Iraqi Journal of Industrial Research, Vol. 10, No. 1 (2023)

Open Access

Iraqi Journal of Industrial Research (IJOIR)

Journal homepage: http://ijoir.gov.iq

Preparing of Nano-Hydroxyapatite from Seashell and Mixed with Gold to Repair Bony Defect of Mandibular Bone in Rabbits

[1]Ghada A. Taqa*, [1]Banan N. Al-Hussary, [2]Nazhan Th. Kashmola

[1]Department of Dental Basic Sciences, College of Dentistry, University of Mosul – Iraq

[2]Al-Kindy Factory/ General Company of Communication Equipment and Power/ Ministry of Industry and Minerals – Iraq

Article information

Article history:
Received: October, 05, 2022
Accepted: April, 12, 2023
Available online: June, 14, 2023

Keywords:
Seashell,
Nanoparticles hydroxyapatite,
FTIR

Corresponding Author:
Ghada A. Taqa
ghadataqa@uomosul.edu.iq

DOI:
https://doi.org/10.53523/ijoirVol10I1ID278

Abstract

The objectives of the present study were undertaken to prepared hydroxyapatite powder from seashell and convert this to nano size and then added to gold nanoparticles. Materials and Methods: The seashells were cleaned and the phosphoric acid was added. The product was inserted in oven then calcined in the muffled furnace, to evaporate CO_2 and getting the white crystalline powder which indicated presence of hydroxyapatite. This powder was converted to nanoparticle. Gold 1% mixed with prepared seashell nano hydroxyapatite. The characteristics of the prepared nano hydroxyapatite from seashell, were studied by the FTIR infrared spectrophotometer. The most biocompatible nano hydroxyapatite estimation by chemical test and examine prepared nano hydroxyapatite from seashell alone or when mixed with gold 1% in vivo to detect the effectiveness on reparing bone defect in mandibulare rabbits. Results: The results of an infrared measurement (FTIR Spectroscopy) for prepared nano hydroxyapatite showed that the chemical structure and band have the same FTIR spectrum of standard nano hydroxyapatite and have the same nano traits as the chemical test showed a yellow precipitation consisting in the nHA seashells. As an indication of biocompatibility and increase Bone Mineral Density by repairing bone defect in rabbits. And when added gold to nano hydroxyapatite, increased the efficacy of bone remodeling and repair bone. Conclusions: The possibility of preparing nanoparticles for hydroxyapatite from seashell are simple and inexpensive feedstock's and can be successfully produced by chemical precipitation technology from seashells with a phosphoric acid solution.

1. Introduction

Bones are specialized forms of connective tissue that can be classified either by their shape: long bones, short bones, flat bones, and irregular bones; or by their place of development into intramembranous and endochondral bone (Nanci, 2017) [21]. Bone preservation and formation is regulated by three main types of cells residing within it: osteoblasts, osteocytes, and osteoclasts. In order to meet the demands of skeletal growth and mechanical function, and to maintain bone vitality and health, bone undergoes a dynamic remodeling process through a process combined with bone formation by osteoblasts and bone resorption by osteoclasts (Han et al.) [12]. Healing large bone defects is a major clinical challenge. Although bone possesses remarkable repair and regeneration, there are many clinical situations in which the size and/or location of the bone defect impairs healing (Verrier et al.) [32]. Bone healing is a meticulously regenerative process, which restores 98% of bone structure. The bone healing process is conventionally divided into three phases: an early inflammatory phase, a repair phase, and a final remodeling phase (Mohammed et al.) [18]. Bone remodeling is an important, dominant, and coordinated lifelong process in the adult skeleton (Nyary and Scammell) [22]. Bone remodeling is therefore essential to maintain bone mass, to repair partial skeletal damage, and to prevent the accumulation of too much old bone. Bones are unique in that regeneration can occur without a fibrous scar. Although skeletal tissue has a strong regenerative capacity, the healing process may fail, resulting in a delayed healing period, (Einhorn and Gerstenfeld) [9]. So surgeons have tried to add substances to the bone matrix to promote and speed up healing and the formation of new bone (Naji et al.) [20]. New bone graft alternatives have been devised in recent decades, such as demineralized bone matrix, bone-forming proteins, calcium phosphate, calcium sulfate, and hydroxyapatite (HA) (Athanasiou et al.) [1]. There has been a growing trend towards using nanomaterials, such as nanoporous hydroxyapatite ($Ca_{10}(PO4)_6(OH)_2$, nHA), to develop bone grafts. These materials, which mimic the nanostructure of natural hard tissues, can be used to regenerate bone and accelerate the healing process of fractured bone (Lu et al.) [17]. Bone grafting has been done to repair bony defects for centuries. Since then, bone grafts and bone grafting procedures have been developed (Hussein and Taqa) [15]. Most of the previous studies indicated that the fabrication of bone grafts using ion-supportednano-hydroxyapatite (nHA) in combination with bioactive polymers can significantly accelerate the healing of bone and cartilage defects and this may be due to the physicochemical similarities between the structures of the compensated bone and bone tissue (Prondvai et al.) [24]. Other types of nanoparticles are gold nanoparticles (GNPs) which are widely used in diagnostics, drug delivery, biomedical imaging, and photothermal therapy due to surface plasmon resonance, fluorescence, and facile surface operation. According to recent studies, gold nanoparticles show a positive effect on osteogenic differentiation of mesenchymal stem cells and osteoblast-like cells (Heo et al.) [13]. Therefore, the purpose of the present study is to Prepare hydroxyapatite from the seashell and then converting it to nanoparticles and adding them to gold, and study effect of nHA when used it as implants to remodeling the lost bone in the mandibular rabbits.

