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Bone Reformative Capability of Nano-chitosan in Rabbits

Thkraa Mahmood Al-bayaty [1], Ziad H. Deleme [2*]

[1] Ministry of Health/ Nineveh Health Directorate / Iraq.
[2] Department of oral and maxillofacial surgery, College of Dentistry, Mosul University / Iraq

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***Correspondence:**
E-mail: Ziaddeleme@uomosul.edu.iq

Abstract

Aims: To evaluate the ability of natural biomaterial nano chitosan for osteogenic regeneration in rabbit animal models. **Materials and methods**: Eighteen male domestic rabbits were used in the study the formation of two bony defects was created in the rabbit's femur, one filled with nano chitosan material, and the other defect was left empty as a control. Densitometry and histomorphometric analysis were measured at, 7 days, 14 days, and 28 days which was used as a parameter. **Results:** The nano chitosan showed lesser radio-opacity than the control with a positive osteogenic effect due to the osteoinduction and osseointegration properties. **Conclusions**: This study supports that nano chitosan natural polymer may have better application prospects for bone repair and acceleration of the healing process.

القدرة الإصلاحية للعظام من الكيتوزان النانوي في الأرانب

الملخص

الأهداف: لتقييم قدرة المادة الحيوية الطبيعية الكيتوزان النانوي على تجديد العظام في نماذج الارانب. **المواد وطرائق العمل:** تم استخدام 18 أرنبًا ذكرا منزليًا في الدراسة الحالية مع احداث عيبين في عظم الفخذ، أحدهما مليء بمادة الكيتوزان النانوي والفجوة الأخرى تركت فارغة كعنصر تحكم. تم استخدام قياس الكثافة والتحليل النسيجي المقاس كمعيار للبحث في 7 أيام و14 يومًا و28 يومًا. **النتائج:** أظهر الكيتوزان النانوي عتامة شعاعية أقل من التحكم مع تأثير إيجابي لتكوين العظم بسبب خصائص الاندماج العظمي والالتئام العظمي. **الاستنتاجات:** تدعم هذه الدراسة أن البوليمرالكايتوسان الطبيعي النانوي قد يكون له آفاق تطبيق أفضل لإصلاح العظام وتسريع التئام الجروح.

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INTRODUCTION

Bone grafting represents a frequently performed procedure to enhance and stimulate bone regeneration in different conditions in orthopedics as well as oral and maxillofacial surgery, including post-traumatic skeletal complications, such as delayed unions, non-unions, mal-unions, and traumatic bone defects as well as other situations including joint fusions, avascular necrosis and various tumours reconstruction procedures. Currently, bone grafting materials are available in the clinical setting to enhance bone regeneration, varying from autologous bone to several bone graft substitutes [1,2,3] . Autogenous bone graft is the old standard graft because it provides osteoinduction, osteoconduction and osteoprogenitors cells, but it still has some certain effects like limited graft volume, donor site morbidity and increasing time of operation .Allogenic bone graft and xenograft are still involved in the clinical use, but they are associated with many problems like the possibility of cross infection and chance of immune rejection of recipient [1,4].

Several countries are currently experiencing an exceedingly high demand for bone grafts and bone tissue engineering solutions [5] . Interestingly, nanotechnology represents one of the outstanding strategies significantly progress the field of bone tissue engineering and resolve existing limitations of conventional approaches including insufficient mechanical strength of scaffolds, impaired cellular proliferation and differentiation and inadequate production of extrinsic necessary to optimize osteogenesis [6]. Nano technology is a rapidly emergent field regarded as the ability to manufacture materials at the nanoscale level [7]. Nanomaterials refers to materials that have been synthesized to have a size with at least one dimension in the range of approximately 1 to 100 nanometres and exhibit unique properties determined by their size which make them able to be used in a wide range of innovative applications. Recently, various micro/Nano carriers, particularly nanoparticles and nanofibers, have become available for enzyme immobilization, Typically, smaller particles provide a larger surface area for the attachment of enzymes [8 & 9].

