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Original Article

Histomorphometric Analysis of Bone Formation After Using Simvastatin Chitosan Nanoparticles as a Local Delivery System in Periodontal Bony Defects in Rabbits

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Abstract

Background: simvastatin (SV) has a characteristic effect on the regeneration of bony tissues and the decrease of bone resorption. Accordingly, this study was conducted to explore the effect of locally applied simvastatin loaded in a chitosan nanoparticles as a delivery system for regeneration of bone in intrabony defects in a rabbit model.

Materials and methods: This trial was carried out on 20 male albino New Zealand rabbits. The rabbits were randomly distributed into 4 groups where each group contained 5 rabbits (n=5) as follows: Group 1 (NC group) designed as the negative control where the rabbits had not undergone any intervention conversely interventional groups where an intrabony defect was created designed as: Group 2 (PC) the positive control where the intrabony defects were covered with gingival flaps only. Group 3 (NP) and group 4 (SP) were received chitosan tripolyphosphate nanoparticles (*CS-TPP* NPs) and medicated SV -CS-TPP NPs respectively. All the animals were euthanized after 6 weeks. Descriptive histological and histomorphometric analysis of specimens stained with hematoxylin-eosin (H & E) and Masson's trichrome stain (M T) was done.

Results: The histomorphometric analysis of area percent of newly bone formation in mm^2 showed statistically significant differences between interventional study groups. SV -CS-TPP NPs treated group showed the highest mean of area percent of newly formed bone 37.33 ± 3.82, Group 3 (NP) 18.57 ± 3.42 , while group 2 (PC) showed the lowest one 6.21 ± 2.30.

Conclusion: Chitosan nanoparticles as a carrier for local application of simvastatin optimizing the new bone formation in intrabony periodontal defects.

Keywords: Simvastatin; Chitosan; Nanoparticles; Bone regenerations; Animal; Bone area

Introduction

The alveolar bone resorption, which decreases the bone height and width needed for placement of dental implant, contributes to problems in restorative dentistry and esthetic encounters clinically in treatment by dental implant [1]. Several therapies have been used to enhance bone regeneration, for instance

medications in the formula of minute molecule, bone tissue engineering and bone replacement usage [2].

It has been documented that statins, particularly simvastatin (SV), a type of small molecule drug and a recognized 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor that is mostly utilized to reduce blood serum levels of cholesterol [3], had a great impact on new bone development via enhancing the expression of the bone morphogenetic protein-2 (BMP-2) gene in bone cells and enhancing the blood vessels formation by indirect increase the discharge of vascular endothelial growth factor (VEGF) [4,5]. Noteworthy investigation fulfillment received confirmed that SV has a distinctive influence on the improvement of bone development and the decrease of bone resorption and fracture hazard [6, 7].

Nevertheless, usage of statins clinically is challenging due to the decreased systemic availability (~2%) and severe adverse reactions for example toxic effect on the liver, acute hepatic failure and occurrences of myalgia [8]. Therefore, local delivery through evading metabolism in the liver could release greater drug amounts at the bone and lessen the unwanted reactions [9].

Though, delivery of simvastatin to bone in order to affect osteoblasts and osteoclasts is challenging [10]. In added context, topical use of simvastatin devoid of regulation for the optimum concentration to be released may have an insignificant wanted outcome or prompt inflammatory reaction [11]. While several materials have been utilized for simvastatin loading, for instance a poly (l-lactic acid)/gelatin fibrous scaffolding [12] and a calcium sulphate scaffolding [13] that have superior effect in engineering of bone tissue, the difficult manufacture procedures, cost, and absence of antibiotic property hinder their local application orally.

Therefore, utilizing scaffolding with biocompatible and antimicrobial activities, which has an organized discharge role and is laden with the poorly soluble medication simvastatin [14], as a local delivery carrier for bone formation can facilitate the drug delivery procedure.

Biodegradable polymers have been highly considered in the construction of drug delivery systems with sustained release property [15] due to their ability for chemical handling and potential to dissociate into pieces that can easily be degraded [16]. Chitosan (CS) has been widely evaluated in medicinal uses due to its antimicrobial , biocompatibility, biodegradability, and minor harmfulness properties [17].