2. Materials and Methods

2.1. Chemical Study

2.1.1. Synthesis of Seashell Hydroxyapatite

The snail sea shell was soaked in water for 24 hours and then washed with deionized water and its surface was mechanically cleaned to remove the dirt stuck to it. Then 100 grams of clean dried shells were weighed and then the dried shells were burned at 900℃ for 1 hour in Furnest muffled furnace, to remove CO2 and obtain white crystalline powder and calcium oxide (CaO). The exact molar ratio needed for a 98% solution of phosphoric acid was added to CaO and left for 24 hours to allow time for the reaction to occur and complete, a strong reaction occurred with a large foam (Figure (1 A & B). Then we wash it with deionized water, then filter the precipitate through paper and leave it for 48 hours at room temperature, then put the precipitate in the oven for 30 minutes at 100 ° C for drying and thus we obtain hydroxyapatite.

2.1.2. Preparation Hydroxyapatite nHA Nanoparticles

Hydroxyapatite nHA nanoparticles were prepared by the mechanical grinding method described by Claudio et al. [6]. Mechanical grinding is a simple technique that does not require high temperature to create nanopowder. In this technique, the coarse feedstock is crushed between a vigorously rammed ball and this leads to the shredding of the feedstock, which results in very fine particles. This process can be performed at room temperature and the rotating speed in the mill has reached 3000 rpm for two hours. In order to equalize the particle size, the materials were separated using a well-defined non-porous membrane and the size of the particles ranged between 27-37.5 nanometers when examined with a scanning electron microscope Transmission Electronic Microscope. 1% of gold for the purpose of treating laboratory animals and knowing its role in repairing and grafting lost bone.

2.1.3. Estimation by Chemical Test (Silver Nitrate Test)

This test is done by added silver nitrate on prepared Hydroxyapatite from seashell to know this compound ortho or di or metaphosphorous by the color of precipitation that formed (Clarence, 1964) [5]. Depending on the method of preparation different crystal forms of silver phosphate can be produced of the same lattice structure the reaction can be used to know the biocompatibility of orthophosphate (Dorozhkin) [8].

2.1.4. Infrared Spectroscopy

The prepared nHA from seashell were characterize by using FTIR-Alfa-Bruker spectrophotometer (Germany) in region (500-4000 cm^{-1}). This measurement was carried out in the University of Mosul, College of dentistry, Iraq.

2.2. Experimental Procedure

2.2.1. Experimental Animals

16 white male rabbits were given general anesthesia using a mixture of intramuscular injections of ketamine (40 mg/kg) and xylazine (8 mg/kg) and waiting for a few minutes until the rabbit lost consciousness. Then the rabbit was placed on its left side on the operating table, and hair was removed from the pinna area. Submandibular using electric hair clippers and sterilized with povidine iodine as an antiseptic.

2.2.2. Surgical Procedure: After the animals were anesthetized within 5 minutes, a small incision (about 5 cm in length) was made in the skin over the submandibular region with the lower border of the beginning of the jaw from the symphysis region using the surgical blade, using a scientific and sterile technique. Part of the jaw bone was removed by drilling a groove (2 mm in diameter and 6 mm in length) in the dental engine at a low speed using a 2.3 mm drill head. The groove was washed with distilled water and dried from the blood using cotton granules. Then the rabbits were divided into 3 groups depending on the type of material that will be placed in the groove where the groove was filled as follows:

1. Group 1: serve as control group consisted from 4 rabbits no treatment. This group were scarified after (30) days.
2. Group 2: filled bony defect with Seashell Nano hydroxyapatite prepared from seashells, group consist from 4 rabbits this group were scarified after (30) days.
3. Group 3: filled bony defect with nano hydroxyapatite prepared from seashells and mixed with gold 1% , group consist from 4 rabbits this group were scarified after (30) days.