Nano-chitosan deacetylate chitin with the degree above 70% 'Chitosan'. [9] Chitin is the wide-spread biopolymer in nature after cellulose. It is the major component of cuticles of insects, fungal cell walls, yeast or green algae [10]. It is also present in crab and shrimp shells contains a number of free amine groups [11]. Nano-chitosan using ionic cross-linking [12] has unique high surface reactivity, bioactive, bio-degradable, biocompatible, nontoxic, low-antigenic and anti-bacterial properties which enable it to be used in several biomedical and pharmaceutical applications. It is reported to have biological properties such as antitumor, antimicrobial, and antioxidant activities.

Moreover, it can be used in pharmaceutical excipient or drug carrier, obesity treatment and as a scaffold for tissue engineering. CTS has been extensively used in bone engineering since it was shown to promote cell growth and mineral-rich matrix deposition by osteoblasts cells in vitro [13 - 15] . The aims of this study are to evaluate the efficiency of Nano-chitosan on accelerating bone healing and generation in rabbits.

MATERIALS AND METHODS

This study had been carried out in the department of oral and maxillofacial surgery, College of Dentistry, University of Mosul, Iraq. Under ethic approval committee reference number UoM.Dent/ A.L.9/21. Eighteen rabbits weighted 1.3-1.5 Kg and aged 3-4 months were chosen. nano-chitosan represent by a package of medical nano chitosan 30gm was bought from SHAANXITOP PHARMA CHEMICAL China), with D.D 95%, viscosity <100cps, insoluble <1%, ash <1%, moisture <10%, heavy metal <10ppm, fineness <80nm, arsenic <0.5ppm, density 0.08g\ml with a white yellowish powder appearance, that ensured by Fourier-transform infrared spectroscopy FTIR (to determine the function groups) and *Scanning electron microscope* SEM (to determine the surface morphology) as shown in (Figures1,2 and 3).

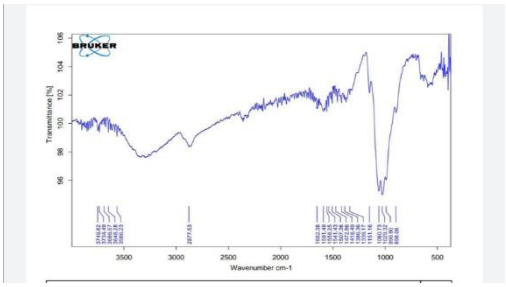

Figure (1): The infra-red spectra (FTIR) of Nanochitosan particles

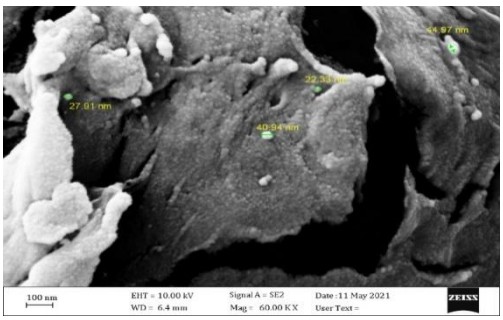

Figure (2): scan electronmicroscope of Nanochitosan magnification power 60.00KX

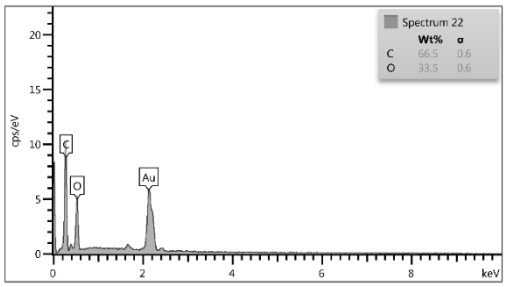

Figure (3): Energy dispersive spectra (EDS) of Nano polymer was used to identify the chemical composition of the Nano chitosan.

