Nanotechnology and its Role in the Treatment of Induced Periodontitis (Experimental Study)

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Abstract: Background: Nanotechnology is emerging as a rapidly growing field with its application in science and technology for the purpose of manufacturing new materials at the nanoscale level. Application of such field in dentistry and periodontitis in particular may have a great potential in the regeneration of tooth supporting structure as the chronic periodontitis is one of the problems affect a major part of the world population. **The aim of current study**: was to regenerate periodontal tissue defect using hydroxyapatite nano particles (HANP) and compare its regenerative potentiality with autogenous bone graft (ABG). **Material and Methods**: Chronic periodontitis was induced in twenty seven, 13–16 months old Beagle dogs. After one month of inducing chronic periodontitis, the animals were distributed randomly into equally three groups: Group I (conventional group): Treated with open flap debridement. Group II (ABG group): Treated with open flap debridement and ABG. Group III (HANP group): Treated with open flap debridement and HANP. Three dogs in each group were sacrificed with overdose of Phenobarbital sodium salt at day 1 and at 6th & 12th weeks after the surgical procedures. Then the specimens were processed for subsequent histologically by H&E and immunohistochemically by Matrix Metalloproteinase 9 (MMP-9) antibody and Collagen -1 (Col-1) antibody. **Results**: At day one after surgical procedure all animals showed signs of gingival inflammation, apical migration of attachment epithelium and destruction of the periodontuim. In group I, after 6 and 12 weeks, the signs of degenerative changes of periodontium became less. Nevertheless, the signs of complete periodontium regeneration were not clearly seen. While treatment with surgical conventional procedure and ABG showed successful tissue regeneration which became more advanced in animal treated with both conventional procedures and HANP. The immunohistochemical & statistical results confirmed these histological findings. As Tukey's post hoc test for both MMP9 and CO-I intensity revealed that the difference between each two groups was statistically significant, except for the difference between group II and group III at the 6th and 12th weeks and between all groups at the day one. **Conclusion:** There was no notable difference between the healing effect of HANP and the ABG which considered the gold standard osseous graft material providing a promising insight into the application of nanotechnology in the periodontal diseases management.
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1. Introduction

Tissue engineering is an emerging interdisciplinary area of research and technology. It is looking for new ways to apply the principles of material science and bioengineering to construct biological substitute that will restore and maintain normal function in diseased and injured tissues [(1,2)].

Periodontitis is a chronic inflammatory disease, characterized by destruction of the tooth supporting apparatus. The process of regeneration of destructed periodontium depends on the migration, adhesion, proliferation and differentiation of periodontal ligament cells, which are the predominant cells of the periodontium and playing a leading role in the homeostasis and regeneration of periodontal tissues [(3-5)]. Many researches have used different approaches to achieve complete regeneration of the attachment apparatus. But their goals have been not achieved till the emergence of recent developments in nanotechnology, which provides a promising insight into the management of periodontal diseases [(6-7)].

Nanotechnology is emerging as a rapidly growing field with its application in science and technology for the purpose of manufacturing new materials at the nanoscale level [(8)]It has achieved tremendous progress in the past decades. Recently, nanomaterials which are materials with basic structural units smaller than 100 nm in at least one dimension have evoked a great amount of attention for improving disease prevention, diagnosis and treatment [(9,10)].

Medical applications of nanotechnology are expected to be of major benefit to society within the next ten years, especially for patients suffering from cancer, cardiovascular diseases, lung, blood and neurological disorders, diabetes,

inflammatory/infectious diseases or orthopaedic problems [10]. Application of such technology in dentistry and periodontitis in particular may have a great potential in the regeneration of tooth supporting structure as the chronic periodontitis is one of the problems affect a major part of the world population [3,7, 11].

Nanotechnology has been greatly utilized for bone tissue engineering strategies. It has been employed to overcome some of the current limitations associated with bone regeneration methods including insufficient mechanical strength of scaffold materials, ineffective cell growth and osteogenic differentiation at the defect site, as well as unstable and insufficient production of growth factors to stimulate bone cell growth to mimic the natural bone nano composite architecture [12,13].

Autogenous bone grafts (ABG) are considered to be the gold standard in bone regeneration because of their osteogenic activity. ABG are harvested from the patient, either from intraoral sites (such as the maxillary tuberosity of a healing extraction site) or extraoral sites (such as the iliac crests, ribs, cranium and tibialmetaphyses). The usage of ABG necessitates consideration of the donor site, procurement technique and handling or processing of the harvested material [14 -16]. Intra oral ABG can be harvested with or without processing to yield graft materials of different forms including; cortical chips, osseous coagulum and bone blend. Many investigators have reported on the clinically successful usage of intraoral autogenous grafts in intrabony defects treatment. Regardless of the intraoral donor site, autografts yield regenerative responses superior to that of surgical debridement alone [17-19]. ABG are non immunogenic and contain osteoblasts and osteoprogenitor stem cells, which are capable of proliferating. Therefore, these grafts are osteoinductive. However, there are limitations to obtaining autogenous grafts such as insufficient oral sites, the requirement for a second surgical site, lengthening of the surgical procedure, morbidity at the donor site and may introduce additional medical complications. This resulted in the development of alternative (synthetic) bone substitutes [16, 20].

Many bone substitutes have been used for periodontal regeneration. Hydroxyapatite (HA), a synthetic calcium phosphate is commonly used in periodontal regeneration and restoration of tooth supporting system. Many reports recommend this material because of its good biocompatibility, osteoconductibility and its chemical and structural similarity to the mineral component of bone [21,22]. This material has a similar composition and structure to natural bone and bind to it directly when implanted. These features have led to HA receiving considerable attention as a graft biomaterial in both the research and clinical fields. However, treatment of periodontal bone loss with HA always results incomplete regeneration of the periodontium. It is believed that failure of regeneration is due to the property of the calcium phosphate material, which has only osteoconductive but no osteoinductive effect on periodontal osseous defects. In addition, its inconsistent cell reactions-which are dependent upon the surface properties limit its clinical use in various situations of bone defect [23, 24]. Currently, development in nanotechnology is the main contributor to periodontal tissue regeneration, through using hydroxyapatite nanoparticles (HANP).The nonmaterials exhibit much better performance properties than traditional materials [25,26]. A fully synthetic nanocrystalline hydroxyapatite (nano-HA) paste, has been introduced for augmentation procedures in osseous defects. The advantages of such nanostructured material in comparison to traditional bulk material are its close contact with surrounding tissues, quick resorption characteristics and high number of molecules on its surface. It was found that undisturbed osseous-integration and complete resorption of nano-HA paste occurs within 12 weeks [11,27]. Newly developed HA made of nanoparticles was introduced and was reported to exhibit better biocompatibility and protein adsorption capacity [28,29]

In the current study we hypothesized that treatment of induced periodontitis in dogs with (HANP) may enhance the differentiation of periodontal ligament cells. So, the aim of this work was to regenerate periodontal tissue defect using HANP and compare its regenerative potentiality with ABG providing a promising insight into the application of nanoparticles in the periodontal diseases management.

2. Materials and Methods

Animals:

Twenty seven, 13–16 months old, Beagle dogs weight approximately 10 kgs were used. Animals were obtained from the Animal House, Faculty of Medicine, Tanta University. They were housed under controlled temperature and lighting conditions, with free access to standard food and water. Throughout the experimental period, the animals were examined daily for general appearance, activity and weight. The experimental protocol was designed in accordance with the guidelines for the responsible use of animals in research as a part of scientific research ethics recommendations[30]. Chronic periodontitis was induced in all animals. The animals were distributed randomly into equally three groups: **Group I (conventional group):** Treated with open flap debridement.**Group II (ABG group):** Treated with open flap debridement and ABG. **Group III (HANP**

group): Treated with open flap debridement and HANP.

Induction of chronic periodontitis

In all dogs, cotton ligatures were tied and placed around the neck of the mandibular right pre-molar teeth under general anesthesia using I.V injection of ketamine hydrochloride (40 mg /kg). The ligature was knotted and placed in subgingival position in the distal aspect of each premolar to induce chronic periodontitis [31,32]. Dogs were fed a soft diet to promote plaque accumulation. After four weeks of undisturbed plaque accumulation, clinical signs of inflammation (edema, redness and bleeding on genital propping) were evident in the periodontal tissues.

Preparation of graft materials

HANP had been prepared by wet chemical method as reported by [33,34]. In brief, at room temperature, HANP was formed through the wet chemical reaction of calcium nitrate, [Ca $(NO_3)2$ H_2O] and Ammonium hydroxide [NH_4) $2HPO_4$]. The grain size was controlled by changing the time and the temperature of HA precipitation, with pH values between 10 and 12. HANP was mixed with dog venous blood prior to delivery into the periodontal defects. ABG was obtained from the mandibular body cancelous bone. Mucoperiosteal flap related to the experimental site was extended, and the compact layer was removed using surgical bone excavator and ABG plugs were particulated using a bone mill.

Surgical Procedure &post operative care

After 4 weeks of induction of periodontitis, all dogs were subjected to sulcular incisions followed by elevation of buccal mucoperiosteal flap in the region of mandibular pre-molar teeth to expose the periodontal defects. The periodontal defects were subjected to root scaling and planning They were irrigated with saline solution to eliminate any debris, and then dried.[32]. Graft materials were condensed to fill the periodontal defects in both group II and III only. Then the flaps were repositioned and sutured covering the periodontal defect.

Post operative care included systemic administration of pain killer (I.M 0.2 - 1.0 mg/kg ketoprofen) once during the first post operative day[35]. Dogs were fed soft diets to reduce the possibility of local trauma to the wound. Plaque control was maintained by daily topical application of a 0.12% Chlorhexidine solution. Sutures were removed 7 days post surgery [31,32]

Euthanasia

Three dogs in each group were sacrificed with overdose of Phenobarbital sodium salt [31] at day 1 and at 6^{th}&12^{th} weeks after the surgical procedures. Mandibles of all sacrificed dogs were dissected carefully keeping the gingival attachment intact with bone. Then, each mandible was split into two halves at the midline, all right experimental sides were processed for subsequent histological and immunohistochemical analysis.

Tissue preparation

Specimens comprising of bone segments carrying the premolar teeth, were fixed in 10% buffered formalin for 24 h and decalcified in 10 % neutral-buffered EDTA at room temperature for three months. Then, they were dehydrated through ascending graded series of ethanol, and processed into paraffin. 5 µm mesio-distal serial sections were cut and mounted on commercially available positively charged glass slides. Representative sections were stained with Harris' haematoxylin and eosin (H&E) [36] for conventional histological assessment using light microscope (Leica ICC50 HD).

Immunohistochemical Staining

Table (1): Antibodies and other reagents.