After that, the incisions of all rabbits were closed with surgical sutures using the intermittent suture technique, and then the wound was sterilized with iodine 4%.

2.2.3. Postoperative Care

Immediately after the surgery, oxytetracycline was given (0.5 ml/kg body weight, intramuscularly) and it was repeated once daily for the next three days with examination of the animals daily and noticing the recovery from the surgery, and the sutures were removed after one week of the operation.

2.2.4. Histologic Procedures:

The histological examination was performed to assess the degree of healing that was reached after 30 days of using hydroxapite prepared from seashell alone or when we mixed it with 1% of gold with the control group (only performing a surgical operation without using any substance). Histological examination was performed in the Department of Pathology at the College of Veterinary Medicine/ University of Mosul by a histological expert, and each slide was examined under a light microscope. (BH2 Olympus) by a histologist and two experienced pathologists.

The process of regeneration and healing of injured bone occurs via several steps, including the development of granulation tissue, along with the differentiation of osteoblasts and osteoblasts, which are responsible for the deposition of osteoblasts and the filling of the bone with compacted bone material and osteoblasts with inflammatory area.

2.2.5. Statistical Analysis

Data were processed and analyzed using SPSS Ver. 18. Analysis of variance (ANOVA) was used to compare the differences between study groups. For histological observations, histopathological analysis was performed by using the Kruskal-Wallis H test for K-independent samples at a probability level of p-value ≤ 0.05 that was considered significantly different.

3. Results and Discussion

3.1. FTIR Infrared Spectrometer Estimation

Estimation of seashell nano-hydroxyapatite bands Determination of the chemical composition of hydroxyapatite prepared from seashells by FTIR spectrophotometer. Infrared spectrophotometer showed the location of the beams (Figure 2) of nano-hydroxyapatite powder prepared from seashells, indicating that the nano-hydroxyapatite prepared from seashells is α-orthophosphate and is biocompatible with human use. The structure of the powder was analyzed using FTIR spectroscopy after dried at 100°C. The IR Spectroscopy for the hydroxyapatite showed some important bands between the region 500-4000 cm^{-1} which is due to P-O, P=O and OH groups. In FTIR analysis, mainly the peaks for PO_4^{-3} and OH^- groups in the hydroxyapatite can be identified these bands confirm the structure of hydroxyapatite. The IR spectrum of raw HA powder are showed. The sharp peak at 925 cm^{-1} can be explained owing to O-H group deformation. Intense PO_4^{-3} peak appeared at 1030 cm^{-1}. Additional phosphate group bands are found in the region 1000, 963, 875, 639,610, 566, 531 and 450 cm^{-1}, An intense PO_4^{-3} peak appeared at 1030 cm^{-1}. The additional phosphate peaks are found in the region 1138, 495 and 450 cm^{-1}. The C-O-C peak at 1206 cm^{-1} and an intense phosphate peak is found is found at 1037 cm^{-1}. The additional phosphate group bands at 718 cm^{-1}. From the results, the IR analysis showed that the synthetic seashell HA have approximately the same important band regions to the standard hydroxyapatite.

3.2. Estimation of the Chemical Structure

Determination of the chemical composition Silver nitrate solution was added to the prepared nano hydroxyapatite powder. A yellow precipitate is formed, which indicates that the hydroxyapatite powder is nanoscale, and this test is evidence of biocompatibility with orthophosphate.

3.3. Histologic Evaluation and Biocompatibility

Histological evaluation and biocompatibility of hydroxyapatite prepared from seashells and compared to the control group. Histological examination was performed by three examiners in a blinded manner. Histological examination revealed that all groups showed healing and bone development but with some variation, examination included description of specimens, assessment of extent of space filling, formation of connective tissues, osteoclasts, lamellar osseous interconnection and evaluation of newly formed vasculature.

Histological examination after 30 days to examine the site of bone removal showed that there was a high density of bone material in the group treated with nano-hydroxyapatite prepared from seashells compared to the control. The bone density was greater when mixing hydroxyapatite prepared from seashells with 1% gold in terms of increased osteoblasts, also showed a dense amount of granulation tissue, blood vessels and bone marrow compared with the control group. The sites of the missing bone showed that the defect area was filled with a substance. The bone is very dense compared to the control group, and the size of the osteoblast is larger than the control (Figures 3, 4, 5 & 6).

