Evaluation of Hydroxyapatite Nanoparticles with and Without Silver Nanoparticles in the Treatment of Induced Periodontitis in Dogs

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Abstract: Background: Regeneration of periodontal tissues affected by chronic diseases comprises a major scientific and clinical challenge. Recent advances in nanotechnology introduce new therapeutic materials for periodontal regeneration. **The aim** of this study was to investigate the efficacy of hydroxyapatite nanoparticles (HANP) with and without silver nanoparticles (AgNP) on regeneration of periodontium after inducing periodontitis in a dog model. **Methods:** Twelve beagle dogs were used in this study divided into three equal groups. Group (GP) I was divided into GP IA (negative control) represented the healthy untreated dogs, GP IB (periodontitis), GP II (HANP treated dogs), GP III (Ag-HA nanocomposites treated dogs). periodontitis was induced by cotton ligatures in the distal aspect of the mandibular right first, second and third pre-molar teeth. Four weeks after inducing periodontitis group I were sacrificed, while the GPs II, III were subjected to conventional periodontal treatment and treated subsequently using HANP and Ag-HA nanocomposites. After 6 weeks of the treatment, the dogs of GPII & III were sacrificed and the specimens were subsequent processed for H&E and Masson trichrome stains, immunohistochemical reaction by Osteopontin (OPN) antibody. These followed by statistical evaluation. **Results:** GP IB showed degradation of periodontal tissues and all histological features of periodontits. GP II revealed obvious periodontal regeneration, while periodontal tissue was almost normal in GP III. The Masson trichrome stain, immunohistochemical & statistical results confirmed these findings. **Conclusions:** Within the limitations of this study, we can conclude that HANP induces the key elements of the true periodontal tissue regeneration, which enhanced aggressively by a combination of AgNP & HANP, offering potential as therapeutic material in periodontal regeneration.

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1.Introduction

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.[1,2] Nanoparticles (NP) are clusters of atoms in the size range of 1–100 nm and include spherical, cubic, and needle-like nanoscaled particles.[3] The use of NP is gaining impetus in the present century; as such particles are reduced from a micrometer to nanometer size. The resultant properties can change dramatically; for example, chemical, physical, optical and mechanical properties, and biological activity are all altered. [2,4,5]

Periodontitis is a chronic inflammatory disease that affects a major part of world's population characterized by inflammation of gingiva and adjacent dental attachment apparatus and end by destruction of periodontal apparatus.[6,7] Many researchers have been used different approaches to achieve complete regeneration of the periodontium but their goals not achieved till appearance of recent developments in nanotechnology which provide a promising insight into the management of periodontal diseases. Periodontal tissue regeneration is the restoration of tooth supporting structures of the periodontium.[8-10] This process depends on the migration, adhesion, proliferation and differentiation of periodontal ligament (PDL) cells, which are the predominant cells of the periodontium and play a leading role in the homeostasis and regeneration of periodontium. [11-13]

Many bone substitutes have been used for periodontal regeneration. Hydroxyapatite, a synthetic calcium phosphate is commonly used in periodontal surgery for regeneration and restoration of lost periodontium. Many reports recommend this material because of its good biocompatibility and osteoconductibility, and due to its chemical and structural similarity to the mineral component of bone. [14-17] However, according to some studies, complete regeneration of periodontium has not always been found if hydroxyapatite is used in the treatment of periodontal bone loss. It is believed that the 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. So currently, developments in

nanotechnology is the main contributor to periodontal tissue regeneration, through using HANP. [14,18-20]

With advances in nanotechnology, a fully synthetic nanocrystalline hydroxyapatite (nano-HA) paste, has been introduced for augmentation procedures in osseous defects. Advantages of such nanostructured material in comparison to traditional bulk material are its close contact with surrounding tissues, quick resorption characteristics and a 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. [19,21,22] In addition, pure silver has been recently engineered into nanometer-sized particles for use in the treatment of wounds. Silver NP have proved to be most effective as it has good antimicrobial efficacy against bacteria, viruses and other eukaryotic micro-organisms. On the other hand, toxicity from silver is observed in the form of argyria, only when there is a large open wound and large amount of silver ions are used. Also, there are no regular reports of silver allergy were documented. [2,4]

The nonmaterials exhibit much better performance properties than traditional materials.[23] In addition, several studies in vitro suggested that nano hydroxyapatite is stimulator for PDL cells proliferation,[17] and AgNP can drive differentiation of fibroblasts and keratinocytes in addition to its antimicrobial effect. [2,23] Therefore the present study assumed that using HANP with and without AgNP in the treatment of induced periodontitis in dogs may enhance the differentiation of PDL cells and therefore, provide a promising insight into the application of NP in the management of periodontal disease.

2.Materials & Methods

Animals:

Twelve beagle dogs aged 13–16 months with an average body weight of 9–10 kg$_s$ were used in this study. 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.[24]

The animals were divided into three GPs each one consists of four dogs. GP I divided into GP IA (negative control GP), the dogs were neither subjected to induced periodontitis (IPD) nor treatment. GP IB (positive control GP), the dogs were subjected to IPD only. GP II (HANP treated GP), the dogs were subjected to IPD, conventional periodontal treatment and treated with HANP (NanoTech Egypt for Photo-Electronics, City of 6 October, Al Giza, Egypt). GP III (Ag-HA nanocomposites treated GP, the dogs were subjected to IPD, conventional periodontal treatment and treated with Ag-HA nanocomposites (NanoTech Egypt for Photo-Electronics, City of 6 October, Al Giza, Egypt).

Induction of periodontitis

In all groups except GP IA, dogs were subjected to induced periodontitis. Cotton ligatures were tied and placed around the neck of the mandibular right first, second and the third pre-molar teeth under general anesthesia using 25% sodium thiopental solution, 0.5 ml/kg (Sigma Tec Pharmaceutical Industries-Egypt). The ligature was knotted and placed in subgingival position in the distal aspect of each premolar in order to induce chronic periodontitis.[25,26]

Surgical procedure & drug delivery

Four weeks after inducing periodontitis GP IB were sacrificed under anesthesia by sodium thiopental salt overdose, while the dogs of GPs II & III were subjected to conventional periodontal treatment. GPs II & III were treated subsequently using HANP and Ag-HA nanocomposites. Then the flaps were coronally repositioned and sutured with 3/0 black sutures in an interrupted manner. A pain killer was given on the first day postoperatively.