Antibody	Source	Cat. no
Primary mouse monoclonal to Collagen I	Abcam, UK	ab6308
Goat Anti-Mouse IgG H&L (HRP)	Abcam, UK	ab6789
Secondary to MMP-9	Abcam UK	Ab38898
Mouse specific HRP/DAB (ABC) Detection IHC Kit	Abcam, UK	ab64259

Immunohistochemical labeling was performed using the avidin–biotin–complex (ABC) method [37]. Representative sections taken from the central part of the defects were deparaffinized in xylene and re-hydrated through a descending series of ethanol concentrations. The sections were washed with TBS (20 mMTris- HCl, 150 mMNaCl, pH 7.4). Then they were incubated in 0.3% H_2O_2 in dH_2O at room temperature (30 min) to inhibit endogenous peroxidase. Antigen retrieval was performed according to the manufacture instructions. Slides were placed in 100 µl blocking solution (Abcam), for 30 minutes at room temperature. Primary antibodies (Table 1) were applied at recommended dilutions at 4ºC overnight. Sections were washed in 1X Phosphate buffered saline (PBS) and then incubated with secondarybiotinylated antibody (in blocking buffer for 1 hour at room temperature in a humidified chamber. (Secondary antibodies and other reagents used in this study listed in table 1). To perform peroxidise visualization; sections were incubated in ABC solution for 1 hour at room temperature. Color reaction was then developed by adding DAB solution (0.5 mg/ml DAB and 0.1% H_2O) onto the sections. When color reaction was satisfactory, it was stopped by rinsing with H_2O for 5-10 minutes, and then sections were counterstained with hematoxylin for 2 minutes. Sections were gradually dehydrated and mounted with coverslips. Immunohistochemical staining was assessed using light microscope.

Histometric and Statistical analysis

Immunostained sections were analyzed for the staining intensity of MMP-9 and Col-1 expression by Image J analysis system (V 1.48S) using light microscope. For statistical analysis,all measurement data are presented as mean ± standard deviation. All statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test according to Gerber et al., (2006) [38]. Values of P <0.05 indicated a statistically significant difference. "SPSS 20" (SPSS Inc., USA) was used for data analysis.

3.Results

Histological Observations

IPD in dogs results in gingival inflammation and destruction of the periodontuim

All groups showed signs of periodontitis at day one after surgical procedure. There was an obvious heavily gingival infilteration with inflammatory cells and apical migration of epithelial attachment. Also, PDL fibers were disorganized with extravasated red blood cells. In addition, cementum and dentine were markedly resorped and osteoclasts-like cells could be detected on bone surface *(Figure1,A,B.)*. In group I, after 6 and 12 weeks, the signs of degenerative changes of periodontium became less. Regeneration of bone defect was seen and repair of cementum defect by cementoid tissue could also be detected *(Figure1, C-D)*. Moreover, PDL fibers re-organization was obvious. Nevertheless, the signs of periodontium complete regeneration were not clearly seen *(Figure1,E-F)*.

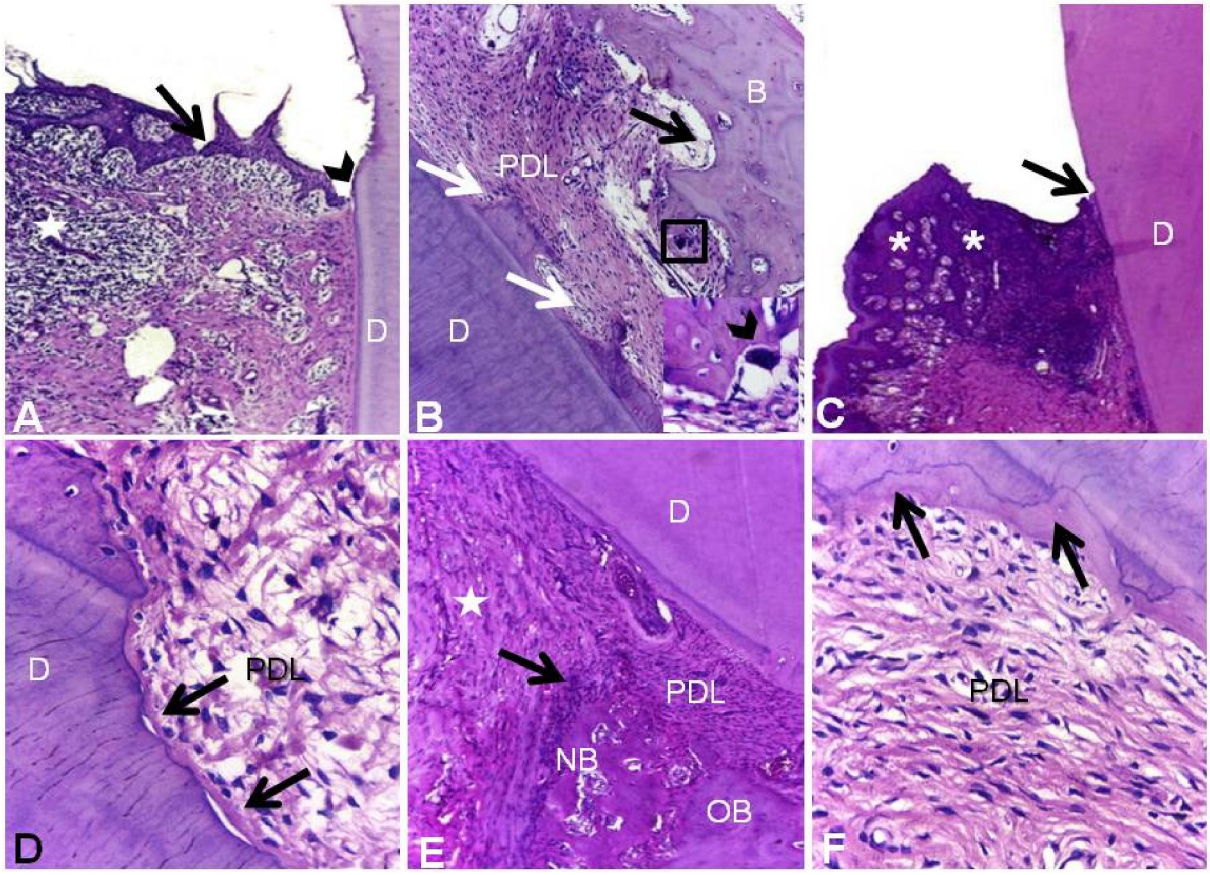

Fig.1: Photographs showing periodontal healing in conventally treated dogs with IPD at day one, 6&12 weeks.(A-B) show healing at day one; (A) Gingival ulceration (Black arrow) with heavily infiltration of the gingival connective tissue with inflammatory cells (white star). Migration of epithelial attachment down to root surface (Arrow head). (B) Marked root and bone resorption (White and black arrows respectively). Higher magnification of black boxed area in inset showing osteoclast in bone cavity (Black arrow head). (C-D) show healing at 6 weeks; (C) Heavily infiltration of the gingival connective tissue with inflammatory cells (white stars) and the level of attachment epithelium still more apically on root surface (black arrow). (D) Repair of root resorption by thin layer of cementoid tissue (Black arrows). (E-F) Show healing at 12 weeks; (E) Bony defect filled mainly by fibrous connective tissue (White stars). New bone (NB) is surrounded by chronic inflammatory cells (Black arrow). (F) Repair of cementum defect by cellular cementum (Black arrows) with less organized PDL fibers. Old bone (OB. Dentine (D) new bone (N) (H&E Original. Magnification. A, B, C and E x 10; D and F X 40).

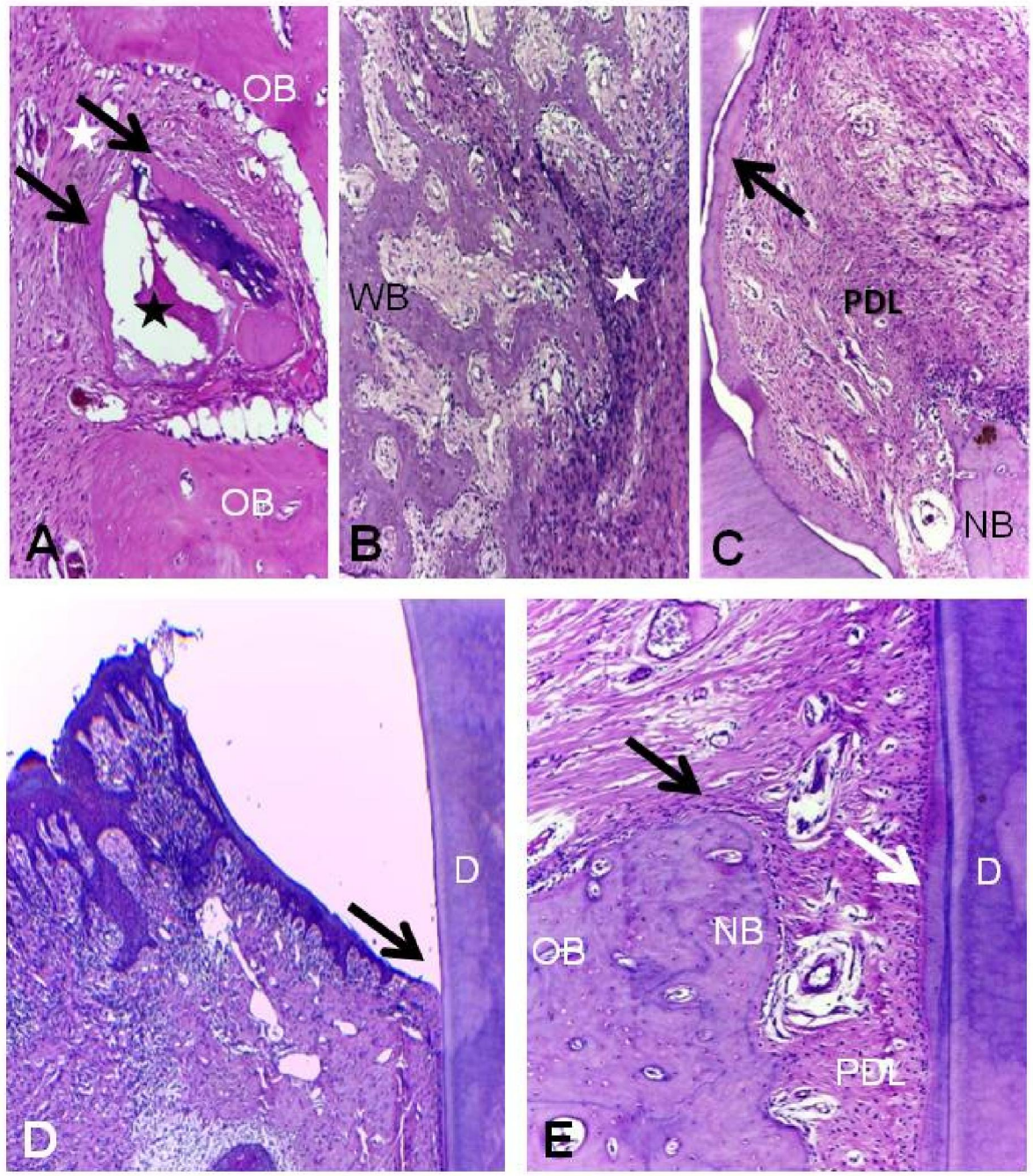

Fig 2: Photographs showing periodontal healing in group II at 6&12 weeks (A,B and C) Show healing at 6 weeks; (A) ABG (Black star) can be noted in the defect area surrounded by new bone spicules (Black arrows) and vascular connective tissues (white star). (B) The bone defect is filled by woven bone (WB) and multiple newly formed blood vessels. Also, moderate infiltration with inflammatory cells and integration of gingival connective tissue (white stars) with newly formed bone are recognized. (C) Repair of root defect by new cellular cementum (Black arrow). In addition, reorganization of functional PDL fibers and reconstruction of alveolar crest of bone (NB) are seen. (D-E) Show healing at 12 weeks; (D) Reattachment of gingival tissue to the tooth surface with normal level of junctional epithelium (Black arrow). (E) Complete replacement of ABG with new bone (NB) lined by active osteoblast cells (Black arrow). Also, repair of root defect by cellular cementum (white arrow) and functional orientation of PDL fibers are observed. Old bone (OB), Dentine (D), New bone (NB) (H&E Original magnification; A, B, D and E X 10, CX40).