Housing and feeding for all rabbits were the same and all rabbits were examined by a veterinary physician to check the animal's health condition. Each rabbit was given general anaesthesia, a mixture of ketamine of 0.6mg/kg and Xylazine of 0.3ml/kg injected intramuscularly. Then the rabbit positioned on his left side and the area over the right femur shaved and cleaned with povidone iodine. A small incision about 1.5 cm was created over the femur bone

near its head by surgical blade no.15 avoiding any trauma to muscle after that the femur bone was exposed by blunt dissection. Two holes of 2 mm diameter and 15 mm apart between holes, created under copious irrigation with distilled water in the femur using 2 mm carbide bure connected to slow motion dental engine. By small plastic scope, about 0.0594g of nano-chitosan add directly to fill one hole and the second hole left empty filled with blood. Suture the inscion by 3/0 silk black simple interrupted suture tachnique, then the rabbits were left to heal at different time intervals. Animals were divided into 3 groups and sacrificed at different time intervals at 7 days, 14 days, and 28 days. After the end of each time interval, the rabbits at each group had been euthanized and a radiographic image for the femoral bone was taken at standard alignment and distance from the X-ray source, the radiographic digital system was Care stream®. The setting of the machine was 60 kV, 10 mA and 0.30 seconds. Measures were managed by drawing a line from the cortical bone crossing the defect by Cs imagining software 7.0.3. Also, the specimen sends for histopathological sections. Histological parameters include:

1. Bone trabecular thickness.

2.The number of osteoblasts.

3. The number of osteoclasts and,.4. The degree of inflammation. Degree of inflammation includes severe inflammation (score 3), moderate (score 2), mild inflammation (score 1), no inflammation (score 0). Statistical analysis done by using SPSS program version 25, Data were presented as means and standard deviation of mean and analysed by independent T-test at a significant level ≤ 0.05.

RESULTS

Radiographic assessment

At 7 days after surgery, the control hole detected with radio-opacity little higher if compare with nano-chitosan hole that (filled by homogenous radiolucent degradable natural polymer). Both the control & nano-chitosan defects borders were clearly detected, showed in (Figure 4).

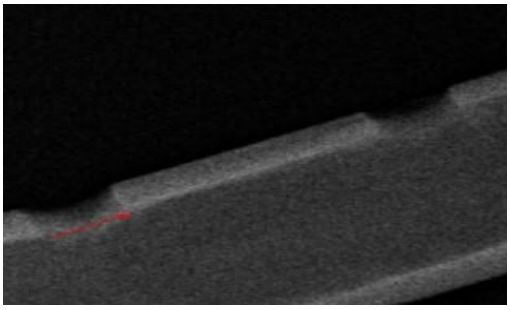

Figure (4): Nano-chitosan and control defect at 7 days after surgery.

At 14 days after surgery, the nano-chitosan group borders were still detected, while the borders of control were barely detected with higher radio opacity than nano-chitosan hole as shown in (Figure 5).

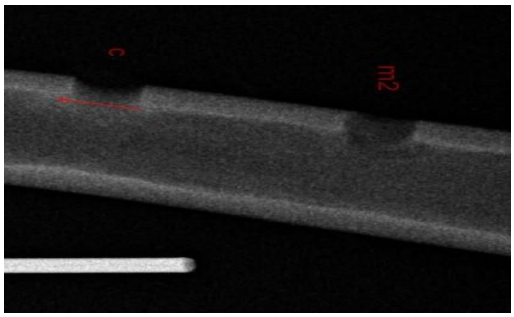

Figure (5): Nano-chitosan and control defect at14 days after surgery.

At 28 weeks after surgery, the borders of both groups were not detected, the control group showed higher radio opacity than nano-chitosan as shown in (Figure 6).

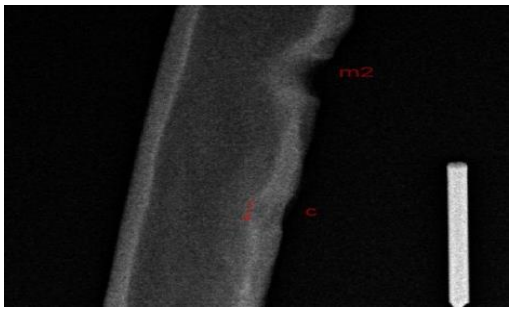

Figure (6): Nano-chitosan and control defect at 28 days after surgery.