Dogs of GPs II & III were sacrificed at 6th week after the surgical procedures. The scarification of dogs were done with an over dose of sodium thiopental. A segment of bone, from the mandibular right half including premolar teeth with the surrounding gingival tissue was processed for subsequent for H&E, Masson trichrome stains & immunohistochemical reaction.

a- H&E and Masson Trichrome stains:

Biopsies comprising right mandibular segments with the first, second and third pre-molars, were fixed in 10% buffered formalin for 24 h. Then, to improve fixation, these segments were cut in smaller parts, each containing half of each premolar tooth with interdenal tissues in between, and fixed for another 24 h. Furthermore. All specimens were decalcified in 10 % neutral-buffered EDTA. The biopsies washed in tap water over night and then dehydrated in ascending grades of alcohol, cleared in xylene and then embedded in low melting point (56˚C) paraffin. A mesiao-distal vertical plane serial sections were processed for H&E and Massion trichrome stain (Leica DM500).[27]

b- Immunohistochemical stains:

Immunohistochemical labelling was performed using the avidin–biotin–complex (ABC) method.[28] Representative sections taken from the central part of the defects were processed for the Osteopontin (OPN) polyclonal antibody (abcam. Cat.No.ab8448)

Evaluation of Immunohistochemical staning:

The immunostaind sections were examined under L.M (Leica DM500). Image software LAS EZ (Leica Application Suite) version 3.0.0 allowed the acquisition and processing of high quality digital

images. Images of the immunostaind sections were analyzed for intensity of OPN expression using the Image J analysis system (Image J 1.48s, Wayne Rasband, National Institute of Health, USA).[29]

Statistical Evaluation

All statistical analyses were performed using the analysis of variance (ANOVA) followed by Tukey's post hoc test according to Mould, 1989.[30] All values were expressed as means and standard deviations. All statistical analyses were done on an IBM PC using the statistical software "SPSS 20" (Statistical Package for Scientific Studies) (SPSS Inc., Chicago, Illinois, USA) for windows.

3.Results

1.1. Haematoxylin and Eosin

In the negative control dogs (GP IA), Specimens revealed healthy periodontal tissue, where the normal attachment apparatus was noticed. The DGJ showed normal architecture, with normal architecture of PDL, alveolar bone, and cementum. Apical to the bone alveolar crest the acellular cementum covered by uniform thin layer of cellular cementum till root apex. (fig. 1).

Periodontitis group (GP IB) revealed destruction of periodontal apparatus. There was an obvious loss of attachment with ulcerated epithelium. The DGJ epithelium developed rete pegs or ridges that protruded into severely destructed connective tissue with moderate to severe inflammatory cell infiltration. Additionally, it characterized by aggressive resorption of cementum and alveolar process by odontoclasts and osteoclasts respectively. (fig. 2).

HANP treated group, specimens revealed regeneration of periodontium. Reorganization of DGJ epithelium was noticed with well organized underling C.T and mild inflammatory cell infiltration. The newly formed cementum was of a cellular type and appeared irregular lining by active cementoblasts. It was separated from old acellulaer cementum by a line of demarcation. Active bone formation was observed around the HANP. The newly formed bone had wide marrow spaces within bone trabecula and lined by active osteoblasts. Repetitive pattern of blood capillaries along the newly formed bone surface was seen (fig. 3).

Ag-HA nanocomposites treated group, specimens revealed advanced regeneration of all periodontal tissues. Normal architecture of DGJ epithelium was observed. The C.T well organized with mild inflammatory cell infiltration. The PDL was more organized and highly vascularized with mild inflammatory cell infiltration and well insertion of Sharpey's fibres into the new root cementum and the new bone. The newly formed a cellular cementum was noticed irregular in previously reabsorbed areas and covered by active cementoblasts. There was a line of demarcation between new and old cementum. The most of the graft particles were completely replaced by advanced bone formation. The newly formed bone trabeculae had wide bone marrow spaces with active osteoblasts and repetitive pattern of blood capillaries lining its surface. In some areas within bone marrow, new bone spicules were formed around the remnant of the graft particles (fig. 4).

1.2. Masson's Trichrome

In the negative control dogs, specimens revealed normal organization of mild staining of fine and coarse collagen fibers with a variable number of fibroblasts. The PDL fibers showed normal arrangement of moderate staining collagen fibers with fibroblasts lying on their long axis (fig. 5). While, Periodontitis group revealed weak staining of fine less organized collagen fibers of gingival C.T with weak fibroblastic proliferation. The PDL fibers showed loss of organization with weak staining of fine collagen fibers and weak fibroblasts proliferation (fig. 6).

HANP treated group, specimens moderate staining coarse collagen fibers with a variable number of fibroblasts in the C.T stroma beneath the DGJ epithelium. Moderate staining coarse collagen fibers of PDL reverted to normal arrangement with a variable number of fibroblasts. Moderate staining of coarse collagen fibers were observed around the HANP. The new bone tissue around the graft particles was connected by moderate staining collagen fibers, in addition to presence of variable number of fibroblasts (fig. 7). **Ag-HA nanocomposites treated group,** specimens revealed normal C.T stroma beneath the DGJ epithelium exhibiting intense staining of coarse collagen fibers with a variable number of fibroblasts. Normal arrangement of intense staining PDL coarse collagen fibers appeared with a variable number of fibroblasts. Intense staining of organic layer lining the alveolar bone and cementum surfaces was observed (fig. 8).

1.3. Immunohistochemical results (Osteopontin antibody)

In the negative control dogs, Immunostaining reaction of OPN revealed weak reactivity in the extracellular matrix of PDL. Mild osteopontin expression was noticed in localized areas adjacent to bone and cementum surfaces. Intense reaction was observed around the blood vessels (fig. 9). While in periodontitis group, immunostaining reaction of OPN revealed weak to mild reactivity in the extracellular matrix of PDL especially in localized areas adjacent to bone and cementum surfaces, while the extracellular matrix close to the resorption areas of cementum showed negative reaction. Intense OPN expression of the osteoclasts and its resorption lacunae was

observed. Intense reaction appeared around the blood vessels (fig. 10).

HANP treated group, immunostaining reaction of OPN revealed intense reaction in the extracellular matrix of PDL with moderate reaction of osteoid layer and cementoid layer lining the new bone and cementum respectively. Intense reaction was observed around the new bone trabeculae and the HANP. Intense reaction was noticed around the blood vessels (fig. 11). **Ag-HA nanocomposites treated group,** immunostaining reaction of OPN revealed intense reaction in the extracellular matrix of PDL next to bone and cementum surfaces with mild reaction lining osteoid layer and cementoid layer of the new bone and cementum respectively. Intense reaction was noticed around the new bone trabeculae and the graft NP. Intense reaction was observed around the blood vessels (fig. 12).