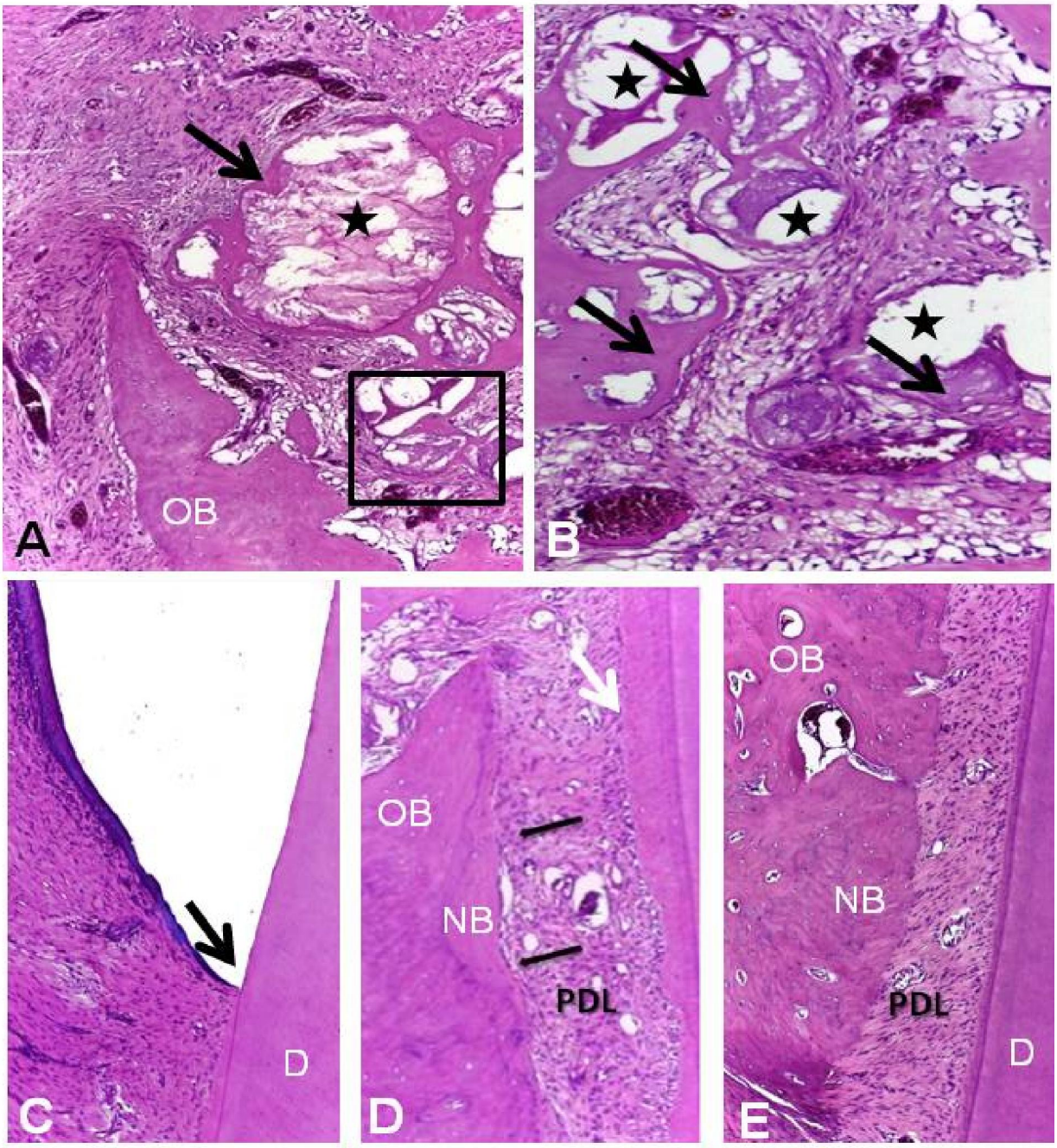

Fig.3: photographs showing periodontal healing in group III at 6&12 weeks. (A-B) Show healing at 6 weeks; (A) Bone defect containing HANP (Black star) surrounded by new bone (Black arrow). (B) Higher magnification black boxed area in (A) Well maintained HANP (Black stars) within the bone defect with vascular connective tissue in growth and spicules of new bone (Black arrows) surrounding the graft material. (C, D and E) Show healing at 12 weeks; (C) Normal architecture of DGJ with normal level of junctional epithelium (Black arrow). (D) Spaces previously occupied with HANP are filled with mature new bone (NB) and newly formed blood vessels. The active osteoblasts are observed on the surface of new bone (black bars) and new cementum is lined by active cementoblasts (White arrow). (E) Shows well organized PDL fibers. Old bone (OB). New bone (NB).Dentine (D).(H&E Original magnification A, C, D and EX 10, BX40)

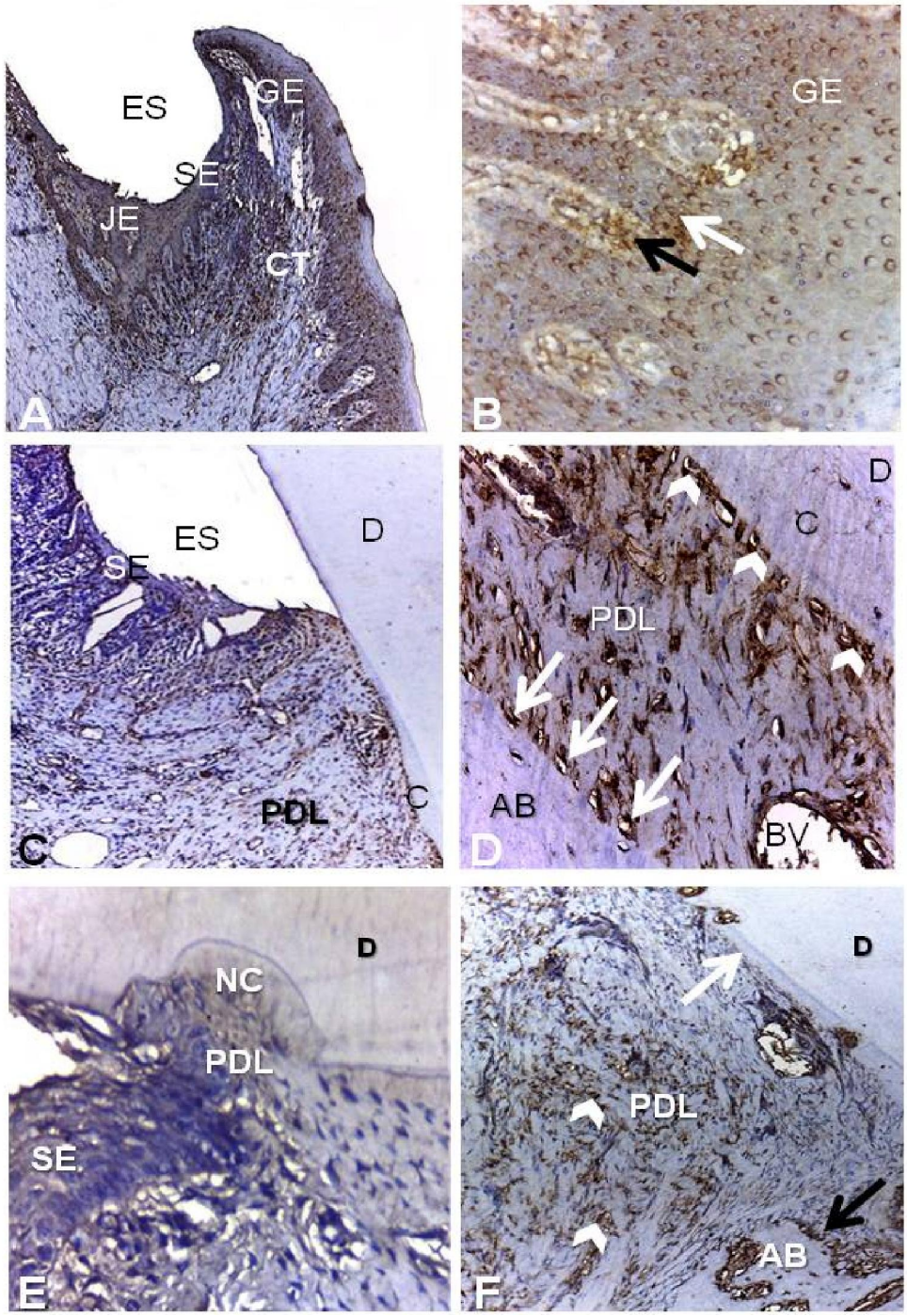

Fig.4: Immunolocalisation of MMP-9 expression during healing in group I at day one, 6th &12th weeks. (A-B) show MMP-9 expression at day one: (A) Intense perinuclear expression of MMP-9 in epithelial cells of DGJ and underlying connective tissue.(B) intense perinuclear stained cells in gingival epithelium and fibroblasts in underlying connective tissue (White and black arrows respectively). (C-D) Show MMP-9 expression at 6th week; (C) Shows moderate MMP-9 perinuclear expression in epithelial cells of DGJ, the underlying CT and PDL. (D) Intense perinuclear MMP-9 reaction in PDL fibroblasts, cementoblast adjacent to cementum surface (white arrow heads) and osteoblasts along bone surface (White arrows). Also, intense MMP-9 reaction surrounding dilated BV is located. (E-F) Show MMP-9 expression at 12th week; (E) Weak MMP-9 expression in epithelial cells of DGJ, the underlying CT and PDL. (F) Mild MMP-9 reaction in cementoblasts next to cementum surface (White arrow). Osteoblasts along bone surface (Black arrow) and fibroblasts (White arrow heads) exhibit moderate MMP-9 reaction. Sulcular epithelium (SE),Gingival epithelium (GE),Junctional epithelium (JE), Enamel space (ES) Dentin (D), Cementum (C), Alveolar bone (AB),Blood vessels (BV), New Cementum (NC). (DAB. Original Magnification A,C and F X 10; B,D and E X 40).