Histopathologic assessment

At 7day the site of the wound of the control group showed granulation tissue characterized by inflammation (score 3) with a scanty amount of new bone formation (bonny spicules) as shown in (Figure 7), while the site of the wound of nano-chitosan group shows the nanomaterial in red color, granulation tissue characterized by inflammation (score 1) and scanty amount of new bone formation (bonny spicules) as shown in (Figure 8).

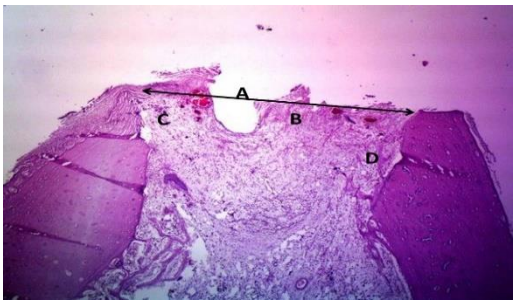

Figure (7): Histological section of control group,7 days after surgery at 40 X magnification. H & E stain.

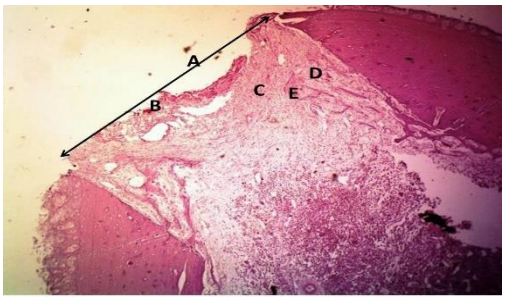

Figure (8): Histological section of Nano-chitosan group, 7 days after surgery at 40 X magnification H& E stain.

At 14 days the site of wound of control group shows granulation tissue characterized by inflammation (score 1) and moderate amount of new bone formation (bonny spicules) as shown in (Figure 9), while the site of the wound of nano-chitosan group shows the nanomaterial in red colour, granulation tissue without inflammation (score 0) and a profound amount of new bone formation (bonny spicules) as shown in (Figure10).

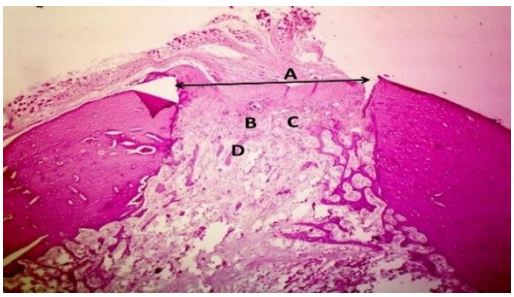

Figure (9): Histological section of control group, 14days after surgery at 40 X magnification. H & E stain.

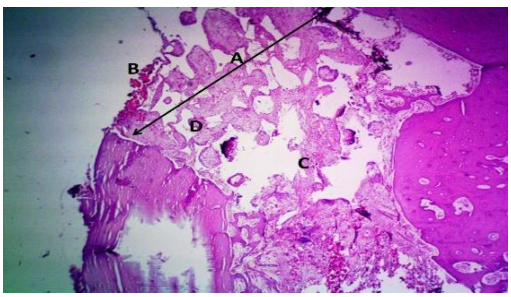

Figure (10): Histological section of Nano-chitosan group, 14 days after surgery at 40 X magnification. H & E stain

At 28 days the site of the wound of the control group was without presence of granulation tissue and inflammation (score 0) and a thin layer of new bone formation (bonny spicules) almost closed the defected area, as shown in (Figure11), while the site of the wound of the nano-chitosan group without the present of granulation tissue and inflammation (score 0) and the nano material was degraded with the present of a profound amount of new bone formation perfectly closed the defected area as shown in (Figure12).

All statistical results represented the mean and standard deviation of mean, significant difference level $p \leq 0.05$.

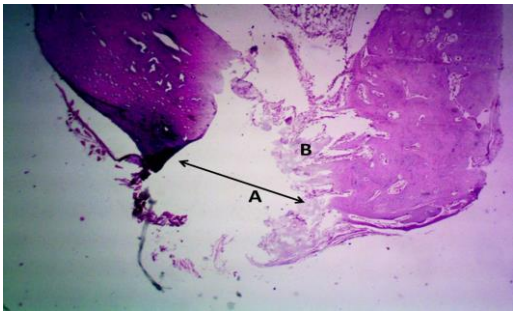

Figure (11): Histological section of control group28 days after surgery at 40 X magnification. H & E stain.