2- Statistical Evaluation

Intensity of OPN immunohistochemical expression:

The greatest mean intensity of OPN immunohistochemical expression was recorded in GP II (HANP treated group), with the least value obtained in GP IA (negative control group) and group II (periodontitis group). Analysis of variance (ANOVA) test revealed an extremely significant difference between GPs (P-value < 0.0001). Tukey's post hoc test revealed that the difference between each two GPs was statistically significant, except for the difference between GP IA and GP IB (tab. 1).

Table 1: Intensity of OPN immunohistochemical expression and statistical significance of the difference (ANOVA) test for group IA & IB in relation to groups II, III at the 6th week.

	Group I	Group II	Group III at 6th week	Group IV at 6th week	Group V at 6th week
Mean	5.12[a]	3.82[a]	11.79[b]	88.02[c]	64.37[d]
S. D	.48	.15	2.23	5.74	2.49
Max	5.61	3.99	13.78	93.46	67
Min	4.66	3.64	9.81	82.58	62.18
F -value	684.25				
P-value	< 0.001***				

*** extremely significant Tukey's post hoc test: Means with different letters are significantly different.

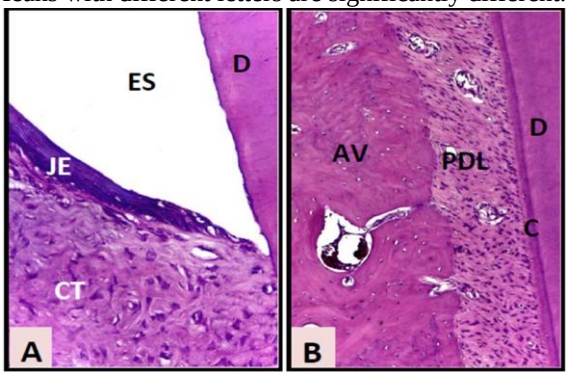

Figure. 1: Photographs of group IA (negative control group) showing healthy periodontal tissue. **A.** Normal architecture of junctional epithelium (JE) supported by well organized CT with mild inflammatory cell infiltration. **B.** Normal histological architecture of root and alveolar bone (AV) with will organized Sharpey's fibres and uniform thickness of PDL. Enamel space (ES), dentin (D), cementum (C) (H&E orig. mag., **A&B** ×40).

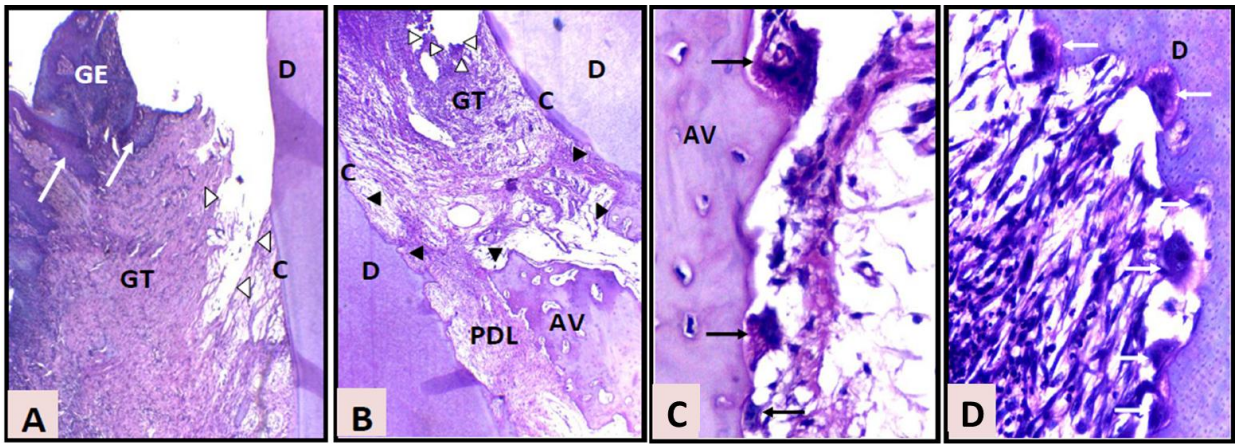

Figure. 2: Photographs of group IB (periodontitis group) showing sever destruction of periodontal apparatus. **A.** Loss of attachment epithelium (white arrow heads). Gingival epithelium (GE) proliferated and migrated (white arrows) into severely inflammatory cell infiltrated granulation tissues (GT). **B.** Cementum (C) and alveolar bone (AV) resorptions (black arrow heads), widening of PDL space with loss of organization and large dilated blood vessels. **C.** Obvious resorption of the alveolar bone (AV) by osteoclasts (black arrows). **D.** Aggressive resorption of the cementum extended into the dentine (D) by odontoclasts (white arrows). Dentin (D) (H&E orig. mag., **A** ×10, **B** ×4, **C&D** ×40).