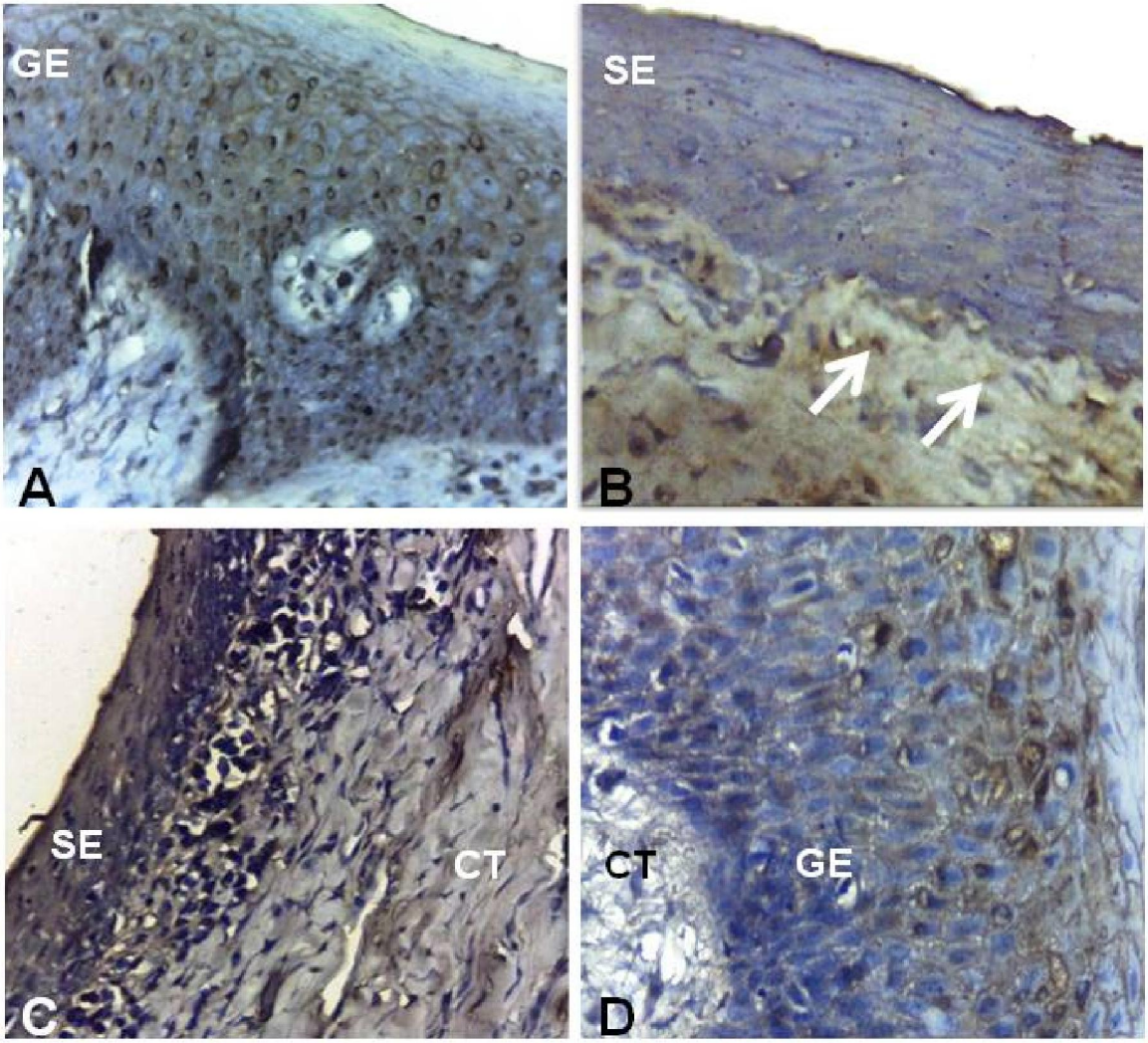

Fig.5: Immunolocalisation of MMP-9 expression during healing in group II at 6th &12th weeks. (A-B) Show MMP-9 expression at 6th week. (A) Moderate prenuclear MMP9 expression in gingival epithelium. (B) weak MMP-9 expression in sulcular epithelium and mild reaction in underlying CT (White arrows). (C-D) Show MMP-9 expression at 12th week. (C) Weak MMP-9 expression in sulcular epithelium and underlying CT. (D) Mild MMP-9 expression in spinous and granular cell layers. Gingival epithelium (GE), Sulcular epithelium (SE). (DAB. Original. Magnification. A and C X10; B and D X 40).

Treatment with surgical conventional procedure and ABG shows successful tissue regeneration

At 6th week in group II, although ABG could be still noted in the defect area, the boundary between ABG and old bone was indistinguishable. PDL exhibited many small blood vessels along with regeneration and re orientation of PDL fibers. Also, the root surface was repaired with cellular cementum*(Fig.2, A,B,C).* At 12th week, DGJ appeared nearly normal with mild inflammatory cells infiltration. Hard tissues demonstrated regenerative ability; ABG was completely resorped and replaced with new bone with no demarcation between new and old bone. Also, continual deposition of new bone with active osteoblasts was evident. In addition, repaired cementum of cellular type was clearly seen *(Fig.2,D, E).*

Treatment with surgical conventional procedure and HANP promote periodontium healing in dogs with IPD

At 6th week in group III, although the grafted biomaterial was still occupying the periodontal defect space, this group showed regeneration of attachment epithelium, with normal gingival tissue free from inflammatory cells infiltration. Also, there was alveolar bone regeneration as most of spaces within the grafted material were packed with loose connective tissue that contained newly formed blood vessels as well as spicules of new bone formation around HANP. In addition, PDL fibers were well oriented and new blood vessels formation was apparent *(Fig.3, A-*

B). After 12th week, all grafted materials were completely resorped and replaced with more mature new bone. Interestingly, newly formed bone was completely integrated with the old one. Moreover, a thick layer of new cementum appeared to be bordering the old native one. Additionally, marrow spaces and PDL were rich in blood vessels *(Fig.3, C- D-E).*

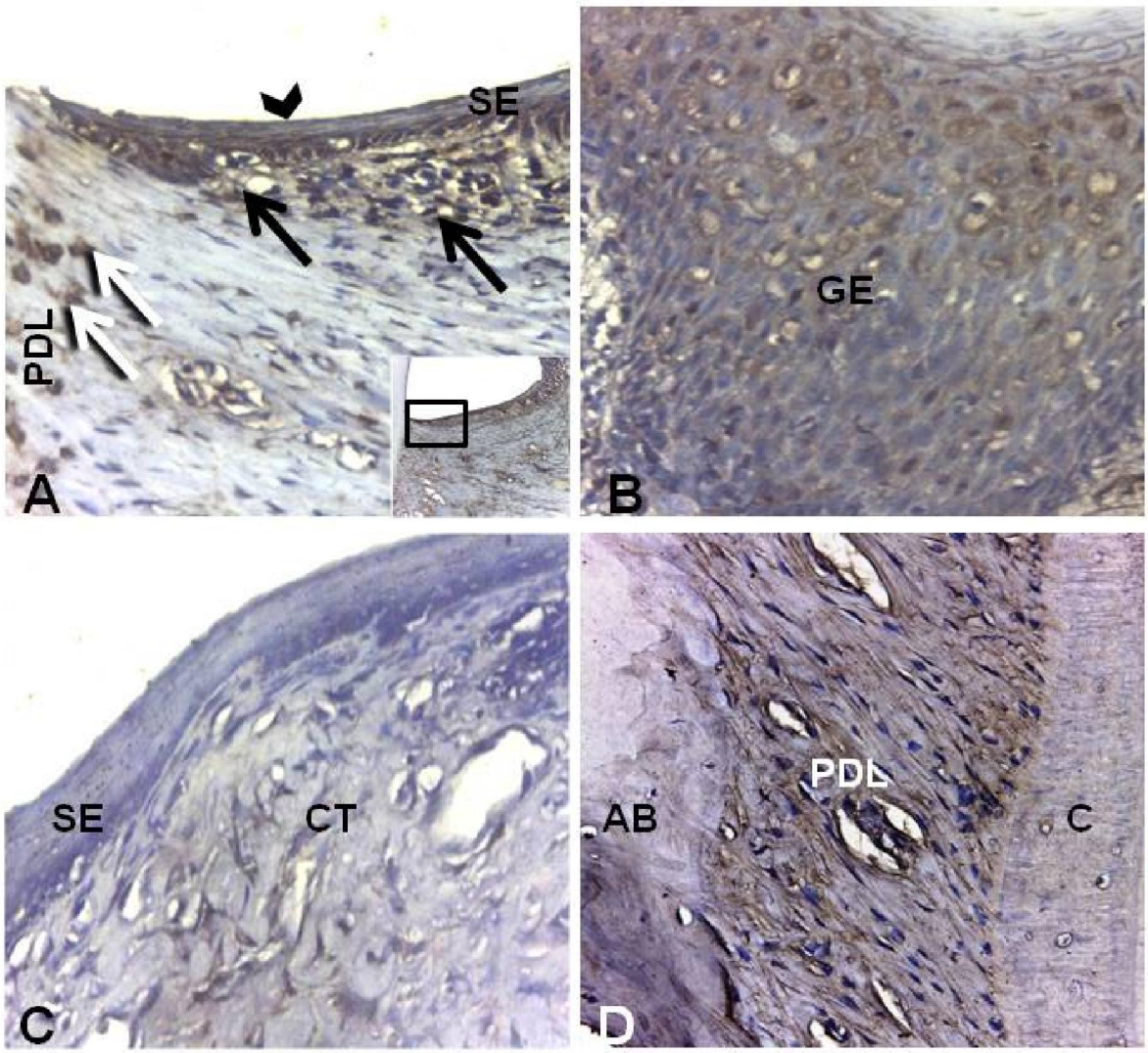

Fig.6: Immunolocalisation of MMP-9 expression during healing in group III at 6th &12th weeks. (A-B) show MMP-9 expression at 6th week; (A) Higher magnification of the black boxed area inset. It shows mild MMP-9 expression in sulcular epithelium, underlying CT and PDL (Black, White arrows and black arrowhead respectively). (B) Gingival epithelium shows moderate perinuclear MMP-9 expression in spinous and granular cell layers. (C-D) Show MMP-9 expression at 12th week;(C) Weak MMP-9 expression in sulcular epithelium. (D) Mild MMP-9 reaction in PDL. Gingival epithelium (GE), Sulcular epithelium (SE), Cementum (C), Dentine (D). (DAB. Original Magnification C X10; A,B and D X 40).