The chemical formula of hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$, which is the main inorganic component of bones and teeth in humans (Rahman, 2019) [25] and hydroxyapatite makes up about 70% of the weight of human bones (Elbasuney) [10]. The prepared synthetic nano-hydroxyapatites are considered as potential biomaterials for bone tissue engineering applications due to their excellent biological properties.

The present work included the preparation of HAn hydroxyapitate nanoparticles from a cheap source of seashell. The synthetic HA derived from different raw materials is of paramount importance as it inherits variable structural and chemical properties (Sundarabharathi et al.) [29]. In this work, there was no sign of an inflammatory reaction in all the complexes except in the control group. This result can be explained by what was mentioned by (Nagaraja et al.) [19] that hydroxyapatite constitutes the main inorganic part of the hard tissue due to its improved osseous properties. By stimulating the activity of osteoclasts and promoting local growth factors, it therefore improves bone healing (Bayani et al.) [2]. HA contains only calcium and phosphate ions, and therefore has no harmful local or systemic toxicity and that the high temperature used to prepare HA burns any organic molecules such as proteins that inhibit immune response and transplant rejection (Dorozhkin,) [8].

In this work, hydroxyapatite was prepared from seashells, which are considered as a source of calcium carbonate with phosphoric acid, and then converted to nanoscale. In a simple and cost-effective way, to convert seashell into a high-value biomedical product from nanoscale hydroxyapatite with its ability to regenerate bones (Usha and Sudhaparimala) [31]. Where prepared nano hydroxyapatite in the treated group showed good mechanical properties that support bone repair and grafting until healing occurs that prevents bone fracture compared to the control group in which animals showed incomplete bone filling, and this may be related to the properties of HA prepared from The coincidence, which led to an increase in the contact area for bone implants, which enhances the entry of the prepared nano-hydroxyapatite into the mineral tissues, and this result can be explained by the opinion mentioned by (Tihan et al.) [30] that the developed hybrid materials containing hydroxyapatite do not show significant cytotoxicity and support diffusion into the cell Thus, this result holds great promise for applications in bone tissue engineering.

Infrared spectra analysis is one of the important techniques used to identify and characterize the chemical composition of compounds. From the results, FTIR analysis showed that nano-hydroxyapatite prepared from seashells has the same properties as standard HA nano-hydroxyapatite. From the results of the chemical test carried out we found that the prepared compound contains orthophosphate depending on the yellow color of the obtained sediment. This may indicate that nano-hydroxyapatite prepared from seashells can be safely used as an alternative to bone graft.

The nHA prepared from seashells succeeded in replacing the missing bone surgically, and it did not show any irritation or injury after surgery in all treated groups. These results can be explained that the use of nano-hydroxyapatite prepared as a bone substitute is useful because it does not cause an immune response and does not cause irritation and it is non-irritating and inflammatory.

The addition of gold to hydroxyapatite prepared from seashells increased the healing of lost bone, and these results were in agreement with other findings indicating that gold-containing injectable hydrogels for tissue regeneration would stimulate bone regeneration and could serve as a biodegradable grafting material to treat a defect (Li et al.) [16]. After 4 weeks of using the nano-hydroxyapatite prepared from the shell at the site of the missing bone, new bone formation appeared in the periphery and covered the entire surface of the prepared nano-hydroxyapatite implant and the entire missing bone was repaired, and this may be related to the bone conduction properties of the prepared nano-hydroxyapatite implant so that it can adhere to Osteoblast cells and eventually form new bone, and these results agreed with the results of other studies that showed that the formation of new bone can be the result of the work of bone marrow from the bone marrow from the ends of the missing bone (Sharifi et al.) [28]. Furthermore, the lower density scores recorded in the control group could be explained by the inability and oligodendrocytes, which are activated during slight osteogenic differentiation, to synthesize and mineralize sufficient bone matrix that can fill the bone defect. Moreover, the lower scores obtained in terms of signs of remodeling in the control group could be explained by the absence of osteoclasts, the cells responsible for this bone healing stage (Bigham-Sadegh et al.) [4]. These results confirm the failure of bone fusion in the control group, in contrast to the group treated with nano-hydroxyapatite prepared from shells.

The results of the histological examination showed the formation of granulation tissue at the site of the missing bone, and the histological results indicated that the areas of the missing bone in the experimental animals in all groups showed healing and different amounts of new bone formation by the HA-prepared nanohydroxyapatite. The surfaces covered with HA hydroxyapatite increase osteoblast cell activity, differentiation, and ossification. This result can be explained by what the scientists mentioned (Hiratsuka et al.) [14] that the process of bone formation increases using hydroxyapatite, as it filled the lost bone place with bone material compared to the control group. This result may be related to HA that promoted bone formation and this result agrees with what the scientist (Orman et al.) [23], that hydroxyapatite has an ideal pore size and properties that support bone tissue growth and osteoblast proliferation therefore, it can be a good candidate for clinical applications due to its low production cost and natural biological origin.