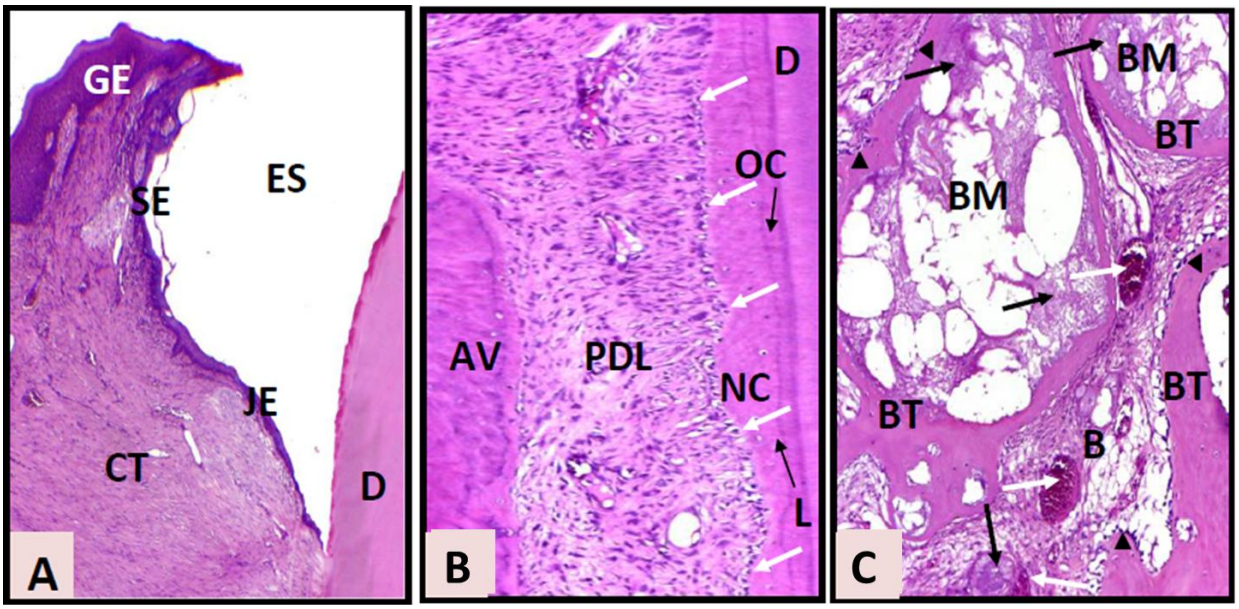

Figure. 3: Photographs of group II (HANP treated group) showing **A.** Reorganization of DGJ epithelium, well organized C.T with mild inflammatory cell infiltration **B.** (Uniform thickness of PDL with high vascularity and mild inflammatory cell infiltration. irregular newly formed cementum (NC) of a cellular type lined by active cementoblasts (white arrows). **C.** Newly formed bone trabecula (BT) with wide bone marrow spaces (BM) containing the HANP (black arrows) with large dilated blood vessels (white arrows), BT lined by active osteoblasts and repetitive pattern of blood capillaries (black arrow heads). Enamel space (ES), gingival, sulcular & junctional epithelium (GE, SE, JE), dentin (D), alveolar bone (AV), line of demarcation (L), old acellular cementum (OC) (H&E orig. mag., **A, B& C** ×10).

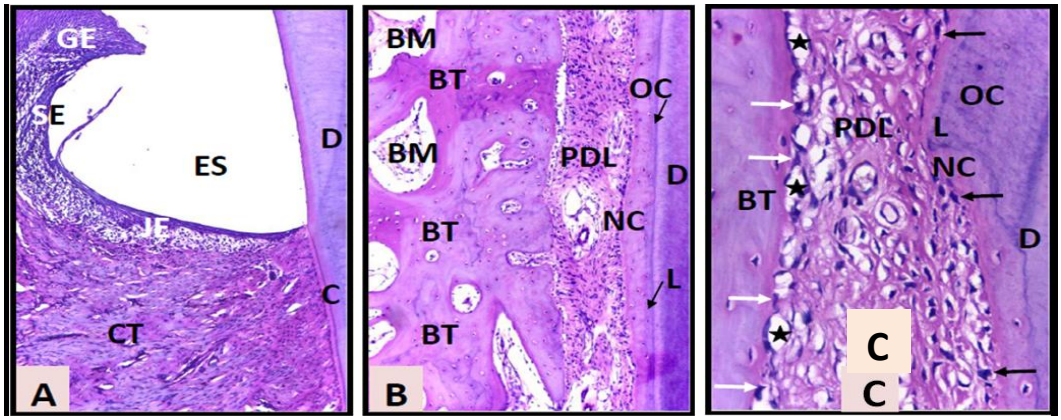

Figure. 4 Photographs of a group III (Ag-HA nanocomposites treated group) showing advanced regeneration of periodontal tissues. **A.** Normal architecture of DGJ with mild inflammatory cell infiltration. **B.** Uniform thickness of the PDL with high vascularity and mild inflammatory cell infiltration. Most of the graft particles is replaced by bone trabecula (BT) surrounding bone marrow spaces (BM). **C.** The PDL with high vascularity. Irregular new cellular cementum (NC) lined by active cementoblasts (black arrows) and separated from old cementum (OC) by line of demarcation (L). Newly formed bone trabeculae (BT) lined by active osteoblasts (white arrows) and repetitive pattern of blood capillaries (stars). Dentin (D), connective tissue (CT), enamel space (ES), gingival, sulcular, & junctional epithelium (GE,SE, JE), (H&E orig. mag., **A & B**×10, **C**×40).

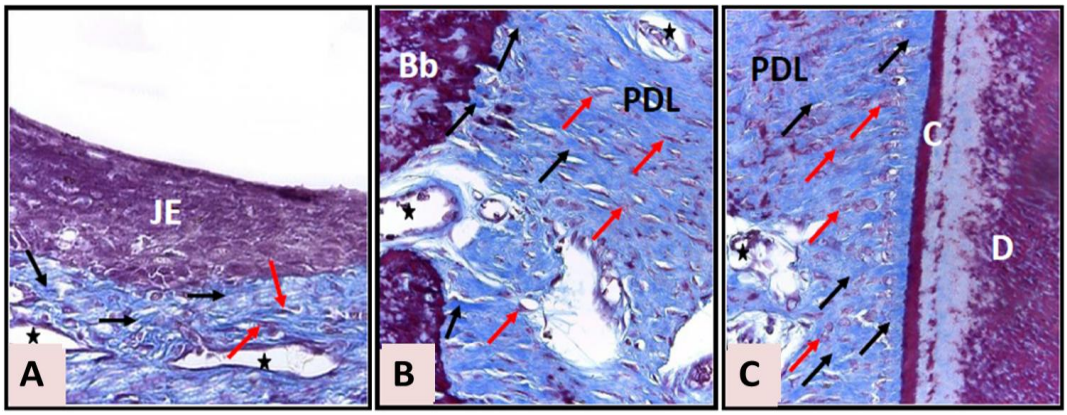

Figure. 5: Photographs of a group IA (negative control group) showing. **A.** Normal organization of moderate staining of fine and coarse collagen fibers (black arrows) with a variable number of fibroblasts in C.T (red arrows). **B&C.** moderate staining of well organized PDL fibers (black arrows) with fibroblasts (red arrows) lying on their long axis. Junctional epithelium (JE), Blood vessels (stars), dentin (D), cementum (C). (MT orig. mag., **A,B, & C** ×40).