Histometric and Statistical analysis

I} Intensity of MMP-9 immunohistochemical expression:

The greatest mean intensity of MMP-9 immunohistochemical expression was recorded at the day one of all groups, with the least values obtained at the 12th week in group II (autogenous bone group) followed by group III (HANP group) at the same period. Analysis of variance (ANOVA) test revealed an extremely significant difference between groups at the same period and between the different periods in the same group (p-value < 0.0001) except

between all groups at the day one where there was no statistical significant differences. Tukey's post hoc test revealed that the difference between each two groups was statistically significant, except for the difference between group II and group III at the 6th and 12th weeks and between all groups at the day one (Tab.2 & Fig.10).

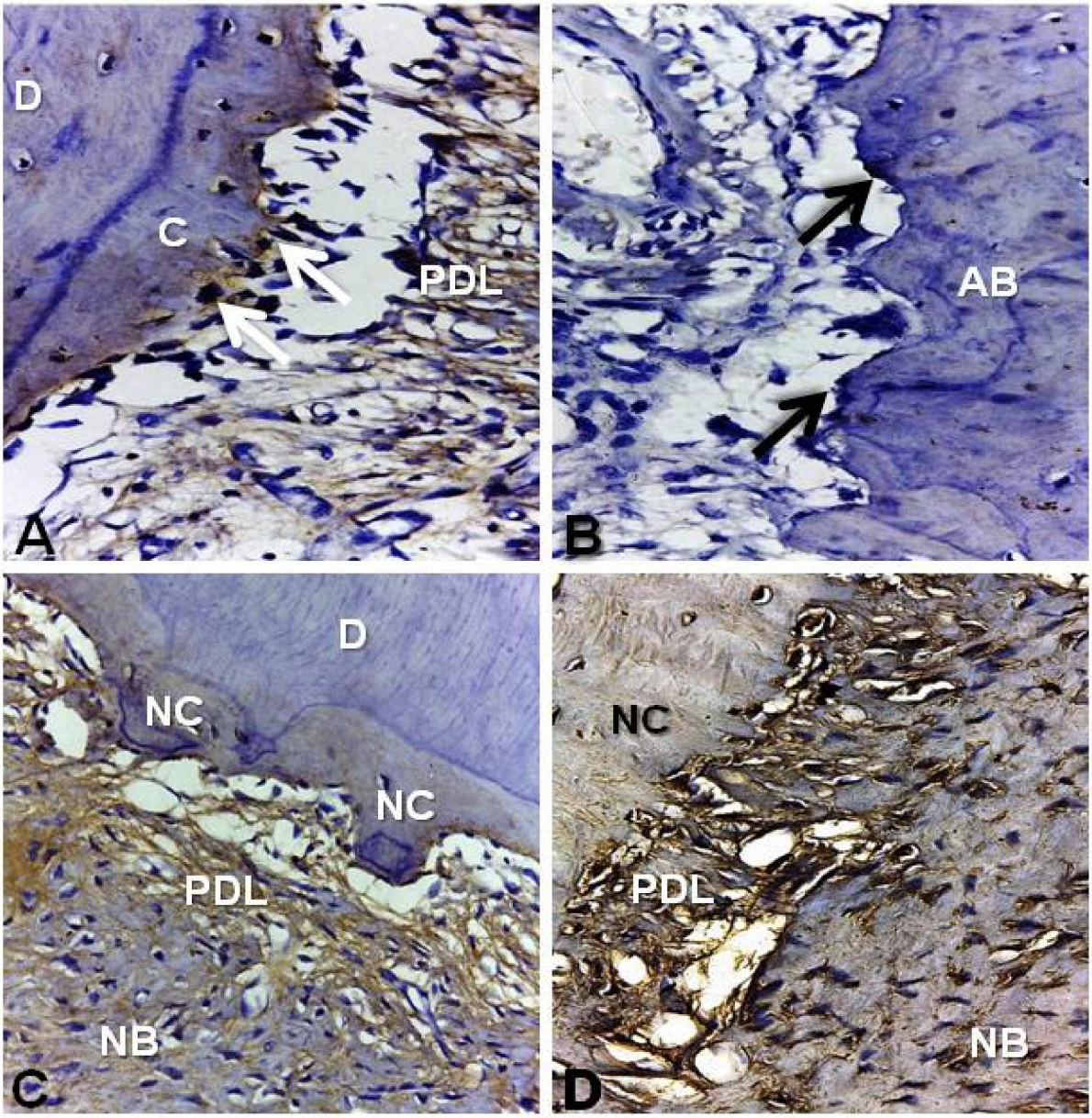

Fig.7: Immunolocalisation of Col-1 expression during healing in group I at day one, 6th &12th weeks. (A-B) Show Col-1 expression at day one; (A) Weak expression of extracellaular matrix of col-1 in degenerated periodontal tissues adjacent to resorpedcementum surface (White arrows). Whereas destructed alveolar bone showed no reaction to col-1 (B). (C) Shows Col-1 expression at 6th week; it Shows moderate expression of extracellular matrix of col-1 in PDL and mild reaction adjacent to new cementum and bone. (D) Shows Col-1 expression at 12th week; it shows intense expression of col-1 in extracellular matrix adjacent to new cementum and bone surfaces. While newly formed cementum and bone show moderate staining. Dentin (D), Cementum (C), Alveolar bone (AB), Newcementum (NC), New bone (NB). (DAB. Original. Magnification (A-D) X 40).

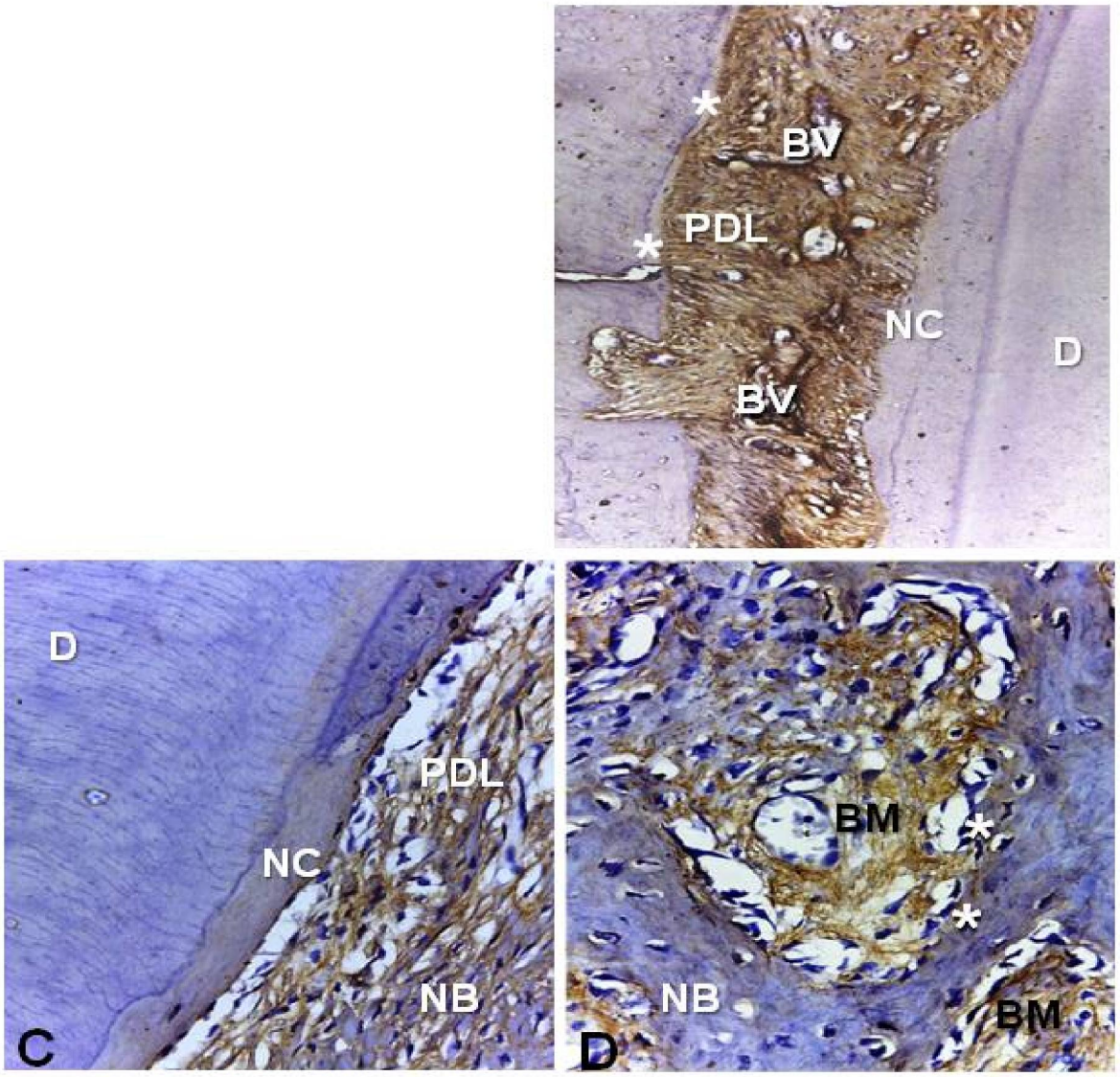

Fig.8: Immunolocalisation of Col-1 expression during healing in group II at 6th&12th weeks.(A) Shows Col-1 expression at 6th week; it shows Intense extracellular matrix expression of col-1 adjacent to new cementum, new bone, PDL and proliferating blood vessels. However, new cementum and osteoid tissue exhibit mild reaction (White asterisks). **(B-C)** Show Col-1 expression at 12th week; (B) Moderate expression of col-1 in new cementum and new bone. Whereas, new PDL shows intense expression. (B) Intense reaction of col-1 in extracellular matrix of bone marrow and a moderate one in osteoid tissue (White asterisks). Dentin (D), New cementum (NC), New bone (NB), Bone marrow (BM).Blood vessel (BV). (DAB. Origina Magnification A>B and C X 40.)

Table (2): Intensity of MMP-9 immunohistochemical expression and statistical significance of the two-way difference (ANOVA) test for groups I, II, and III at the periods day one, 6th week, and 12th week.