Chitosan nanoparticles (CSNPs) can be gained through ionic crosslinking interactions between negatively charged ions on sodium tripolyphosphate (TPP) and the protonated amine groups on chitosan. Several trials have got heparin-loaded CSNPs through the microemulsion technique, and the resulted particles demonstrated controlled drug discharge following changing into the chitosan/TPP molar percentage [18]. Furthermore, the earlier investigation established that chitosan can be manufactured into membranes for usage in guided bone regeneration (GBR) [19]. By means of a barrier membrane, chitosan membrane has the moderating capability to actually exclude tissues of non-osteogenic origin related to hindered bone healing that give rise to inadequate bone development [20], however it is not adequately osteoconductive by itself to stimulate prompt regeneration of bone during the early period of healing [21]. Medications that have the capacity to stimulate bone development may increase chitosan effectiveness in bone regeneration in a synergistic way.

A previous study indicated that a local delivery system of poly (d,l-lactide-co-glycolide

acid)-lovastatin-chitosan-tetracycline nanoparticles might be useful adjunctive therapy for periodontal regeneration [22].

In our previous study [23], we mainly reported the radiographic changes in the bone after using simvastatin chitosan nanoparticles for bone regeneration in periodontal intrabony defects. However, the current research was carried out to investigate the histomorphometric analysis of newly formed bone following application of this nano-delivery system in a rabbit model.

Materials and Methods

Materials

Simvastatin (SV) was a kind gift (Global Napi Pharmaceuticals, Egypt). Low molecular weight chitosan (CS) and tripolyphosphate (TPP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). All other chemicals were of pure analytical grade.

Preparation of SV CS-TPP nanoparticles by ionic gelation technique

SV CS-TPP nanoparticles were formulated adopting the ionic gelation method using TPP as a gelation agent [24]. Saturated SIM ethanolic solution (5 mg / 0.5 mL) was added to 9 mL CS solution in 1% v/v acetic acid (0.34%, pH 3.5). Then, 1 mL of cross-linking agent TPP solution (1.15%) was added dropwise followed by homogenization for 8 min at 12,000 rpm (UltraTurrax→ T-25, IKA, Germany) then stirring was continued at 600 rpm (WiseStir→ MSH-20D, Daigger, USA) for 2 h. The formed nanoparticles (NPs) were then separated by centrifugation (Sigma Laborzentrifugen→ 1-14, Germany) at 18,000 rpm for 20 min [23].

The size of the SV CS-TPP was in the nano range (106 nm), with a zeta potential of +43.3 mv that conferred stability to the formula. The drug entrapment efficiency in the NPs in phosphate buffered saline (pH = 7.4) was high (98.78%), whereas the drug release pattern from the NPs was controlled over 14 days that guaranteed the simvastatin delivery in a therapeutic dose needed to support bone regeneration [25].

Animals

Ethical approval of the current study protocol obtained from Cairo University Institutional Animal Care and Committee (CU-IACUC) and was registered with (CU III F 85 19) code.

This study was performed on 20 male albino New Zealand rabbits ranging in age from 3-4 months, and weighting 2-2.5 kg approximately. The rabbits could access freely the food to chow diet and drink water during the duration of the research conduction. Before starting the study, two weeks was allowed as a period of accommodation for the animals. In isolated cages, the rabbits were retained under constant conditions of 60 ± 10 % humidity, 25 ± 2°C temperature and a 12/12-h light/dark sequence following the guidelines for the care and use of laboratory animals (Institute of laboratory animal resources, 1996). All the designed experimental steps were performed at the animal housing unit at Faculty of Pharmacy, Cairo University.

Study design

The rabbits were randomly allocated into 4 groups where each group contained 5 rabbits (n=5) as follows: Group 1 (NC group) served as the negative control where the rabbits did not receive any intervention however rabbits in Group 2 (PC) served as the positive control where the created bony defects were only covered with gingival flaps. The animals' created bony defects in group 3 (NP) and group 4 (SP) were treated with non-medicated (CS-TPP NPs) and medicated NPs (SV CS-TPP NPs) respectively. Euthanasia of all animal was attempted after 6 weeks.