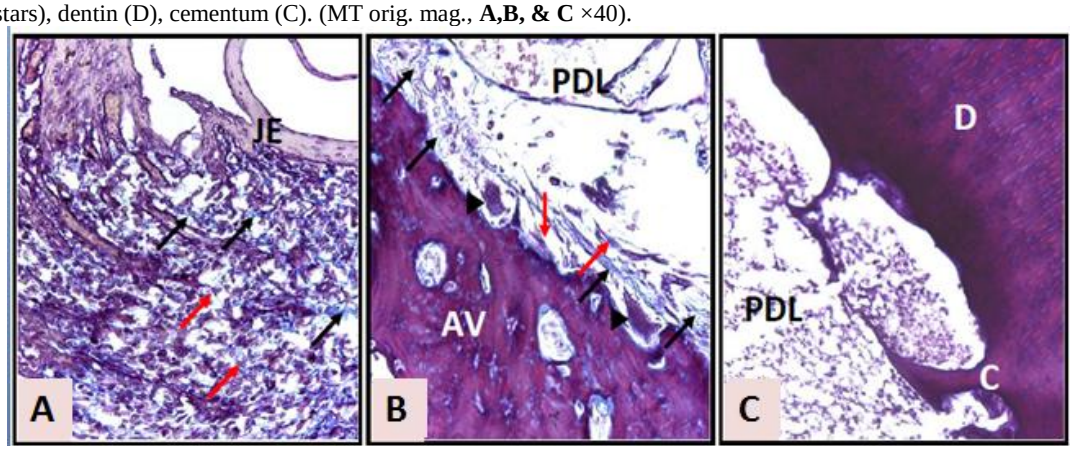

Figure. 6: Photographs of a group IB (periodontitis group) showing. **A.** Weak staining of fine less organized collagen fibers (black arrows) with weak fibroblasts proliferation (red arrows). **A&B.** Loss of PDL organization with weak staining of fine collagen fibers (black arrows) and weak fibroblasts proliferation (red arrows). Alveolar bone (AV), osteoclasts (black arrow heads), cementum, dentin (D), Junctional epithelium (JE) (MT orig. mag., **A, B**, **& C** ×40).

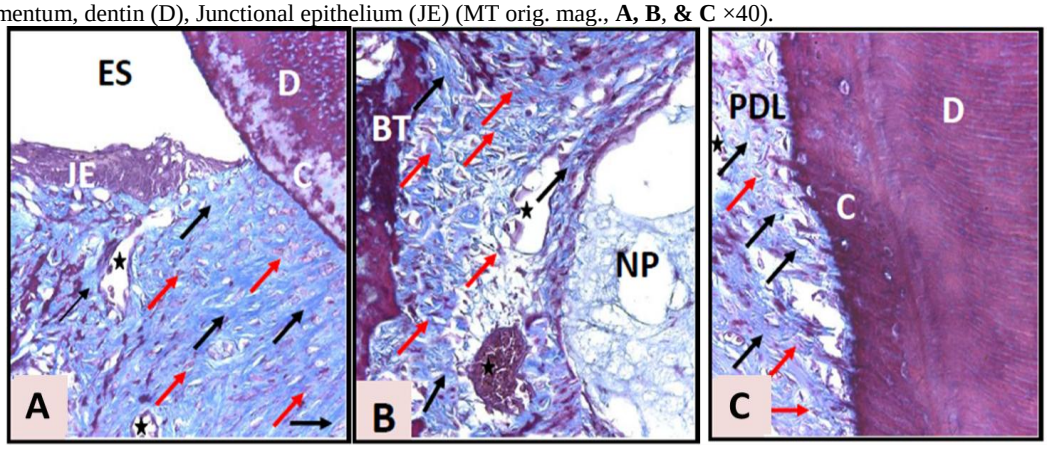

Figure. 7: Photographs of a group II (HANP treated group) showing. **A.** Well organized C.T stroma beneath the junctional epithelium (JE) with moderate to intense staining coarse collagen fibers (black arrows), a variable number of fibroblasts (red arrows **B.** Moderate staining coarse collagen fibers (black arrows) with a variable number of fibroblasts (red arrows) in between HANP and bone trabeculae (BT). **C.** Moderate to intense staining coarse collagen fibers (black arrows) of PDL with a variable number of fibroblasts (red arrows). Dentin (D), new cellular cementum (C), enamel space (ES), blood vessels (stars) (MT orig. mag., A&B×40).

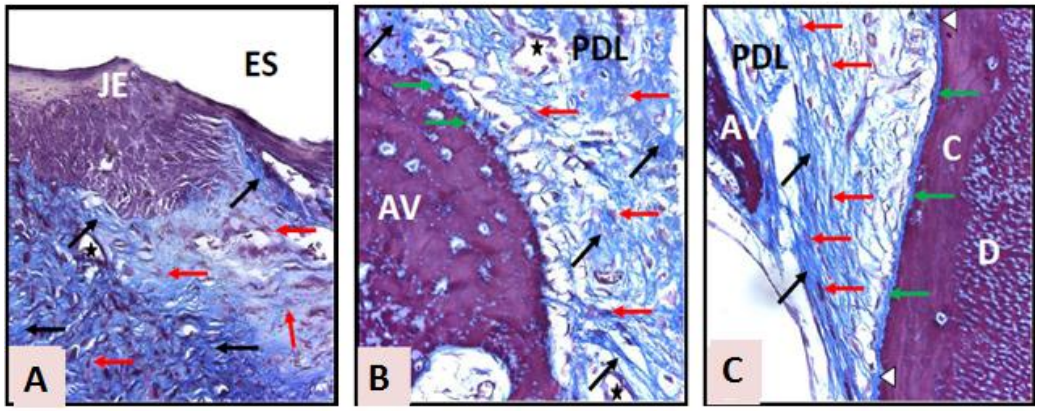

Figure 8: Photographs of a group III (Ag-HA nanocomposites treated group) showing. **A.** Normal C.T stroma beneath the junctional epithelium (JE) with intense staining coarse collagen fibers (black arrows), and a variable number of fibroblasts (red arrows). **B&C.** Moderate staining of PDL collagen fibers with normal arrangement and a variable number of fibroblasts. Intense staining of organic layer lining the alveolar bone (AV) and new cellular cementum (C) surfaces (green arrows). Dentin (D), enamel space (ES), blood vessels (stars) (MT orig. mag., **A, B, &C**×40).

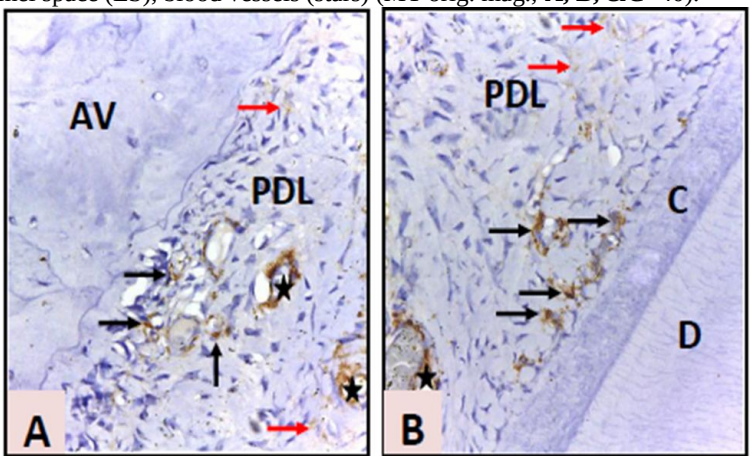

Figure. 9: Photographs of a group IA (negative control group) showing weak OPN immunostaining reaction of extracellular matrix of PDL (red arrows). **A.** Mild OPN expression in localized areas (black arrows) adjacent to alveolar bone surface (AV). **B.** Mild OPN expression in localized areas (black arrows) adjacent to cementum surface (C). Intense reaction around the blood vessels (stars). Dentin (D) (DAP orig. mag., **A & B** ×40).