	Groups				
Duration	**Group I**	**Group II**	**Group III**	**F-value**	***p*-value**
Day one	39.05 ± 3.72	39.05 ± 3.72	39.05 ± 3.72		
6 weeks	28.25± 1.52	7.94 ± 0.49	9.13 ± 0.22		
12 weeks	20.44 ± 0.83	5.24 ± 1.1	7.28 ± 0.51	586.018	0.001***
F-value	110.214				
***p*-value**	0.001***				

** Extremely significant

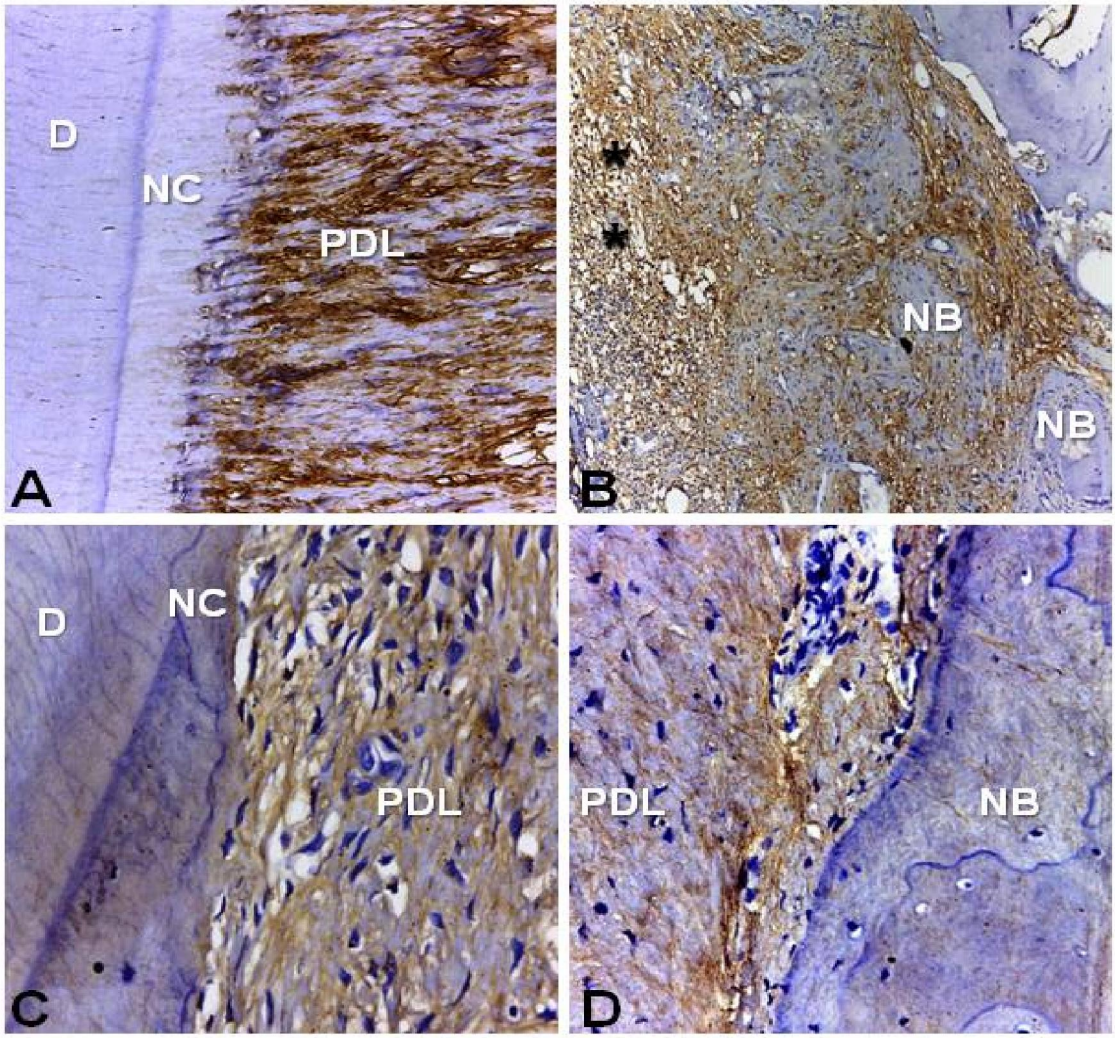

Fig.9: Immunolocalisation of Col-1 expression during healing in group III at 6th &12th weeks. (A-B) Show Col-I expression at 6th week; (A) Intense expression of col-1 in extracellualar matrix ahead of new cementum and PDL. On the other hand, new cementum with inserted Sharpey's fibers show moderate reaction. (B) Intense reaction in extracellular matrix along the new bone and around the HANP (Black asterisks). (C-D) Show Col-1 expression at 12th week; (C) Moderate expression of col-1 in extracellualar matrix in front of new cementum and PDL. (D) Moderate col-1 expression in extracelluar matrix ahead of new bone. Dentin (D), New cementum (NC,) New bone (NB). (DAB. Original Magnification A,C and DX 40, B X 10).

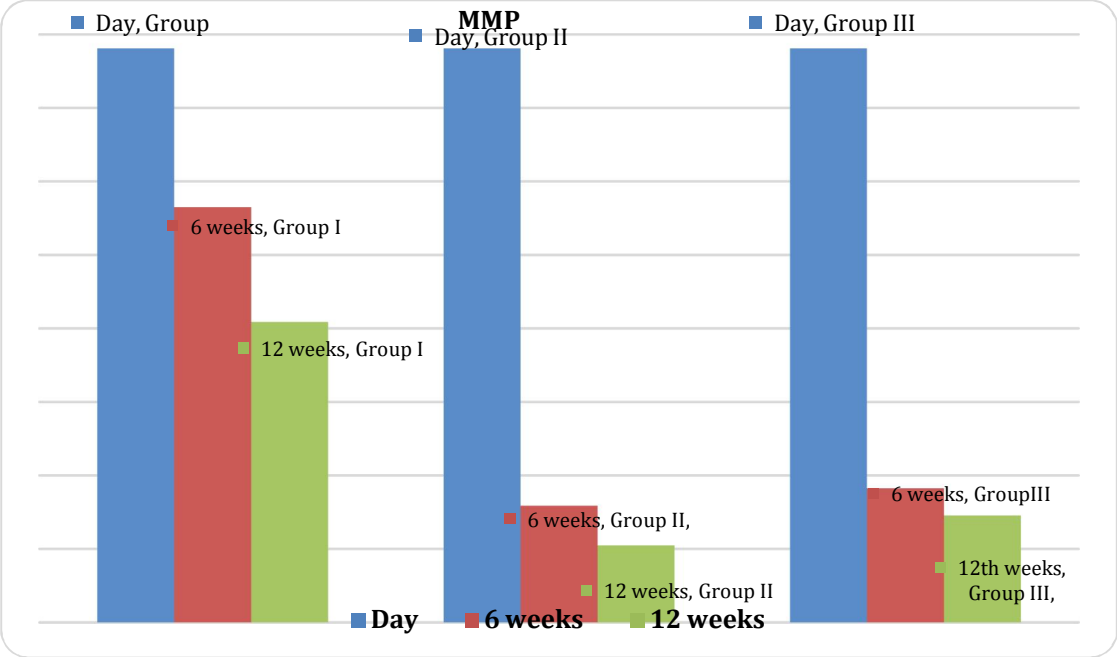

Fig. 10: Column chart representing mean intensity of MMP-9 immunohistochemical expression for groups I, II, and III at the periods; day one, 6th week, and 12th week.

II- Intensity of col- I immunohistochemical expression:

The greatest mean intensity of Col-1 immunohistochemical expression was recorded in group II at 12th week followed by group III at the same period, with the least value obtained in all groups at the day one. Analysis of variance (ANOVA) test revealed an extremely significant between groups at the same period and between the different periods in the same group (*p*-value < 0.0001) except between all groups at the day one where there was no statistical significant. Tukey's post hoc test revealed that the difference between each two groups was statistically significant, except for the difference between group II and group III at the 6th and 12th weeks and between all groups at the day one. (Tab.3 & Fg.11)

Table (3): Intensity of Col-1immunohistochemical expression and statistical significance of the two-way difference (ANOVA) test for groups I, II, and III at the periods day one, 6th week, and 12th week.

	Groups				
Duration	**Group I**	**Group II**	**Group III**	**F-value**	***p*-value**
Day	8.46 ± 4.18	8.46 ± 4.18	8.31 ± 4.81	512.407	0.001**
6 weeks	26.26 ± 5.63	88.44± 8.04	81.45 ± 5.40		
12 weeks	45.02 ±12.60	114.38± 3.85	100.48 ± 6.02		
F-value	180.054				
***p*-value**	0.00 1**				

** Extremely significant

4.Discussion

Over the last few decades, the intra osseous periodontal defects were treated by wide range of graft materials to promote the regeneration of affected periodontium. These materials could enhance the proliferation and attachment of PDL cells which are essential for repair of damaged periodontium[22].Recently, the synthetic hydroxyapatite nanocrystals had been developed as a promising class of graft material for bone defect regeneration [39, 40].

The present study hypothesized that using HANP as a graft material may enhance the proliferation of periodontal ligament cells and accelerate the periodontal healing after ligature induced chronic periodontitis in dogs. The results were compared with ABG which is considered the gold standard material for bone substitute. Periodontal healing was evaluated using qualitative histological and quantitative immunohistochemical analysis at day 1and 6th& 12th weeks after initiation of treatment.

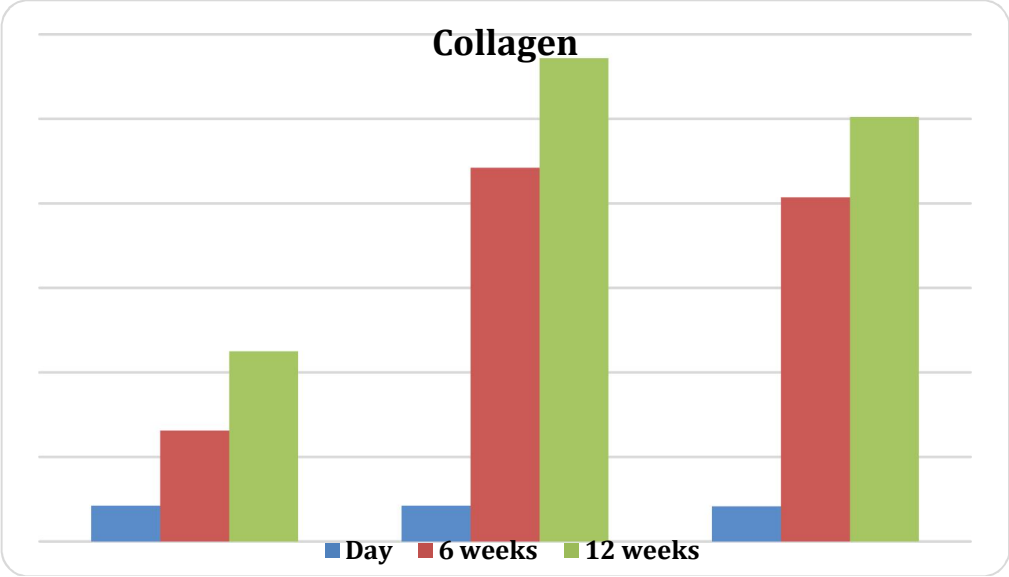

Fig.11: Column chart representing mean intensity of Col-1immunohistochemical expression for groups I, II, and III at the periods day one, 6th week, and 12th week.