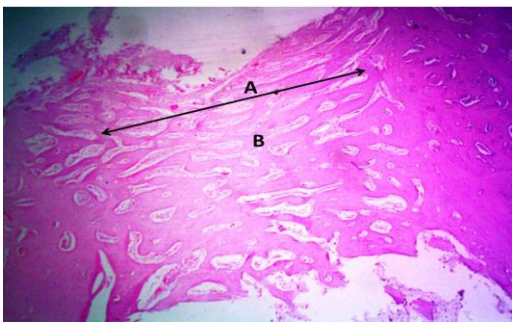

Figure (12): Histological section of Nano-chitosan group28 days after surgery at 40 X magnification. H&E stain.

Radiographic results

Statistical analysis of radiographic results showed that there was a statistically no significant difference between defect filled with the control group and the nano-chitosan group at 7days, 14, 28 days as shown in (Table 1).

Table (1): Statistical analysis of radio graphical results, represented as mean ± standard error of mean at significant level

	DAY 7	DAY 14	DAY 28
Control	58.83±5.94 a	56.83±8.04 a	71±2.14 a
Nano-Chitosan	49±5.13 b	46±3.18 a	67.5±2.96 a

Histological results

1. Bone surface area: Throughout the periods of study, the nano-chitosan nanomaterial showed greater bone surface area formation and statistically there was a highly significant difference as shown in (Table2).

Table (2): Statistical analysis of new formed bone area represented as mean ± standard error of mean at significant level.

	DAY 7	DAY 14	DAY 28
Control	2940.82± 143.08 a	12623.42± 328.06 a	19404.66± 551.60 a
Nano-Chitosan	11787.22±507.80b	26586.56±507.80b	62501.6± 2271.68 b

2. Osteoblast numbers found: The osteoblast numbers at the nano-chitosan defect showed an increase in number than in the control defect with a statistically highly significant difference between groups at 7 days, significant difference at14 days, and no significant difference between groups at 28 days as shown in (Table 3).

Table (3): Statistical analysis of osteoblast represented as mean ± standard error of mean at significant level 0.05.

OSTEOBLAST NO./ 40x field			
	DAY 7	**DAY 14**	**DAY 28**
Control	7±0.7a	18.2±0.8a	23±1.4 a
Nano-Chitosan	12.42±0.9a	22.14± 14 b	26± 1.4 b

3. Osteoclast number found: The osteoclasts numbers at the nano-chitosan defect showed an increase in number than the control defect with a statistically no significant difference between groups at 7days, highly significant difference at14days, and significant difference at 28 days as shown in (Table 4).

Table (4): Statistical analysis of osteoclast represented as mean ± standard error of mean at significant level 0.05.

OSTECLAST NO./40x field			
	DAY 7	**DAY 14**	**DAY 28**
Control	0.5± 0.02 a	3.5± 0.4 a	5±0.6a
Nano-Chitosan	0.85± 0.1b	7± 0.5b	7.14± 0.5 b