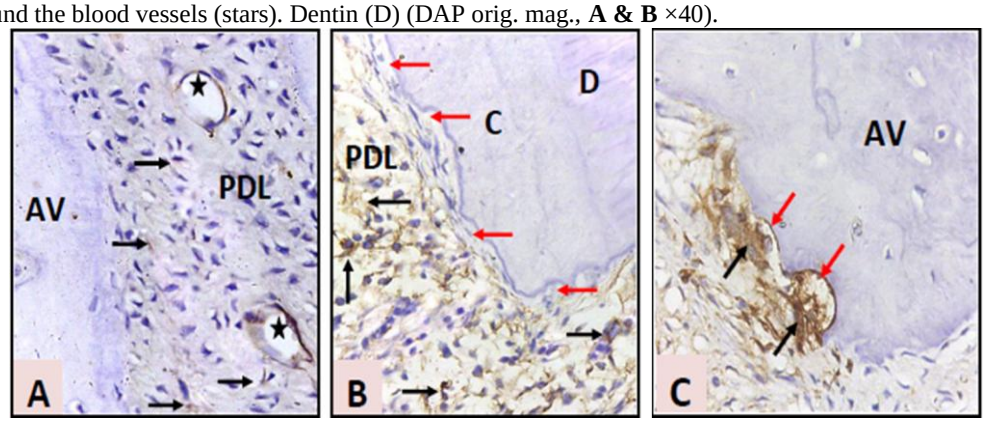

Figure. 10: Photographs of group IB (periodontitis group) showing **A.** Weak to mild OPN expression in the PDL extracellular matrix in localized areas (black arrows) adjacent to alveolar bone surface (AV). **B.** Mild OPN expression in the extracellular matrix (black arrows) in areas next to cementum (C). Negative reaction in the extracellular matrix (red arrows) close to the resorption areas of cementum. **C.** Intense OPN expression in the osteoclasts (black arrows) and its resorption lacunae (red arrows). Intense reaction around the blood vessels (stars). Dentin (D) (DAP orig. mag., **A, B,& C** ×40).

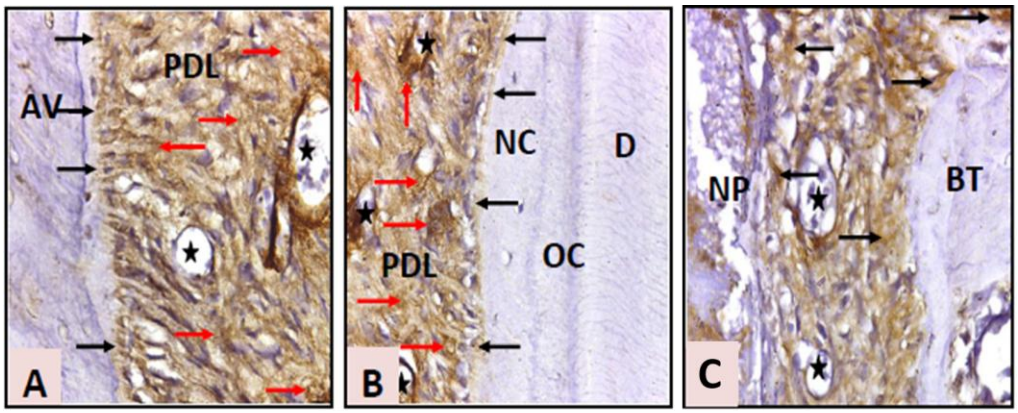

Figure. 11: Photographs of a group II (HANP treated group) showing **A.** Intense OPN expression in the extracellular matrix (red arrows) of PDL with moderate reaction of osteoid layer (black arrows) lining the new bone. **B.** Intense OPN expression in the extracellular matrix of PDL (red arrows) with moderate reaction of cementoid layer (black arrows) lining the new cementum. **C.** Intense OPN expression in the extracellular matrix (black arrows) surrounding the HANP (NP) and Bone trabeculae (BT) Intense reaction around the blood vessels (stars). Dentin (D), alveolar bone (AV), old cementum (OC), new cementum (NC) (DAP orig. mag., **A,B& C** ×40).

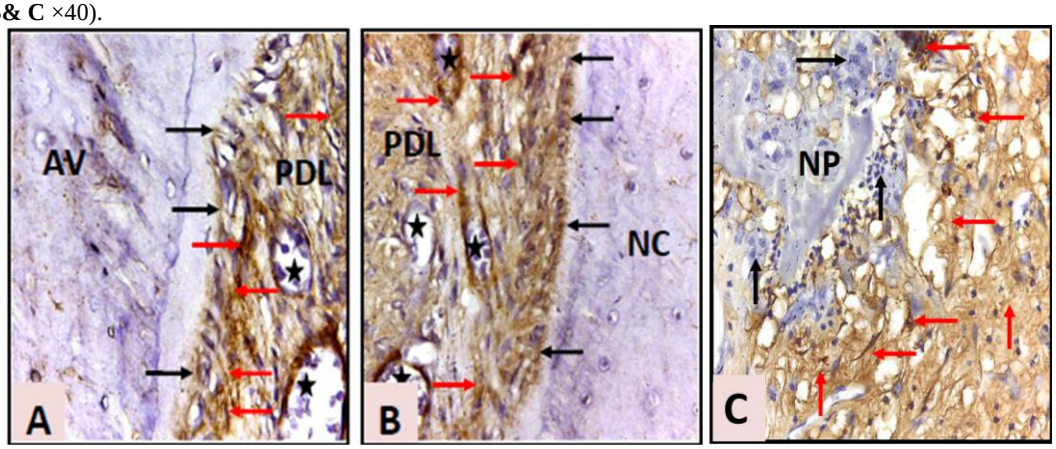

Figure. 12: Photographs of a group III (Ag-HA nanocomposites treated group) showing **A.** Intense OPN expression in the extracellular matrix (red arrows) of PDL with mild reaction lining the osteoid layer (black arrows) of the new bone. **B.** Intense OPN expression in the extracellular matrix (red arrows) with mild reaction lining cementoid layer (black arrows) of the new cementum. Intense reaction around the blood vessels (stars).**C.** Intense OPN expression (red arrows) surrounding area of high cellularity (black arrows) close to remnants of the graft nanoparticles (NP). Alveolar bone (AV), new cementum (NC) (DAP orig. mag., **A, B& C** ×40).