The results of the histological examination of the infiltration of inflammatory cells at the site of the bone defect were found only in the control group, while there was no infiltration of inflammatory cells in all the hydroxyapatite groups, whether it was in its normal form or its nano-form mixed with gold. As hydroxyapatite has an anti-inflammatory effect, it does not provoke any inflammatory response, and therefore, no excess inflammatory cells accumulate at the site of the operation. This result can be explained by what the author Shapovalova et al. [27] reported. The author showed that HA can stimulate the release of anti-inflammatory cytokines indicating the presence of potential anti-inflammatory properties.

The healing process shows an increase in pre-existing circulating blood vessels that are believed to transport osteoblasts to the site of the missing bone (Ripszky et al.) [26]. This may be appropriate to the bone which may lead to the formation of new bone and the remodeling of the newly formed bone. The scientists explained that the pore size and the porosity of hydroxyapatite play an important role in bone growth, as well as the proliferation of osteoclasts. Therefore, the nano-shape increased the capacity of the prepared hydroxyapatite more than its normal form (Gredes et al.) [11].

Histological examination showed in the group in which gold was mixed in a small percentage with hydroxyapatite prepared from seashell after 30 days after the operation, it filled the defect of the missing bone and increased bone density more than hydroxyapatite when it was alone, as was found in the histological examination results for the infiltration of inflammatory cells at the site of the defect. The osteoblasts were only in the control group, while there was no infiltration of inflammatory cells in all groups of hydroxyapatite alone or nano-hydroxyapatite with gold. The inability to detect inflammatory cells in the hydroxyapatite nanoparticles group could be due to the anti-inflammatory effect of the hydroxyapatite and gold used, and this result can be explained by the opinion mentioned by the scientist (de Araújo et al.) [7], who said that gold nanoparticles have analgesic and anti-inflammatory effect.

(A) (B)

Figure (1). (A & B): Preparation of sea shell Hydroxyapatite.

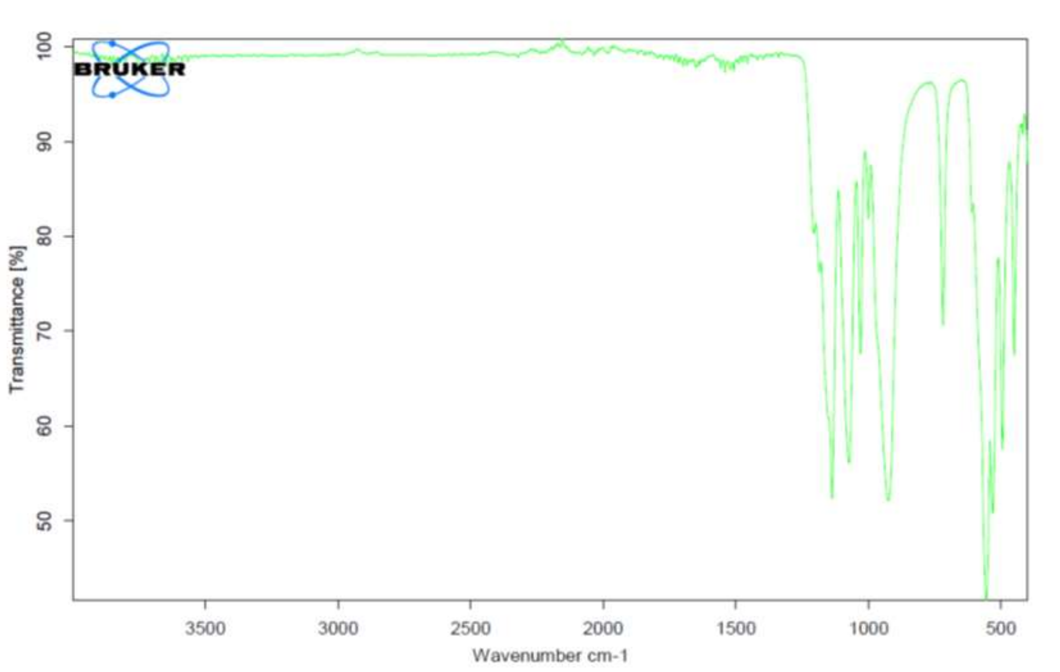

Figure (2). Chemical structure of hydroxyapatite prepared from seashells by infrared spectrophotometer).