Beagle dogs are considered the best animal model used in periodontal researches; their periodontium and teeth size are nearly similar to human. In addition, their surgical manipulation is easier compared to other animal models [41,42]. In the present study, chronic periodontitis was induced using cotton ligature in sub gingival area around the neck of premolar teeth.The use of ligature for induction of periodontitis elicited periodontal inflammation and destruction after one month through plaque accumulation and increased microbial biofilm around the cervix of the teeth [43].

Matrix Metalloproteinases (MMPs) are attractive genes for periodontal disease. They have multiple roles in the host response to infection progression, leucocyte recruitment, cytokine and chemokine processing and matrix remodelling[44,45]. The MMP-9, also known as gelatinase B which is an important member of MMPs family and highly expressed during periodontitis [46]. Whereas Collagen- I is the major extracellular component matrix (ECM) protein of the PDL and provides a near ideal microenvironment for the PDL cells to attach, proliferate and form a PDL spindle like morphology. In this study, periodontitis and the regenerative effect of treatment modalities were evaluated through the expression of both MMP-9 and Col-1 respectively [47]

Histological findings in all groups at day one reveled severe destruction of DGJ,periodontal tissues and resorption of both root and alveolar bone. In addition to, gingival ulceration and apical migration of attachment epithelium along the affected root surface. These epithelial change are characteristic features for changing from junctional mode to pocket formation as reported by Muller and Schroeder 1982[48]. Moreover These results are in agreement with Graves *et al.,* 2008, Preshaw *et al.,* 2008 Minkle *et al.,* 2014[49-51] who observed alveolar bone and root absorption in ligature induced periodontitis after fourteen days of induction. due to accumulation of plaque and microorganism on teeth and surrounding gingival tissue leading to gingivitis that later may develop into periodontitis as a results of production of inflammatory cytokines [e.g. interleukins (IL), tumor necrosis factor-α (TNF-α), prostaglandin E2) and enzymes [including the Matrix Metalloproteinases (MMPs)].

In conventionally treated animals positive signs of regeneration of destructed periodontal tissues were observed after 6th and 12th weeks of initiation of treatment. Nevertheless, signs of complete regeneration of periodontium were not clearly seen. These result was confirmed by Bartold *et al.,* 2000 and Chacko 2014[52,53] who observed minor regeneration of the periodontium was occurred in the early phases and spontaneous regeneration does not occur without therapeutic intervention.

In the present study, ABG implanted into periodontal defects produced favorable results at 6th&12th weeks after initiation of treatment compared with conventionally treated animals. Our histological observations of periodontal regeneration with AGB revealed new bone and cementum regeneration with re-orientation of the periodontal ligaments fibers. In addition, the boundary between old and new bone was indistinguishable. These results correlate with Sumer et al 2013 and Alhadad *et al.,* 2014 [54,55] who concluded that both; autogenous bone and bony glass

grafts were equally effective in treating Grade II furcation involvement as they provided statistically significant improvement in all of the clinical and radiographic parameters tested compared with open debridement alone control group.

Moreover Kim *et al.,* 2005 [56] reported that ABG implanted into periodontal defects, have been reported to produce favorable clinical results compared with conventional treatment alone. However, they observed small resorption pits in most teeth.

On the other hand, Ellegaard and Loe, 1971[57] reported that grafts of intraoral cancellous bone and marrow did not show a significant difference in the clinical outcome when compared with the conventional treatment. Also, Renvert *et al.,* 1985[58] found limited differences between grafted and non grafted sites except in deep defects.

The positive effect of ABG was further confirmed immunohistochemicaly. The MMP-9 expression was decreased in intensity from day 1 to 6th& 12th weeks after the surgical operation. In contrast to Collagen I expression which increased over the same previous time points, indicating reduction of inflammatory reactions and regeneration of periodontium. This agrees to a large extent with Kim *et al.,* 2005 [56] as they reported no evidence of inflammatory reactions, root resorption, or ankylosis with ABG, confirming ABG as a successful graft material. Also the localization and distribution of Col-1 expression in PDL extracellular matrix - in present study- are in agreement with Christgau *et al.,* 2007[59] who studied the expression pattern of Col-1 and other extracellular matrix in furcation defect of dogs following guided tissue regeneration.

In the present study, NHAP showed bone formation represented by new bone spicules, 6 weeks after initiation of treatment. This result is in agreement with the study performed to evaluate the influence of nHA on the adhesion, proliferation and differentiation of alveolar bone-derived cells. The increased expression of BMPs and osteoinductive biomarkers suggested that nHA might stimulate the proliferation and differentiation of local alveolar osteoblasts and thus encouraging bone regeneration at alveolar bone regeneration sites. Moreover SEM showed that nHA had a rougher surface than the control substrate and favored the adhesion and proliferation of human osteoblasts. [59-61]

Therefore, the nanHP might act as promoter of bone regeneration at the bone defect site in two ways: by inducing osteogenic differentiation [26,34].and/or by inducing the secretion of specific factors, such as BMPs and several important markers of osteogenesis like alkaline phosphatase, collagen -1, osteocalcin and osteonectin[60].

In the current study, NHAP were completely resorped with new bone completely integrated with the old one after 12 weeks. This in agreement with Thorwarth 2005 and Spies *et al.,* 2008 [28,62] who reported that undisturbed osseous integration and complete resorption of nano HA graft within 12 weeks.

The re-organization of PDL fibers with NHAP was recognized in the current study. This matches with Seo *et al.,* 2004 [63] who reported that human PDL contains a population of multipotent postnatal stem cells, providing a unique reservoir of stem cells from an accessible tissue resource.Moreover Kasaj *et al.,* 2008[22] demonstrated that the nHA has a capability to promote PDL fibrobast proliferation and osteogenic differentiation.

Noteworthy, the nanoparticles accelerate the periodontal healing through fibroblasts proliferation and collagen formation. Moreover the fibroblasts have an important role in re-epithlization, collagen fiber synthesis, extracellular regeneration, remodeling of wound and release of such endogenous growth factors FGF and VEGF.[22]

Interestingly In the current study, immunohitochemically, MMP-9 expression decreased with time progression indicated the regenerative effect of NHAP which further confirmed with increased Col-1 expression. These resultsare consistent with Itagaki *et al.,* 2008[64] who examined the expression of Matrix Metalloproteinases (MMPs), type I collagen and osteocalcin during bone healing in a rat calvarial experimental defect model. They found that there are correlation between their expression during healing of bone defect.

These Qualitative results of immunolocalization of both MMP9 and COL-1 were confirmed by further Quantitative results. The expression of MMP-9 was significantly recorded at the day one of all groups, with the least values obtained at the 12th week in group II followed by group III at the same period. Moreover the difference between each two groups was statistically significant, except for the difference between group II and group III at the 6th and 12th weeks and between all groups at the day one These results were in agreement with Carnerio *et al.,* 2009[65] who recognized the expression of MMP9 was significantly higher in periodontitis than in normal sample. The significant increase of MMP9 after 4 months of inducing periodontitis is confirmed by Rayan *et al.,* 2000 [44] who stated that increased activity of MMPs was seen in chronic inflammatory conditions including periodontitis and they destroy extracellular matrix components like collagen, gelatin, laminin, fibronectin and proteoglycans. In contrast the expression of COL-1 was significantly increased in group II at 12th week followed by group III at the

same period, with the least value obtained in all groups at the day one. These results are in agreement with Xin *et al.,* 2010 [66] who found an inverse relationship between the level of expression of MMP and COL-1 in periodontitis with.diabetic patient.

In conclusion, the present study indicates that, treatment of periodontitis in dogs with either ABG or HANP leads to an enhanced periodontium regeneration when compared with the conventional treatment alone. However, there was no notable difference between the healing effect of HANP and the ABG which considered the gold standard graft material. This makes HANP a more favorable graft material as it is available, relatively cheap and does not necessitate a second surgical site for autogenous graft.

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References:

1. Kong L, Peng Z, Li S, Bartoold M. (2006): Nanotechnology and its role in the management of periodontal diseases. Periodontology 2000; 40: 184–196.
2. Cardoso D, Jansen J, Leeuwenburgh S. (2012): Synthesis and application of nanostructured calcium phosphate ceramics for bone regeneration. J Biomed Mater Res, Part B.:100:2316–2326.
3. McCulloch C, Bordin S. (1991): Role of fibroblast subpopulations in periodontal physiology and pathology. J. Peridontol. Res; 26:144-154.
4. McCulloch C. (1995): origin and function of cells essential for periodontal repair: the role of fibroblasts in tissue homeostasis. Oral Disease; 1:271-278.
5. Miguel S, Rupf S, Frenzel J, Eschrich K. (2003): The effects of extracts from periodontopathic bacteria on human periodontal fibroblasts stimulated with mineralization supplements. J. Oral Sci; 45: 127-137.
6. Nevins M, Camelo M, Nevins L, Schenk R, Lynch S. (2003): Periodontal regeneration in humans using recombinant human platelet-derived growth factor-BB (rhPDGF-BB) and allogenic bone. J.Peridontol; 74: 1282-1292.
7. Segundo E, Quintanar A, Perez V, Guerrero D. (2005): Preparation and characterization of triclosan nanoparticles for periodontal treatment. Int. J. Pharm; 294: 217–232.
8. Albrecht M, Evan C, Raston C. (2006): Green chemistry and the health implications of nanoparticles. Green Chem; 8: 417–432.
9. Kelly. B, Bogaert. P (2008): Medical nanotechnology in Europe. RAJ Pharma.; 451-458.
10. Rai M, Yadav A, Gade A. (2009): Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances; 27: 76–83.
11. ShirmohammadiA,Chitsazi M, Lafzi A. (2009): A clinical comparison of autogenous bone graft with and without autogenous periodontal ligament graft in the treatment of periodontal intrabony defects. Clin. Oral Investig; 13: 279-86.
12. Kim H. (2007): Biomedical nanocomposites of hydroxyapatite/ polycaprolactone obtained by surfactant mediation. J Biomed Mater Res.; 83:169–177.
13. AlpaslanE, Webster T, (2014): Nanotechnology and biotechnology to increase tissue growth: a summary of in vivo studies. International Journal of Nanomedicine 9: 7–12
14. Garrett S, Bogle G. (1994): Periodontal regeneration with bone grafts. Curr Opin Periodontol; 168–77.
15. Helm G, Dayoub H, Jane J. (2001): Bone graft substitutes for the promotion of spinal arthrodesis. Neurosurg Focus; 10:E4.
16. Prabhahara K, Bari K, Motaktla N, Prenmasta T. (2014): Comparison of β-tricalcium phosphate and autogenous bone graft with bioabsorbable membrane and autogenous bone graft in the treatment of intrabony periodontal defects: A clinico-radiographic study. J. Dr. NTR. University. Health Science 3: 28- 36.
17. Mellonig J. (1992): Autogenous and allogeneic bone grafts in periodontal therapy. Crit Rev Oral Biol Med.; 3:333–352.
18. Rosenberg E, Rose L. (1998): Biologic and clinical considerations for autografts and allografts in periodontal regeneration therapy. Dent Clin North Am.; 42:467–490.
19. Kiyokawa K, Kiyokawa M, Hariya Y, Fujii T, Tai Y. (2002): Regenerative treatment of serious periodontosis with grafting of cancellous iliac bone and gingival flaps and replanting of patients teeth. J Craniofac Surg.; 13:375–381.
20. Sumer M, Keles GC, Cetinkaya BO, Balli U, Pamuk F, Uckan S. (2013): Autogenous cortical bone and bioactive glass grafting for treatment of intraosseous periodontal defects. Eur J Dent.7: 6-14.
21. Bartold P, McCulloch C, Narayanan A, Pitaru S. (2000): Tissue engineering: a new paradigm for periodontal regeneration based on molecular and cell biology. Periodontol; 2000. 24: 253–269.
22. Kasaj A, Willershausen B, Reichert C, Röhrig B, Smeets R, Schmidt M. (2008): Ability of nanocrystalline hydroxyapatite paste to promote human periodontal ligament cell proliferation. J. Oral Science; 50: 279-285.
23. Okumura M, Ohgushi H, Dohi Y, Katuda T, Tamai S, Koerten H. (1997): Osteoblastic phenotype expression on the surface of hydroxyapatite ceramics. J Biomed Mater Res;37:122-129.
24. Sukumar S, Dřízhal I. (2008): Bone grafts in periodontal therapy. Acta. Medica.; 51:203–207.
25. Sun W, Chu C, Wang J, Zhao H. (2007): Comparison of periodontal ligament cells responses to dense and nanophase hydroxyapatite. J. Mater. Sci:Mater. Med. 18: 677-683.
26. Wang H, Li Y, Zuo Y, Li J, Ma S, Cheng L. (2007): Biocompatibility and osteogenesis of biomimetic nano-hydroxyapatite/polyamide composite scaffolds for bone tissue engineering. Biomaterials;28:3338-3348.
27. Chris J, Verdonschot N, Schreurs B, Buma P. (2006): The use of a bioresorbablenano-crystalline hydroxyapatite paste in acetabular bone impaction grafting. Biomaterials; 27: 1110-1118.
28. Thorwarth M, Schultze S, Kessler P, Wiltfang J, Schlegel K. (2005): Bone regeneration in osseous defects using a resorbablenanoparticular hydroxyapatite. J. Oral Maxillofac. Surg. 63:1626-1633.
29. Kong L, Peng Z, LI D, Bartoold M. (2006): Nanotechnology and its role in the management of periodontal diseases. Periodontology 2000; 40: 184–196.
30. Knight A. (2011): The costs and benefits of animal experiments; Houndmills, Basingstoke; New York, N.Y.: Palgrave Macmillan
31. Nociti F, De Toledo C, Machado M, Stefani C, Line S, Gonçalves R. (2001): Clinical and microbiological evaluation of ligature-induced peri-implantitis and periodontitis in dogs. Clin Oral Implants Res.;12:295-300.
32. Sato S, Fonseca MJV, Del Ciampo JO, Jabor JR, Pedrazzi V. (2008): Metronidazole-containing gel for the treatment of periodontitis: an in vivo evaluation. Braz. Oral Res.; 22: 145-150.

33. Diaz M, Barba F, Miranda M, Francisco G, Torrecillas R, Moya1 J. (2009): Synthesis and Antimicrobial Activity of a Silver-Hydroxyapatite Nanocomposite. Journal of Nanomaterials. ID 498505, 6 pages.
34. Paz A, Guadarrama D, López M, Jesús E, Brizuela N, Aragón J. (2012): A comparative study of hydroxyapatite nanoparticles synthesized by different routes. Quim. Nova; 35: 1724-1727.
35. KuKanich B1, Bidgood T, Knesl O (2012): Clinical pharmacology of nonsteroidal anti-inflammatory drugs in dogs. Vet Anaesth Analg 39:69-90.
36. Kumar G & Kiernan J. (2010): Education Guide:Special Stains and H & E Dako North America, Carpinteria, California. Second Edition.
37. Christgau M, Caffesse R, Newland J, Schmalz G, D'Souza R. (1998): Characterization of immuno-competent cells in the diseased canine periodontium. J Histochem Cytochem.; 46:1443-54.
38. Mould R. (1989): introductory medical statistics. 2nd ed., Adam Hilger, Bristol and Philadelphia, pp. 17.22.126.
39. Gerber T, Holzhuter G, Gotz W. Bieenengrabe V, Henkel K, Rumpel E (2006): Nanostructuring of biomaterials- a pathway to bone grafting substitute. Eurp J. Trauma 23,132-140.
40. Taghi M, Shirmohammadi A, Faramarzie M, Pourabbas R, Rostamzadeh A. (2011): clinical comparison of nano-crystalline hydroxyapatite (Ostim) and autogenous bone graft in the treatment of periodontal intrabony defects. Med. Oral Patol. Oral Cir. Bucal; 16: 448-453.
41. Wikesjö U, Kean C, Zimmerman G. (1994): Periodontal repair in dogs: supraalveolar defect models for evaluation of safety and efficacy of periodontal reconstructive therapy. Journal of Periodontology. 65:1151–1157.
42. Struillou X, Boutigny H, Soueidan A, Layrolle P. (2010): Experimental Animal Models in Periodontology: A Review.The Open Dentistry J; 4: 37-47.
43. 43-Jin Q, Cirelli J, Park C, Sugai J, Taba M, Kostenuik P, and Giannobile W. (2007): RANKL Inhibition through osteoprotegerin blocks bone loss in experimental periodontitis. J Periodontol.; 78: 1300-1308.
44. -Ryan ME, Golub LM. (2000): Modulation of matrix metalloproteinase activities in periodontitis as a treatment strategy. Periodontol 2000. 24:226-38.
45. -Chou SH, Feske SK, Simmons SL, Konigsberg RG, Orzell SC, Marckmann A, Bourget G, Bauer DJ, De Jager PL, Du R, Arai K, Lo EH, Ning MM. (2011): Elevated peripheral neutrophils and matrix metalloproteinase 9 and bio- markers of functional outcome following subarachnoid hemorrhage. Transl Stroke Res.; 2, 600–607.
46. -Uitto VJ, Overall CM & McCulloch C. (2003): Proteolytic host cell enzymes in gingival crevice fluid. Periodontol 2000.; 31: 77–104.
47. -Watanabe T, Kubota T. (1998): Characterization of fibromodulin isolated from bovine periodontal ligament. J Periodontal Res.; 33:1–7.
48. Muller-G, Schroeder H. (1982): The pocket epithelium: a light- and electronmicroscopic study. J.Periodontol.; 53: 133-144.
49. Graves D, Fine D, Teng Y, Van Dyke T, and Hajishengallis G. (2008): "The use of rodent models to investigate hostbacteria interactions related to periodontal diseases," Journal of Clinical Periodontology; 35:89–105.
50. Preshaw PM. (2008): Host response modulation in periodontics.:Periodontol 2000.;48:92–110.
51. MinkleGulati, Vishal Anand,1 VivekGovila, and Nikil Jain (2014): Host modulation therapy: An indispensable part of perioceutics Indian Soc Periodontol; 18: 282–288.
52. Bartold P, McCulloch C, Narayanan A, Pitaru S. (2000): Tissue engineering: a new paradigm for periodontal regeneration based on molecular and cell biology. J. Periodontol. 24: 253–269.
53. Chacko N, Abraham S, Rao H, Sridhar N, Moon N, Barde D (2014): A Clinical and Radiographic Evaluation of Periodontal Regenerative Potential of PerioGlas: A Synthetic, Resorbable Material in Treating Periodontal Infrabony Defects. J Int Oral Health.6:20-26.
54. Sumer M, Keles GC, Cetinkaya BO, Balli U, Pamuk F, Uckan S. (2013): Autogenous cortical bone and bioactive glass grafting for treatment of intraosseous periodontal defects. Eur J Dent. 7:6-14.
55. Elhaddad S, AbdElrazak M, Saudi H, Ghoraba N, (2014): Evaluation of bioactive glass and autogenous bone in the treatment of Grade II furcation involvement: A randomized controlled trial. J of Interdisciplinary Dentistry 4: 13-23
56. Kim C, Choi S, Cho K, Chai J, Wikesjö U, Kim C. (2005): Periodontal healing in one-wall intra-bony defects in dogs following implantation of autogenous bone or a coral-derived biomaterial. J Clin. Periodontol.; 32:583-589.
57. Ellegaard B, Löe H. (1971): New attachment of periodontal tissues after treatment of intrabony lesions. J Periodontol.; 42:648-52.
58. Renvert S, Garrett S, Shallhor RG, Egelberg J. (1985): Healing after treatment of periodontal intraosseous defects. III. Effect of osseous grafting and citric acid conditioning. J. Clin. Periodontol.; 6:441-445.
59. Christgau M1, Caffesse RG, Schmalz G, D'Souza RN: (2007): Extracellular matrix expression and periodontal wound-healing dynamics following guided tissue regeneration therapy in canine furcation defects.J Clin.Periodontol. 34:691-708.
60. Thian E, Huang J, Ahmad Z, Edirisinghe MJ, Jayasinghe SN, Ireland (2008): Influence of nanohydroxyapatite patterns deposited by electrohydrodynamic spraying on osteblast response. J BiomedMater, 85:188–194.
61. Pilloni1 A, PompaG, Saccucci M, Di Carlo G, Rimondini L, Brama M, Zeza B, Wannenes F, Migliaccio S (2014): Analysis of human alveolar osteoblast behavior on a nano-hydroxyapatite substrate: an in vitro study.*BMC Oral Health* 14:22-25.
62. Spies C, Schurer S, Gotterbarm T, Breusch S (2008): Animla study of the bone substitute material Ostium within osseous defect in Gottingerminipigs. Z OrthpUnfall, 146: 64-69.
63. Seo B, Miura M, Gronthos S, Bartold P, Batouli S, Brahim J, Young M, Robey P, Wang C, Shi S. (2004): Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet; 364: 149–155.
64. Itagaki T, Honma T, Takahashi I E chigo S,Sasno Y (2008): Quanititave analysis and localization of mRNA transcript of type -1 collagen, Osteocalcin and MMP2, MMP8, MMP13 during bone healing in rat calvarial experimental defect model.The Anatomical Record 291:1038-1040.
65. Carneiro E, Menezes R, Garlet G, Garcia R, Bramante C, Figueira R, Sogayar M, Granjeiro JM (2009): Expression analysis of matrix metalloproteinase-9 in epithelialized and nonepithelialized apical periodontitis lesions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 107:127-32.
66. Xin L. Lin Z, Na W, Xiaohong F, Liangjia B, (2010): Periodontal Ligament Remodeling and Alveolar Bone Resorption During Orthodontic Tooth Movement in Rats with Diabetes. Diabetes Technology & Therapeutics. 12:65-68.