4.Discussion

The main hypothesis of the present investigation are, first, HANP may promote periodontal regeneration in dogs with induced periodontitis, second, the presence of AgNP with HANP in the form of Ag-HA nanocomposites may enhance periodontal regeneration through the newly developed properties of this nano-material.

The experimental model of periodontitis employed in the current study was induced by using sublingual cotton ligatures. To induce chronically inflamed periodontal tissues similar to the situation in humans. The use of ligatures for induction of experimental periodontitis elicits periodontitis after one month through two combined mechanisms: an inflammatory process induced by increased microbial biofilm and plaque formation around the cervix of the teeth and an acute physical irritation factor as a consequence of the subgingival placement of the ligature.[31] Other studies on periodontal healing in animals used surgically created acute periodontal defect models without previous bacterial contamination. [32,33] In contrast, it is known that the integrity of cementum is chemically altered by disease.[34,35]

In the current study, histological examination of GP IB (periodontitis group) showed degradation of periodontal C.T and alveolar bone resorption by osteoclasts, these findings are in agreement with Di Paola *et al.*, (2011) [36] and Graves *et al.*, (2008) [37]. As the previous studies had reported,[38-40] we also observed a significant increase in the inflammatory infiltration, attachment loss, and root resorption by multinucleated giant cells, so-called odontoclasts. This

resorptive activity has already been described previously by different studies.[41-43] The disturbance of the cementum is thought to result in a weakened PDL attachment, which is more susceptible to infection, by periodontal pathogens.[44]

In the present study, histological observations of periodontal soft tissues results of GP II showed soft tissues regeneration which become advanced GP III, where DGJ reorganized, DGJ epithelium appeared with no proliferation or migration and moderate inflammatory cell infiltration. These results revealed that HANPs may have a therapeutic benefit on periodontal epithelium as discussed by Kawai *et al.*, (2011)[45], they hypothesized that calcium-based nanoparticles can acutely decrease open wound size via contracture. This effect is mediated by the release of ionized calcium into the wound bed, which occurs when the pH-sensitive nanoparticles disintegrate in the acidic wound microenvironment.

These results of dentogingival regeneration of GPs II & III were supported by other studies as, Kim1 *et al.*, (2007) [46] who evaluate the amount of new periodontal attachment clinically by measuring the probing pocket depth, also Nyman *et al.*, (1982) [47] who suggests that new attachment can be achieved by cells originating from the PDL.

The reduced inflammation in GP III can be related to the fact that the antibacterial activity of Ag nanoparticles within Ag-HA nanocomposites which play an important role in subsiding of inflammation. This suggestion is confined with those reported by Nadworny *et al.*, (2008) [48] when he found that AgNPs had direct anti-inflammatory effects and improved the healing process in porcine model of contact dermatitis also, it is in accordance with Lansdown, (2002) [49] and Castellano *et al.*, (2007) [50] who attributed the antibacterial activity of Ag ions to its highly reactive as it binds to tissue proteins and brings structural changes in the bacterial cell wall and nuclear membrane leading to cell distortion and death. Silver also binds to bacterial DNA and RNA by denaturing and inhibiting bacterial replication.

The advanced regeneration of DGJ in GP III can be related to the effect of AgNP on triggering the differentiation, maturation, proliferation and migration of keratinocytes, as suggested by Liu *et al.*, (2010) [51] and Liu *et al.*, (2013) [52]. They studied the skin wound healing by using AgNP, and concluded that topical application of AgNPs, accelerated the re-epithelization process during wound healing. Although at this point, the underlying mechanism through which silver operates is still unclear. One possibility may be the Notch signaling pathway, which is one of the most conserved and commonly used communication channels in animal cells. Studies have demonstrated that this pathway is indispensable for cells in various stages of maturation, including terminal differentiation of keratinocytes.[53, 54] Indeed, there is also a possibility that AgNP can promote the differentiation and maturation of keratinocytes through the stimulation of skin stem cell.[55,56]

Regarding periodontal hard tissue regeneration, the GP II showed hard tissue regeneration which appeared more pronounced in GP III. The newly formed cellular cementum appeared irregular in previously reabsorbed areas and there was a line of demarcation between new cellular cementum and old acellular cementum. The most of the graft particles were completely replaced by advanced bone formation in GP III, but in GP II still amount of the graft particles present surrounded by regenerated bone tissue..

Zhang *et al.*, (2007) [57] &Yang *et al.*, (2013) [58], found that HANP-coated scaffolds can be considered to be potentially useful biomaterials for periodontal regeneration. Also, Tala *et al.*, (2013) [59] observed that using of a HANP-Polylactic acid composite material containing a high concentration of HANP may be a useful material for GTR membrane as it will not only act as a barrier, but may also be able to enhance bone regeneration by delivery of biologically active molecules.

On contrary, Lee *et al.*, (2012) [60], when he studied the periodontal tissue reaction to customized nano-HA block scaffold in one-wall intrabony defect in dogs. He found minimal bone regeneration within the defect sites that received the nano-HA block. His exegesis for the minimal bone formation at the experimental sites can be attributed to two factors: absence of a barrier membrane and the slow biodegradation process of the nano-HA block.[61]

The study results indicated that the HA appears to have a specific effect on osteoinduction. As, it was seen surrounded by active new bone spicules and osteogenic cells. This result is confirmed by Wang *et al.*, (1999) [62]. He found that HA promote bone reconstruction and providing support for the formation of microvessels and the attachment of host osteocytes in the early stage of bone defect repair. The stimulation function of HA crystal makes osteocytes active and forms zones of ossification around HA particles, namely, polycentric osteogenesis. HA can provide crystal nuclei to accelerate osteogenesis in the course of bone mineral deposition.

In addition to, many studies reporting the inductive effect of HANP on PDL cells appeared. Shimauchi *et al.*, (2013) [63] found that HANP may regulate the differentiation of human PDL cells via a mechanosensitive signaling pathway and enhance the expression of BMP-2 in PDL cells. This novel mechanism of the action of HANP may offer the promise of new strategies for bone and periodontal

tissue engineering. Lock & Liu, (2011) [64], their results indicated that incorporation of HANP into nanocomposite scaffolds could promote both adhesion and osteogenic differentiation human mesenchymal stem cells similar to a short peptide of BMP-7. Moreover, Zhou *et al.,* (2007) [65] found that amount of total proteins detected from cells cultured on HANP films was significantly higher than that on conventional HA films.

