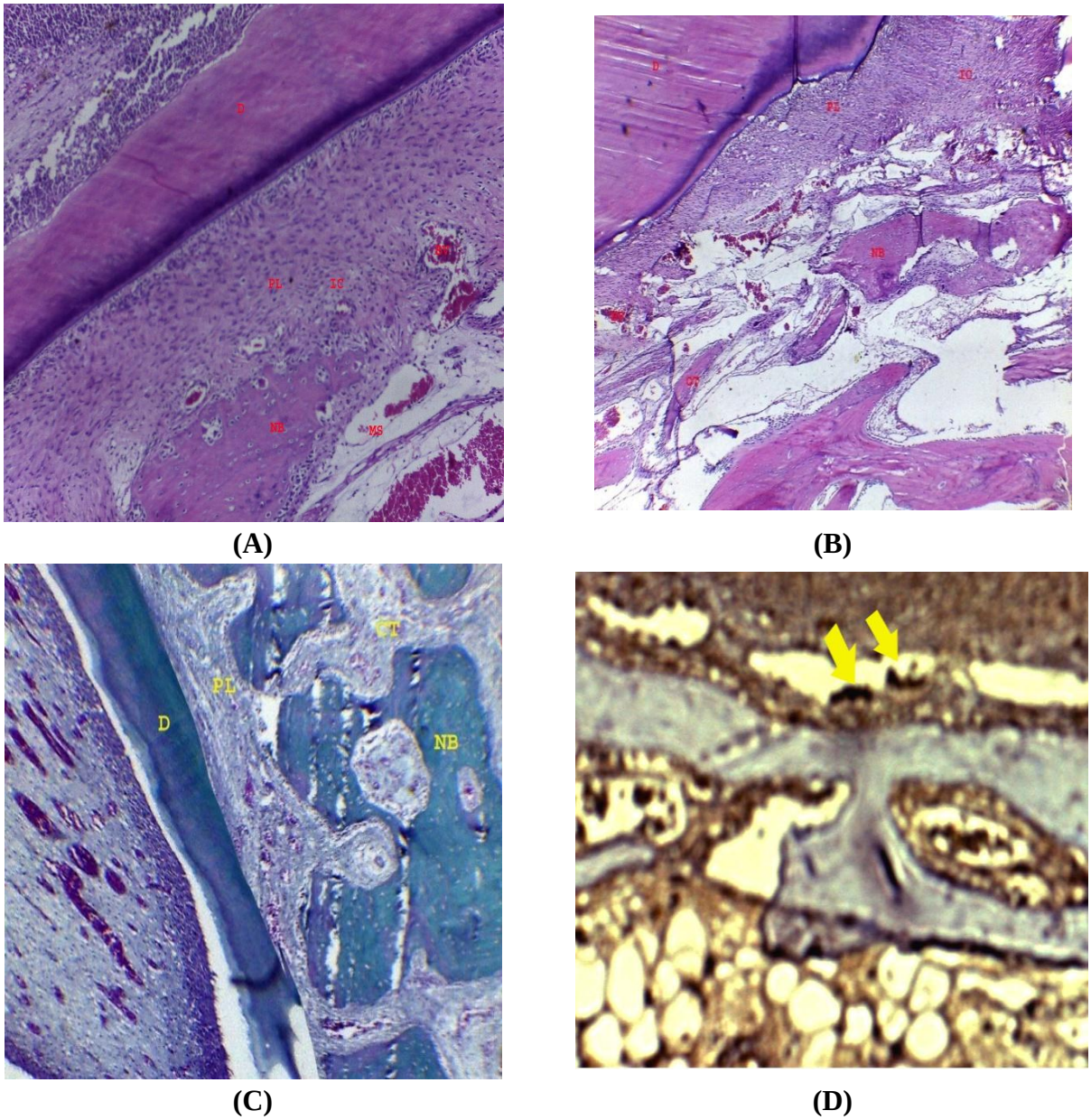

Figure (7) A :H&E section of control specimen at 6 weeks showed newly bone spicules scattered in the defect center and new bone formationNB at the peripherywithmild inflammatory cells infiltration IC.B:Higher magnification, showed the trabeculae of this bone lined by apparent osteoblastic activity. Non functionally oriented collagen fibers of the Periodontal ligament PL, andthin layer of cementum. C: Masson and trichrome section showed osteoid bone formation within the collagenous fibers.D: immunohistochemical staining of osteopontin, showed more expression of osteopontin than at 3 weeks related to blood vessel walls, the periodontal ligament and fibroblasts

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Intensity of Osteopontin immunohistochemical expression:

For G I, osteopontin is intensely expressed at both 3- and 6-weeks' periods specially around blood vessel, around bone spicules and inside the bone marrow.

Whereas in G II a considerable amount of osteopontin was observed at both 3 and 6 weeks postoperative adjacent to the newly formed osteoid of the defect, osteoblast, and around the bone trabeculae were also stained positive.

In G III (control group). The greatest intensity of Osteopontin immunohistochemical expression was recorded at 3 weeks with the least expression was shown at the 6th week in it was more limited to blood vessel walls, the periodontal ligament and fibroblasts.