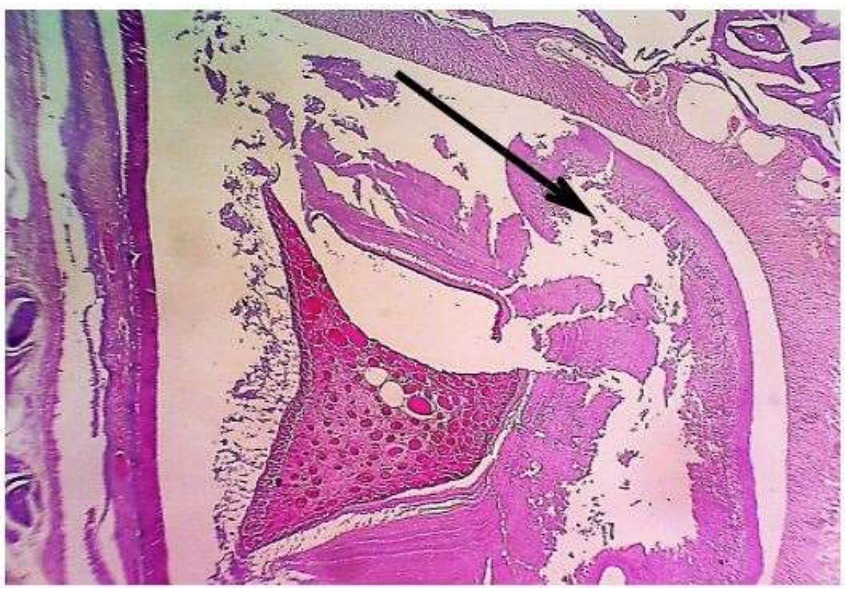

Figure (3). Light micrograph of rabbit's mandibular bone from control group at 30 days showed newly bone formation, lamellar bone trabecular (→). Staining H&E. Magnification 4 X.

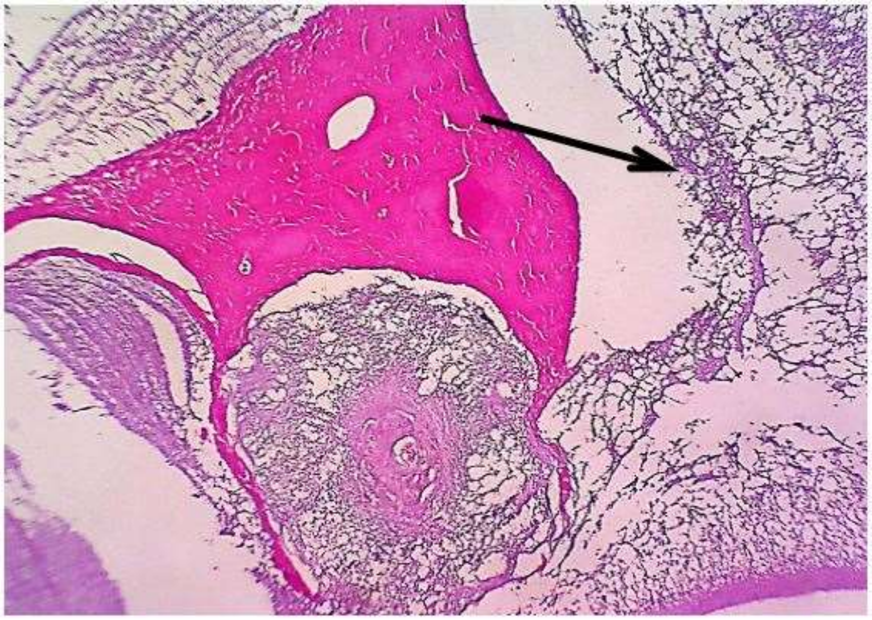

Figure (4). Micrograph of the mandible bones of rabbits from the control group at 30 days' new bone formation, lamellar bone (B.L).