The histochemical results of MT stain in GP IA revealed proper arrangement of mild to moderate staining of fine and coarse collagen fibers with active fibroblasts in gingiva and PDL. On contrary GP IB revealed mild staining of fine collagen fibers with weak fibroblast proliferation in gingiva with nearly absent of collagen fibers adjacent to cementum resorption areas. These results confirming the H&E results, where the deterioration of normal periodontal apparatus structure appeared after inducing of periodontitis which appear clear in the mild staining of collagen fibers and their absence adjacent to cementum resorption areas in GP IB. These findings are confined with previous studies.[66,67]

The H&E histological results of periodontal regeneration are confirmed through the MT histochemical stain. As the results of GP II revealed moderate to intense staining coarse collagen fibers with a variable number of fibroblasts were seen. The collagen fibers became well oriented with intense staining coarse fibers with a variable number of fibroblasts in GP III. These results demonstrate enhancement of regeneration of C.T collagen fibers and fibroblast proliferation in Ag-HA nanocomposites treated dogs (GP III) more than HANP treated dogs (GP II).

These findings indicated that NP of the two graft materials affect the healing of DGJ connective tissue directly or indirectly via collagen synthesis and fibroblast proliferation during granulation tissue formation and the early tissue remodeling phase of periodontal healing and regeneration. This explanation appears to be corroborated with the observation of many previous studies.[25,112] In addition to, fibroblasts have been known to play important roles in reepithelization, collagen fiber synthesis, extracellular matrix regeneration, remodeling of wounds, and for the release of such endogenous growth factors as FGF, PDGF, TGF-β, and VEGF, as reported by different studies.[67-69]

Regarding the advanced C.T regeneration in GP III, the presence of AgNPs may have a key role in this advancement. Through preparing an inflammatory free environment which enhance fibroblast maturation and proliferation. This explanation was supported by Liu *et al.,* (2010) [51], as he reported that AgNP could stimulate cell fibroblast maturation and proliferation. In addition to, Liu *et al.,* (2013) [52] revealed abundant collagen in skin wound healing by AgNP. Furthermore, his morphological observation showed that the microstructure of collagen in AgNPs-treated healed skin demonstrated more organized and compact collagen fibrils alignment, and showed the closest resemblance to normal skin.

Regeneration of PDL in GP III was more advanced than GP II This observation appeared in the two follow up periods of the study. This confirm the role of AgNP in fibroblast maturation and proliferation as supported by other previous studies.[51,70,71] In addition to, These regeneration of PDL are supported by different in vitro studies; Kasaj *et al.,* (2008) [72] found that PDL cells (PDLCs) were able to proliferate in the presence of nano-HA paste; Kasaj *et al.,* (2012) [73] results indicate that nano-HA paste is a strong stimulator of PDL attachment. Furthermore, his data suggest that nano-HA paste acts as a stimulator of cell migration and proliferation. The data of the current study and their supporting in vitro studies strongly suggest that the PDLCs specially fibroblasts play a key role in periodontal tissues regeneration.

Regarding the immunohistochemical results, the periodontium of GP IA revealed weak OPN expression. However, GP IB (periodontitis group) revealed weak to mild OPN expression. These findings are in consistent with those of Jäger *et al.,* (2008), [74] he found a few cells in the PDL showing immunoreactions of OPN in cases of periodontits. while the result of OPN in GP IA disagree with Ivanovski *et al.,* (2001) [75] as he found strong OPN expression in the extracellular matrix of the healthy PDL. While in the extracellular matrix and the cells of the gingival C.T stroma OPN expression was weak as in the present study.

Although GP IB revealed weak OPN expression, the osteoclasts and its resorption lacunae revealed intense OPN expression as reported by Christgau *et al.,* (2007) [76]. This means that OPN has a role in facilitating the adhesion of osteoclasts to the bone and it resorption. This explanation was confirmed by Chellaiah *et al.,* (2003) [77], who found that OPN has functions in osteoclast migration to sites of resorption and it is crucial for normal resorption and bone turnover. In addition, Giachelli and Steitz, 2000 [78] reported that OPN is a potent regulator of clastic cells via interaction with their integrin receptors influencing cell adhesion and activation.

Regarding immunohistochemical results of GPs II & III, intense expression of OPN was appeared. The relation of increase OPN expression with healing and regeneration conditions and decrease with periodontitis as reported in many studies.[74,79-81] Sculean et al.,2002 [81] demonstrated that the expression

of OPN was stronger in the regenerated tissues than in the intact ones. Sculean *et al.*, (2003) [80] following surgical periodontal therapy found that OPN expression was intense at the border near the newly formed cementum and bone as revealed in the current study.

Moreover, the present study revealed moderate reaction of OPN expression in osteoid layer and cementoid layer lining the new bone and the new cementum respectively. Provides further evidence that this molecule plays an important role in the mineralization and/or remodelling process of root cementum and alveolar bone as proved by Christgau *et al.*, (2007) [76]. The primary event of new cementoid deposition was a strong accumulation of OPN along the root surface. This corresponds to previous observations in cementogenesis and cementum regeneration. [39,40,42,82] Although the exact role of OPN in cementum formation is not yet known, it seems to promote cell attachment to the root surface.[76]

Qualitative results of the immunohistochemical expression was confirmed by the quantitative results. The statistical analysis of OPN expression among the all GPs revealed that OPN expression increased significantly in GP II with the least value obtained in GP IA and GP IB. These results confirm the qualitative data, where the treatment with graft NP showed significant levels of periodontal regeneration. This means once an environment for periodontal regeneration has been created, the expression of extracellular matrix molecules associated with the healing process displays the same pattern irrespective of treatment modality as stated by Sculean *et al.*, (2002) [81]. These results also supported by; Lao *et al.*, (2006) [35] study who showed lack of OPN staining along previously diseased human root surfaces and discussed a possible negative influence on the ability for periodontal regeneration.

Conclusions

Based on the results of the present study, it can be concluded that, recent advances in nanotechnology currently enable the design of HANP and AgNP which play an important role in periodontal regeneration. HANP induce the key elements of the true periodontal tissue regeneration. The periodontal regeneration induced by HANP could be enhanced aggressively by a combination of AgNP & HANP, considering the limitations of this study, offering potential as therapeutic material in periodontal regeneration.

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