Surgical protocol

The surgical procedures for the creation of bony defects in the animals were done in accordance with a previous study conducted by Lee et al., 2016 [22]. First, Animals were anesthetized using a combination of ketamine chloride [Ketam 500 mg vial, manufactured by Egyptian Int. Pharm. Ind. CO] (35 mg/kg) and xylazine [Xyla-ject 25 mg vial, ADWA CO. S.A.E 10th of Ramadan City Egypt] (5 mg/kg) injected intramuscularly. The upper left first premolar was locally anesthetized using local anesthesia to decrease local bleeding (Mepivacaine HCl 2%, Levonordefrin 1:20000, 1.8 mL). Following the elevation of buccal & palatal mucoperiosteal flaps, the three-wall unilateral intra bony defect was done at the mesial surface of upper first premolar utilizing size- 4 round bur at low speed under irrigation with sterile saline. The defect depth was standardized to be (4×4×5 mm) regarding bucco-lingual, mesio-distal dimensions and depth apical to the alveolar crest, respectively. Bony defects of rabbits in group I were not subjected to these surgical procedures or any intervention. Rabbits in group II were only covered with gingival flap. Meanwhile rabbits in group III and group IV received non-medicated CS-TPP NPs and SV CS-TPP NPs respectively. Tension-free wound primary closure was accomplished following repositioning and suturing using resorbable suture (Vicryl®5-0; Ethicon Products, Amersfoort, The Netherlands).

All animals were placed on analgesic (Ketofan® 2mL amp., manufactured by Amriya Pharm. Ind., Alex., Egypt) and (Flumox®, 500 mg vial, manufactured by Egyptian Int. Pharm. Ind. CO) for postoperative pain and infection control respectively given intramuscularly along the 3 days after the surgery. Lettuce was used as the nutrition during this period to decrease to effect of the surgical procedures. All wounds were treated topically by 2% chlorhexidine gluconate three times per week throughout the study period.

Histological preparation

All the animals were sacrificed 6 weeks postoperatively by overdose of ketamine injected intramuscularly. The upper jaw was separated, and then the operated posterior area in the upper jaw including teeth, bone, and soft tissue was harvested and fixed in 10% neutral buffered formalin (4% formaldehyde in phosphate buffered saline). Then the specimens were decalcified using EDTA 125 gm in one liter distilled water and sodium hydroxide as a buffer for 3 weeks.

The samples were then washed, dehydrated in ascending grades of ethyl alcohol and finally treated by a clearing agent as xylol followed by embedding in paraffin.

Then, serial sections 5μm representing the entire upper left first premolar from coronal to apical aspects in the buccal-lingual plan, throughout the mesial- distal extension of the tooth were prepared for histological staining. Hematoxylin-eosin (H & E) were used to stain these sections. In addition, Masson's trichrome stain (M T) were used to stain some sections.

After examining the sections throughout the whole defect using light microscope, 3 tissue sections were selected from the central aspect of the defect for histomorphometric analysis.

Histomorphometric analysis:

The Histomorphometric measurements were performed by means of a light microscope equipped with a computer-assisted image analysis system. The following measurements were recorded for the regenerated bone on the mesial surface of the upper first premolar by a senior oral pathologist in Oral Pathology Department, Faculty of Dentistry, Cairo University who was masked about the slides of the groups being evaluated.

The area percent of the newly formed bone was measured with Leica Quin 500 analyzer computer system, (Leica Microsystems, Switzerland). To outline the areas of bone trabeculae, the cursor was used , then the outlined areas were marked by a green binary color that could be measured by the computer. The measured

units (pixels) converted into actual micrometer units by the image analyzer program . Then, area percent of the newly formed alveolar bone trabeculae were measured in 10 different fields in each group using magnification (· 200). Mean and standard deviation (SD) values were calculated for each group.

Statistical analysis

Statistical analysis was done using Statistical Package for Social Sciences, Version 18.0 for Windows. Continuous variables were analyzed as mean values ± standard deviation (SD) in case of normal data.

The analysis of variance (ANOVA) test was used to compare the mean of area percent of newly formed bone between study groups. Tukey post hoc analysis was done for pairwise comparison of the study groups. P value of ≤0.05 was considered statistically significant.

Results

The present study was conducted on 20 male albino New Zealand rabbits where all of the animals continued the duration of the study without any drop out or complications.

Descriptive histology

In group 1 (NC) where the animals did not receive any treatment or intervention, there was normal periodontal structures, the alveolar bone was composed of bony trabeculae as shown in Figure 1 (A, B). But in group 2 (PC) where the animals had the created bony defects covered by the gingival flap only without treatment, there was an empty defect devoid of bone regeneration activity as presented in Figure 1 (C). In addition to thin bands of fibrous tissue formation in Figure 1 (D).