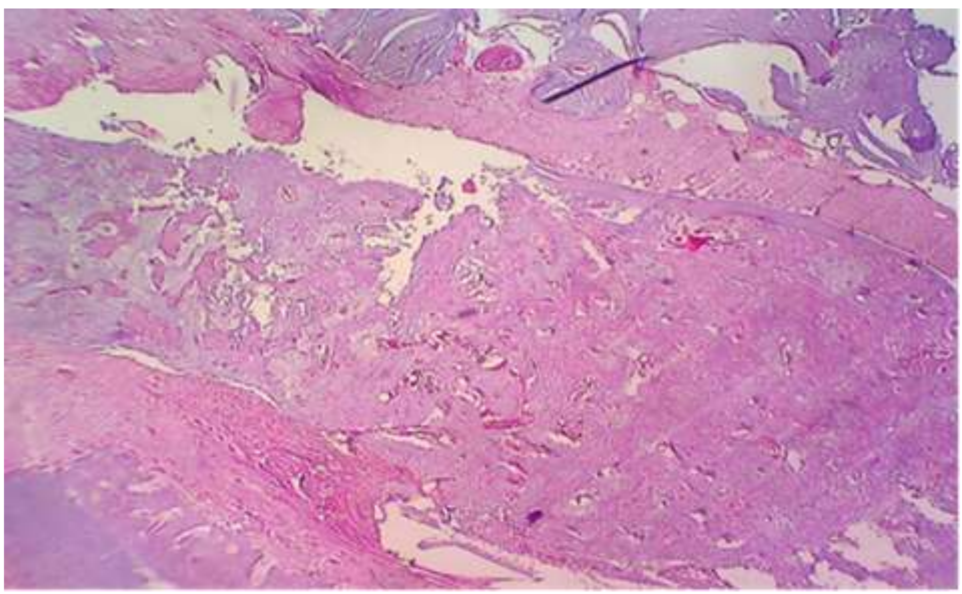

Figure (5). Micrograph of the mandible bone of rabbits 30 days after treatment with nano-hydroxyapatite prepared from seashell showed an increase in connective tissue (DCT), abundance of connective tissue (D.C.T) and abundance of bone lamella (B.L).

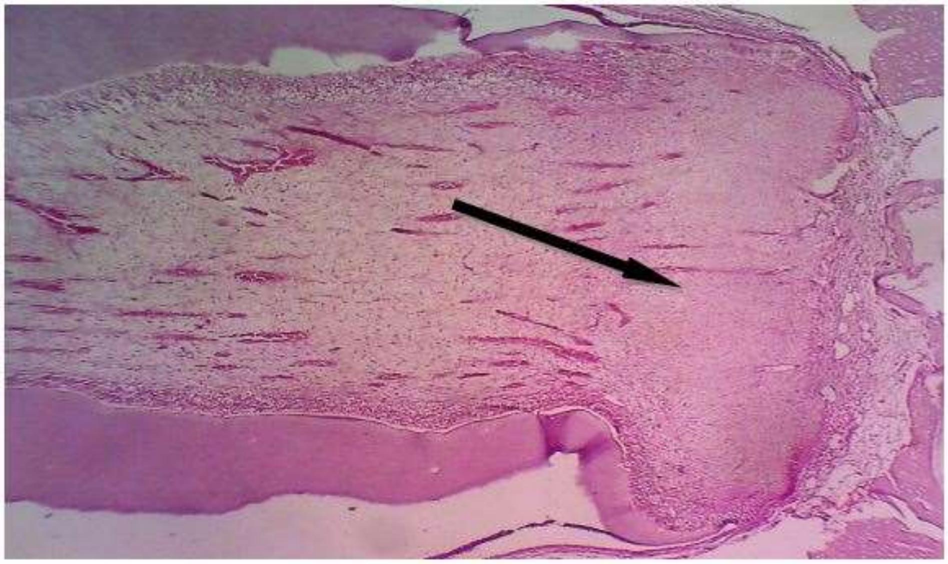

Figure (6). Light micrograph of rabbit's mandibular bone of 1% Gold with Seashell nHA group at 30 days showed space filling with granulation tissue and new bone formation
(→) Staining H&E.

4. Conclusions

We conclude from this study that giving the implant of nano-hydroxyapetite prepared from seashells alone or mixed with gold has repaired and grafted the bone when used in place of the missing bone in the mandible. The ability of nano-hydroxyapetite prepared from shells to repair and replace lost bone without any side effects. This promises to open horizons for doctors and dentists in replacing lost or broken bones in short periods of time and from natural biological materials. It serves two purposes, the first is the production of osteoblasts in large quantities and at a low cost, and the second purpose is at the same time preserving the environment by making use of the marine crustaceans that died in the aquatic and marine environment.

Acknowledgement: The authors are very grateful to the University of Mosul/ College of Dentistry for their provided facilities, which helped improving the quality of this work.

References

[1] V. T. Athanasiou, D. J. Papachristou, A. Panagopoulos, A. Saridis, C. D. Scopa, and P. Megas. "Histological comparison of autograft, allograft-DBM, xenograft, and synthetic grafts in a trabecular bone defect: an experimental study in rabbits", *Med Sci Monit,* vol. 16, no. 4, pp. 24-31, 2010.