Discussion:

In recent years, a great amount of innovative research has focused on finding new materials for tissue regeneration. As a natural bone substitute eggshell has been used in maxillofacial reconstructive surgery.8,10 Due to the biocompatibility of hen's eggshell, it was recommended as a graft material; the chemical composition of $CaCO_3$ derived from eggshell is expected to include the chemical structure of bone as well, making it a very affordable bone replacement.11

Mature rabbits were selected, due to the ease of their handling and their size, as well as the minimal differences in bone composition and density between human and rabbit. Compared to primates and some rodents, rabbits have faster skeletal change and bone turnover12. Moreover, their short regeneration time, small size, lower cost, easy access, and handling compared to monkeys and dogs13. In recent years, rabbit models have been used to test new bone substitute biomaterials. The most common implantation sites are calvaria14, bilateral tibiae and distal femur15

While the chronic model does produce a damaged root surface similar to that seen in periodontitis, it is not comparable to naturally occurring periodontitis.16 As a result, this research used an acute model of surgically produced periodontal lesion.

Due to the very limited histologic studies done on the periodontium of the rabbits. Hence the present study was conducted on the periodontium of the rabbits to investigate the regenerative potential of eggshell bone substitute to regenerate the intrabony periodontal defect.

All the animals were completed the stages of research, with no undesirable symptoms associated with the use of the tested materials, and the healing process proceeded in its normal course.

Results of the current study showed that Both used bone graft have osteoconductive properties and are effective in healing of the periodontal defects with the maximum amount of the total regeneration was seen in the HA group

In general, at 3 weeks' period of healing the observed mild new woven bone formation with non-functionally oriented collagen fibers came in agreement with observations seen in earlier studies.17-19

In group I, the observed bone spicules were surrounded with osteoclasts indicating bone remodeling activity, additionally, remnant of the HA particles was observed at 3 weeks of the healing period indicating the slow resorption of the HA particles giving enough time for the bone replacement and remodeling activity with minimal inflammatory cells infiltration.

This result is confirmed by Wang et al.,20who discovered that HA aid in bone rebuilding by promoting the development of microvessels and the attachment of host osteocytes during the early stages of bone defect healing.

At 6 weeks the defect was nearly filled with highly cellular newly formed lamellar bone with reversal lines this came in agreement with the finding of D'lima et al21 who reported advanced osteogenesis with organized periodontal ligament fibers in periodontal defect treated by HA based material (Chitra granules) in the treatment of intrabony defect in dogs at six months.

On the other hand, group II (eggshell treated group) early healing with vascular fibrous connective tissue, small new bone spicules started at the periphery of the defect were observed. Some area showed Osteoclasts in their Howships lacunae were observed around the spicules of woven bone, disorganized PL fibers, and inflammatory cells infiltration. At 6 weeks more new bone formation of woven pattern and fibro-cartilaginous bone formation with little inflammatory cells indicating the increased osteoconductive activity of the granules

Additionally, no remnants of eggshell graft granules were observed in any specimen indication the rapid biodegradation of the graft. It was reported that biodegradation of bone substitutes is necessary to initiate the process of bone deposition.22-23

Many studies proved the possibility of using eggshell powder as an effective and safe alternative to other bone grafting materials, which is confirmed by the current study.8,9,11

Our results are in consistent with the study of Avinash and Malaiappan24. who compared the effectiveness of eggshell powder as a graft material and membrane versus demineralized bone matrix and type II collagen membrane. They confirmed the possibility of using eggshell powder along with membrane as a potential graft material.

The results of the current study are also in concordant with Uraz et al 201310 who evaluated the efficacy of eggshell as a bone graft substitute in bone healing in rats. They determined that eggshell-derived graft alternatives in gel and powder form are biocompatible and may increase new bone growth.

Another study was conducted on rabbits using the histological, histomorphometrical radiological, and clinical findings to evaluate eggshell powder as a biocompatible material provide osteoproductive activity, where there was satisfactory ossification in the grafted site compared to the control site25

Results of the current study are not agreed with other studies such as that has been conducted by Baliga et al.,26 who states that egg shell powder enhance bone regeneration in the defect margins only. AlsoDurmuş et al.,27 used ostrich eggshell powder as graft materials and eggshell membranes he observed limited bone regeneration on the 90th day follow up period.

The calcified eggshell includes an organic matrix that makes up around 3% of the total weight of the eggshell. Additionally, this organic component comprises proteoglycans and proteins such as ovocleidin 116, ovotransferrin, ovalbumin, ovocalyxin-32, ovocleidin-17, osteopontin (OPN), and lysozyme, all of which are capable of altering the form and precipitation rate of eggshell calcite crystals9.

Histologic results were confirmed by the immunohistochemical analysis of the expression of osteopontin (OPN) the marker of bone regeneration, it is involved in many vital biological processes such as inflammation, immunity and wound healing.28,29 It was found that both group I& II resulted in more expression of OPN than in control group this confirms that eggshell granules has osteogenic potential more or less comparable to HA bone graft.

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Conclusion:

Within the limitation of this study we concluded that Eggshells powder can enhance new bone formation and periodontal regeneration in the treatment of intrabony periodontal defects in rabbit's model. It must be placed in the center of attentionas it is easy to obtain and an inexpensive source of grafting material with reasonable biocompatibility and regenerative ability However more advanced and intricate researches and experiments should be done to confirm its osteogenic potential in human and more histomorphometric studies on the periodontium of the primate's animals.

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