In group 3 (NP), the created bony defects were treated with non-medicated (CS-TPP NPs) NPs. Bone regeneration was detected at the apical extent of the induced intrabony defect associated with dense fibrous connective tissues along with more narrowing of the defect compared to group 2 [Figure 1 (E,F)]. In case of group 4 (SP), where the induced bony defects were treated with the medicated NPs (SV CS-TPP NPs), numerous communicating bone trabeculae enclosing fibrovascular bone marrow spaces were observed. The newly formed bone trabeculae were dense with highly cellular stroma of the connective tissue [Figure 1 (G, H)].

Histomorphometric analysis:

Regarding the area percent of newly formed bone, the analysis of variance (ANOVA) test done after checking normality of data, it showed that the difference between the means in the groups was statistically significant ($P < 0.001$) (Table 1 & Fig.2).Tukey's post hoc test revealed that there was a statistically significant difference between the interventional groups (G2,G3,G4). While, there was no statistically significant difference between the normal control G1 and the positive control G2. (Table1)

Discussion

Local application of statins in bony tissues combined with several vehicle systems has been investigated in many experimental studies. Simvastatin acts by enhancing osteoblast activity while hindering the osteoclast activity. This happens by stimulating ostoblastic cell differentiation by the bone morphogenetic proteins BMPs (through competition of TNF-a-to-Ras/Rho/ mitogen-activated protein kinase) and enhancement of BMP-Smad activity (BMP-Smad signaling) [26]. Also statins increase alkaline phosphatase activity, the expression of bone sialoprotein, osteocalcin, type I collagen and the anti-inflammatory property by decreasing the formation of interleukin 6 (IL-6) and 8 (IL-8) [22]. Chitosan (CS) has been widely studied in the medical filed because it is a natural biocompatible, biodegradable material with lowered toxic effect and showing an antimicrobial potential.