DISCUSSION

Radiographic results revealed for nano-chitosan lower radio opacity if compared with the control group at (1st week) with no significant differnces post opprativly which appeared as a homogenous radiolucency [(16)]. According to the visibility of the bone ;the bone mineralization should be at least 40% other wise is not visible in radiograph [(17)]. Also there is no Ca+2 in composition of nano chitosan [(18)] . The mean radiological grade at 14, 28 days that increases with no significant differences could be explained by bone formation has all ready begun in the defect site by stimulation of biodegradable, osteoinduction nature of material, our study agreement [(7,19 & 20)] In response to bone cells differentiation and bone tissue formation(osteogenesis), related to nature of chitosan which is polycationic biopolymers that can easily interact with poly anionic molecules such as many proteins, DNA, or phospholipids., the presence of both hydroxyl and amino groups and modification (nano) provides many options for polymer [(8)], nano chitosan can stimulate osteoblast proliferation , maturation , bone ECM mineralization, regulation of these osteoblast differentiation-related gene expression and Ca-bending protein, possess a high surface area , porosity that provides good cell adhesion as well as these pores act as channels permitting migration of cells deep into the scaffold permitting new vascularization, nutrients transportation and cells accumulation also the topography of implanted material may facilitate cells accumulation [(21,22)] . Chitosan by decreased IL-6and TNF-α that play a role in bone resorption will increase the number of osteoprotegerins that inhibit RANK interaction with RANKL. This condition results in the inhibition of osteoclast genesis and acceleration in bone repair [(13)] . The properties of osteoinductivity and osteointegratability of chitosan will stimulate new bone growth and balance the number of osteoclasts and osteoblasts so that efficient bone tissue repair can occur.

Throughout the periods of study histological examination showed greater bone formation of nano-chitosan material of nano particles size show approximately greater bone formation than control . The surface nanotopography in scaffolds plays a critical role in modulating cell growth and attachment, proliferation and differentiation, because the undulating changes at the nanoscale level affect the covalent anchoring density of stem cells, also play role in cell adhesion by controlling adhesion size and changing adhesion-related signaling, surface nanotopography of biomaterial scaffolds could orchestrate osteogenesis by modulating the local immune microenvironment. Macrophages, such as cell shape, proliferation, adhesion and phenotype, induce the release of pro-inflammatory cytokines by macro- phages, which in turn regulate the osteogenic processes (the biomimetic hierarchical nanointerface can facilitate Nano-chitosan macrophage polarization and interleukin-4 secretion to promote stem cell osteogenesis and endogenous bone regeneration) [(23).] In response to the degradation of biomaterial (i.e. by the solubility of it and by resorption activity of osteoclast) a strong base because it has primary amino groups with a pKa value of 6.3. The existence of the amino groups shows that pH considerably changes the charged state and properties of chitosan [(8)] . At low pH, these amines get protonated and become positively charged and that causes chitosan to become a water-soluble cationic polyelectrolyte. On the other hand, as the pH increases to 6 and above, the amines of chitosan become deprotonated and the polymer loses its charge and becomes insoluble. The soluble-insoluble transition occurs at its pKa value around a pH between 6 and 6.5. As the pKa value is highly dependent on the degree of N-acetylation, so is the solubility of chitosan, that solubility influences the pattern of ostoblastic resorption activity in terms of shape and distribution of resorption lacunae. [(24)]. The inflammatory reaction at the defect treated with nano chitosan was lesser than that seen in the control group which showed sever inflammatory response at 7days and this may be due to anti-inflammatory action of chitosan and its effect on pro inflammatory cytokines. These results agreed with Sadowska *et al.* [(23)].

CONCLUSION

In conclusion, nano-chitosan material accelerated bone formation and showed a high potential capacity for bone regeneration with low inflammatory reaction.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