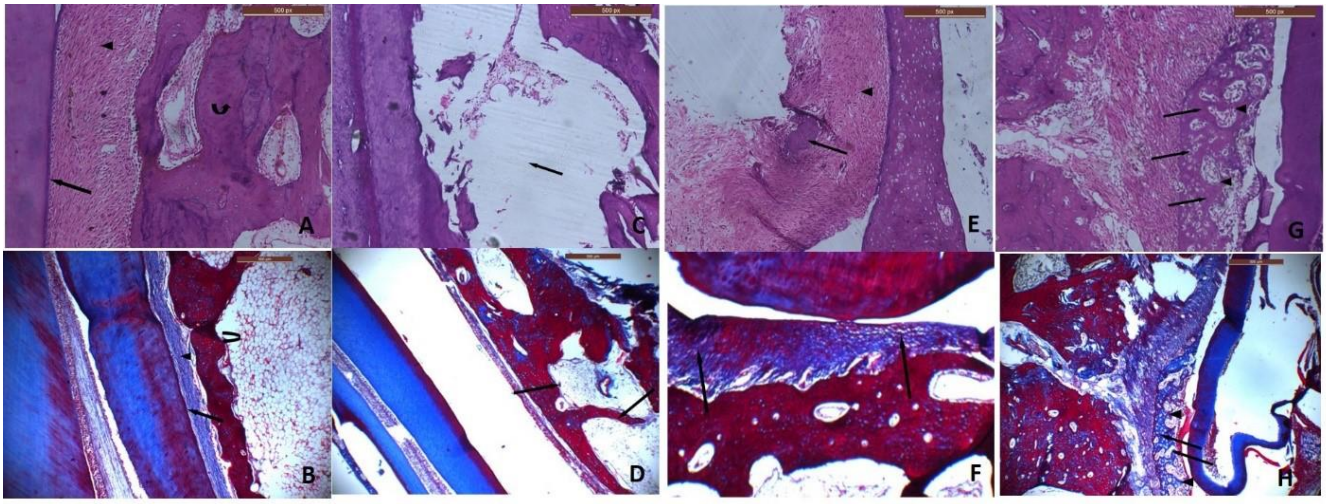

Figure (1): A: Photomicrograph of normal periodontal structures in group 1 (straight arrow = cementum), [arrow head = periodontal ligament (PDL)], and (curved arrow = alveolar bone) (H & E × 100). B: Photomicrograph of normal periodontal structures of experimental tooth (straight arrow = cementum), (arrow head = PDL), and (curved arrow = alveolar bone) in group 1 (MT × 40).C: Photomicrograph showing empty defect in group 2 (straight arrow) (H & E × 100). D: Photomicrograph showing thin bands of fibrous tissues formation (straight arrows) in group 2 (MT × 40).E: Photomicrograph showing newly formed bony spicule (straight arrow) with dense fibrous connective tissues (arrow head) in group 3 (H & E × 100). F: Photomicrograph showing abundant granulation tissues formation (straight arrows) in group 3 (MT × 40). G: Photomicrograph showing multiple interconnecting bone trabeculae (straight arrows) and narrow marrow spaces (arrow heads) in group 4 (H & E × 100). H: Photomicrograph showing multiple interconnecting bone trabeculae (straight arrows) and narrow marrow spaces (arrow heads) in group 4 (MT × 40).

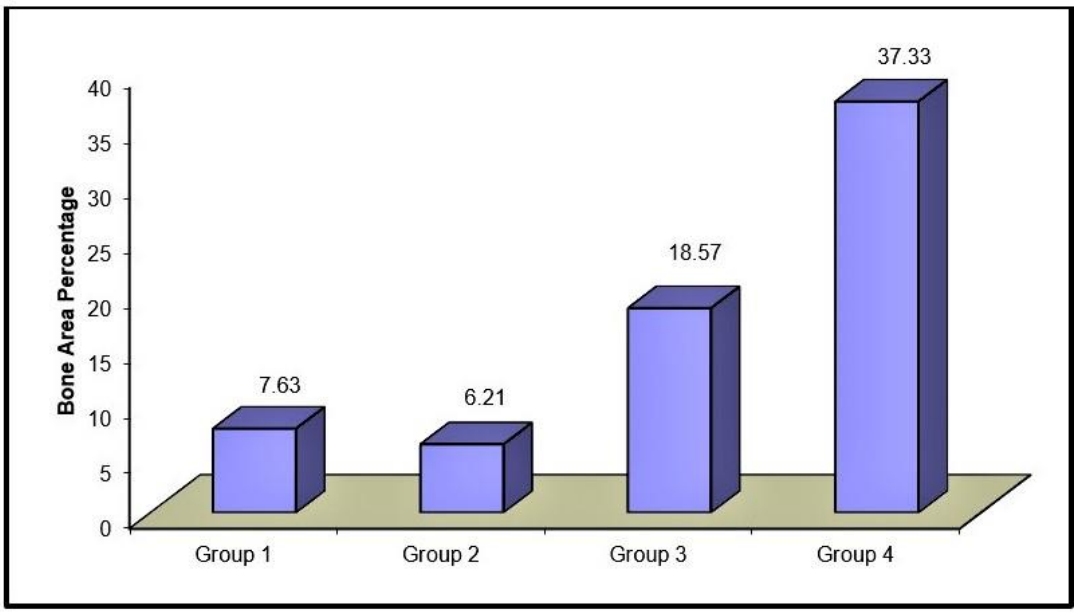

Figure (2): Bar chart showing the mean area percent of the new bone formation the study groups.

Table1: Comparison of mean of area percent of newly formed bone between study groups using one way ANOVA test.

Groups	Mean ± SD in mm^2	Range	P-value
Group 1	7.63^{a} ± 1.65	5.64 – 9.84	
Group 2	6.21^{a} ± 2.30	3.09 – 9.37	<0.001*
Group 3	18.57^{b} ± 3.42	14.57 – 21.92	
Group 4	37.33^{c} ± 3.82	32.05 – 42.67	

*significant difference at ($p<0.05$).
Tukey post hoc test results identified in the table: means with different superscript letters (a, b, c) are significantly different.

Statins have been described by a lot of researchers to possess a local anabolic influence on bones. The optimum dose of statins and the use of the proper vehicles need further research. Smaller doses of statins don't affect bone regeneration, while high doses cause a local inflammatory reaction. Higher local concentrations of statins can be cytotoxic owing to a severe decrease of cholesterol formation, which is essential for the cell membranes integrity [22]. In 2019 Petit et al. in their systematic review studied the role of statins in periodontal therapy, they reported that statins could act as an adjuvant to periodontal treatment. However, further researches are still needed to optimize the recipe of their type, dose, and delivery system could be crucial to attain the optimum treatment response [27].

Accordingly, the current investigation was conducted to study effectiveness of simvastatin chitosan nanoparticles for bone formation in intrabony periodontal defects of rabbits as a local nano delivery system.

In this research the animal model for intrabony defect was described before in several studies [28-30] , ensuring the accessibility and feasibility for surgical procedures.

The results of this study showed that the animals in group 4 (SV CS-TPP NPs) had the highest area of bone formation where it recorded the highest mean value compared to the other study groups. However group 3 non-medicated (CS-TPP NPs) had higher mean value compared to group 2 (PC).

The results of this study are in accordance with the previous study [31] denoted that locally applied simvastatin combined with suitable carrier can be beneficial for bone regeneration in bony defects of rabbits' femurs. In addition the former trial [22] investigated the ability of poly (d,l-lactide-co glycolide acid)-lovastatin-chitosan-tetracycline nanoparticles in bone regeneration in dog model showed significant newly bone formed in the experimental group treated with these nanoparticles compared to the control group did

not treated with the nanoparticles and covered with the flap using micro-CT. Besides, the preceding trial [32] SV loaded -nanostructured lipid carrier in the form of nanoparticles significantly stimulates bone formation in calvarial defect of the rabbit. Furthermore, the recent study [33] reported that 4 mg SV CS NPs formulation enhanced new bone formation compared to other formulations used in rats cranial defect model.

The results of the present investigation are in contrast with the earlier study [34] examined simvastatin combined to chitosan nanofiber membranes for regeneration of bone in calvarial defects of rats which revealed no statistically non significant differences in results of new bone formation between control group received non-loaded chitosan membranes and experimental group received chitosan membranes demonstrated by the histomorphometric and micro-CT analysis. This can be explained by the difference in the type, dose of defect and animal model between the two studies.

We can explain the bone formation capacity of chitosan in our study that was noted the results of group 3 CS-TPP NPs as the chitosan has hydrophilic surface facilitates the adhesion, proliferation, and differentiation of the cellular element. Additionally, it has hemostatic and mucoadhesive properties as its amino groups carry positive charged [35]. In addition, chitosan - a natural polymer – was used for the preparation of the nanoparticles and is known to be a biocompatible and biodegradable polymer. Chitosan biodegradability is essential for being used as a drug delivery system. It is also degradable and its micro/nanoparticles may take place by lysozyme, which is available in human body [36,37].

Poth et al. [38] studied the degradation of chitosan-TPP nanoparticles invitro, in lysozyme solution at 37 °C and CS-TPP , it was found that nanoparticles degrade with a particle size reduction of 40% within 4 days. This may give an explanation to the disappearance of any residual particles in the bony defects after 6 weeks of drug application in the present study.

In contrast to our findings, in previous studies by Alididi et al. and Marei et al. [39,40] revealed that chitosan alone has no osteoinductive or osteoconductive activities, but only if conjugated with other molecules or biomaterials, such as growth factors, hydroxyapatite or mesenchymal stem cells. The difference could be attributed to nanoparticles used in our study, scaling the particles down to the nano size fully exploit the characteristic quantum and surface properties of the material [41].

Future research could investigate the effectiveness of locally applied statins in chitosan nanoparticles as a carrier in different bone defects whether periodontal or with dental implants in different forms according to shape and size of the defect, whether nanoparticle, nanofiber, or a membrane.

Conclusion

Both non-medicated (CS-TPP NPs) and medicated (SV-CS-TPP NPs) were biocompatible and exerted stimulation of new bone formation, however SV-CS-TPP NPs exhibited significant one. Simvastatin chitosan nanoparticles might be used as a local delivery system for enhancement of bone regeneration. Simvastatin fortified the bone regeneration capacity of chitosan nanoparticles and proved the synergistic effect of this promising local nano delivery system in treating periodontal bony defects in rabbits.

Conflict of interest: No conflict of interest.

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Ethics: This study protocol was approved by Cairo University Institutional Animal Care and

Committee (CU-IACUC) and was registered with (CU III F 85 19) code.

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