REFERENCES

1. Roden RD. Principles of Bone Grafting. Oral Maxillofac Surg Clin North Am.

2010;22(3):295-300. doi:10.1016/j.coms.2010.06.001

2. Nyary T, Scammell BE. Principles of bone and joint injuries and their healing. Surg (United Kingdom). 2018;36(1):7-14. doi:10.1016/j.mpsur.2017.10.005
3. Fernandez de Grado G, Keller L, Idoux-Gillet Y, et al. Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management. J Tissue Eng. 2018; 9: 204173141877681. doi:10.1177/2041731418776819
4. Lobb DC, Degeorge BR, Chhabra AB. CURRENT CONCEPTS Bone Graft Substitutes: Current Concepts and Future Expectations. J Hand Surg Am. 2019;44:497-505.e2. doi:10.1016/j.jhsa.2018.10.032
5. Amini AR, Laurencin CT, Nukavarapu SP. Bone tissue engineering: recent advances and challenges. Crit Rev Biomed Eng. 2012; 40(5).
6. Sullivan MP, McHale KJ, Parvizi J, Mehta S. Nanotechnology: current concepts in orthopaedic surgery and future directions. Bone Joint J. 2014; 96(5): 569-573.
7. Qi L, Xu Z, Jiang X, Hu C, Zou X. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr Res. 2004; 339(16): 2693-2700. doi:10.1016/j.carres.2004.09.007
8. Divya K, Jisha MS. Chitosan nanoparticles preparation and applications. Environ Chem Lett. 2018; 16(1): 101-112. doi:10.1007/s10311-017-0670-y
9. Li-Ming Zhao1, Lu-E Shi2, Zhi-Liang Zhang2, Jian-Min Chen2, Dong-Dong Shi2 JY and Z-XT. PREPARATION AND APPLICATION OF CHITOSAN NANOPARTICLES AND NANOFIBERS. Brazilian J Chem Eng. 2011;28:353-362.
10. Einbu A, Vårum KM. Characterization of chitin and its hydrolysis to GlcNAc and GlcN. Biomacromolecules. 2008; 9(7): 1870-1875.
11. Wang X, Xing B. Importance of structural makeup of biopolymers for organic contaminant sorption. Environ Sci Technol. 2007; 41(10): 3559-3565.
12. Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro-and nanoparticles in drug delivery. J Control release. 2004; 100(1): 5-28.
13. Ma Y, Liu P, Si C, Liu Z. Chitosan nanoparticles: preparation and application in antibacterial paper. J Macromol Sci Part B. 2010; 49(5): 994-1001.
14. Zhu L, Luo D, Liu Y. Effect of the nano/microscale structure of biomaterial scaffolds on bone regeneration. Int J Oral Sci. 2020; 12(1): 1-15.

15. Labah D. The Validity of Nano-chitosan/ Nano-Hydroxyapatite as a Promoter of Bone Healing in Ovariectomized Rats. Egypt Dent J. 2017; 63(3): 2389-2402.
16. Martins EAN, Baccarin RYA, Moraes APL, Mantovani CF, Machado TSL, Hagen SCF. Evaluation of chitosan-glycerol phosphate in experimental osteochondral joint defects in horses. J Mol Genet Med S. 2015;4:862-1747.
17. Haghighat A, Hekmatian E, Abdinian M, Sadeghkhani E. Radiographic evaluation of bone formation and density changes after mandibular third molar extraction: a 6 month follow up. Dent Res J (Isfahan). 2011; 8(1):1.
18. Zargar V, Asghari M, Dashti A. A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications. ChemBioEng Rev. 2015; 2(3): 204-226.
19. Rahman MM, Shahruzzaman M, Islam MS, Khan MN, Haque P. Preparation and properties of biodegradable polymer/nano-hydroxyapatite bioceramic scaffold for spongy bone regeneration. J Polym Eng. 2019;39(2):134-142.
20. Zhao D, Yu S, Sun B, Gao S, Guo S, Zhao K. Biomedical applications of chitosan and its derivative nanoparticles. Polymers (Basel). 2018; 10(4).
21. S. G, T. G, K. V, Faleh A. A, Sukumaran A, P.N. S. Development of 3D scaffolds using nanochitosan/silk-fibroin/hyaluronic acid biomaterials for tissue engineering applications. Int J Biol Macromol. 2018;120:876-885.
22. Mohammadpour Dounighi N, Eskandari R, Avadi MR, Zolfagharian H, Mir Mohammad Sadeghi A, Rezayat M. Preparation and in vitro characterization of chitosan nanoparticles containing Mesobuthus eupeus scorpion venom as an antigen delivery system. J Venom Anim Toxins Incl Trop Dis. 2012; 18(1): 44-52.
23. Sadowska JM, Wei F, Guo J, Guillem-Marti J, Ginebra M-P, Xiao Y. Effect of nano-structural properties of biomimetic hydroxyapatite on osteoimmunomodulation. Biomaterials. 2018; 181: 318-332.
24. Puttini I de O, Poli PP, Maiorana C, et al. Evaluation of osteoconduction of biphasic calcium phosphate ceramic in the calvaria of rats: Microscopic and histometric analysis. J Funct Biomater. 2019; 10(1) :7.