[2] M. Bayani, S. Torabi, A. Shahnaz, and M. Pourali, "Main properties of nanocrystalline hydroxyapatite as a bone graft material in treatment of periodontal defects", *Biotechnology & Biotechnological Equipment*, vol. 31, no. 2, pp. 215-220, 2017.

[3] G. N. Bernard, "Healing and repair of osseous defects", *Dent.clin. of north America,* vol. 3, no. 5, pp. 12-15, 1991.

[4] A. Bigham-Sadegh, I. Karimi, M. Shadkhast, M.H. Mahdavi, "Hydroxyapatite and demineralized calf fetal growth plate effects on bone healing in rabbit model". *J. Orthop. Traumatol,* vol. 9, no. 16, pp.141–149, 2015.

[5] A. D. Clarence, Modern inorganic pharmaceutical chemistry.1st Ed., ohn welly and sons, IN, London, 1964, E-book].

[6] L. Claudio, D.E and Castro, S.M. Brian Department of Chemical Engineering, (chapter one) Tulane University, vol. 396, pp. 124-128, 3rd Ed. JE Hampsey New Orleans, Louisiana, USA. 2005 [E-book].

[7] R.F.de Araújo Júnior, A.A de Araújo, J.B. Pessoa, F. P. F. Neto, G.R. da Silva, A. L. C. L. Oliveira, and L.H. Gasparotto, “Anti-inflammatory, analgesic and anti-tumor properties of gold nanoparticles”, *Pharmacological Reports,* vol. 69, no. 1, pp. 119-129, 2017.

[8] S.V. Dorozhkin, “Self-setting calcium orthophosphate (CaPO4) formulations and their biomedical applications”, *Adv Nano-Bio. Mater. Dev,* vol. 3, pp. 321-421, 2019.

[9] T.A. Einhorn, and L.C. Gerstenfeld, “Fracture healing: mechanisms an intervention”, *Nature Reviews Rheumatology*, vol. 11, no. 1, pp. 45-54, 2015.

[10] S. Elbasuney, “Green Synthesis of Hydroxyapatite Nanoparticles with Controlled Morphologies and Surface Properties Toward Biomedical Applications”, *Journal of Inorganic and Organometallic Polymers and Materials,* vol. 1, no. 8, pp. 11-15, 2019.

[11] T. Gredes, M. Wrobel-Kwiatkosk, M. Dominika, T Gedrange, and C. Kunert-kelli, “Osteogenic capacity of transgenic flax scaffolds”, *Biomedical technology,* vol. 57, no. 1, pp. 53-58, 2012.

[12] Y. Han, X. You, W. Xing, Z. Zhang, and W, Zou, “Paracrine and endocrine actions of bone—the functions of secretory proteins from osteoblasts, osteocytes, and osteoclasts*”. Bone research,* vol. *6,* no. 1, pp. 1-11, 2018.

[13] D. N. Heo, W. K. Ko, M. S. Bae, J. B. Lee, D. W. Lee, W. Byun, and I. K. Kwon, “enhanced bone regeneration with a gold nanoparticle–hydrogel complex”, *Journal of Materials Chemistr,* vol. 2, no. 11, pp. 1584-1593, 2014.

[14] T. Hiratsuka, M. Uezono, K. Takakuda, M. Kikuchi, S. Oshima, S. T. Sato, and K Moriama, “Enhanced bone formation onto the bone surface using a hydroxyapatite/collagen bone-like nanocomposite.”. *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 10, no. 2, pp. 391-398, 2020.

[15] A.A. Hussein, and G.A Taqa, “The Impact of Natural Calcium Carbonate and Ubiquinone on Bone Mineral Density in Rabbits”, *Journal of Applied Veterinary Sciences*, vol. 6, no. 4, pp. 15-22, 2021.

[16] H. Li, S. Pan, P. Xia, Y. Chang, C. Fu, W. Kong, Z. and Qi, “Advances in the application of gold nanoparticles in bone tissue engineering”, *Journal of Biological Engineering*, vol. 14, no. 1, pp. 1-15, 2020.

[17] Z. Lu, S.I. Roohani-Esfahani, and J. Li, H. Zreiqat. “Synergistic effect of nanomaterials and BMP-2 signalling in inducing osteogenic differentiation of adipose tissue-derived mesenchymal stem cells”, *Nanomedicine,* vol. 11, no. 21, pp. 9-28, 2015.

[18] Z.S. Mohammed, G.A. Taqa, and M.S. Sulaiman, “Evaluating the local and systemic effects of silicon dioxide on healing of mandibular bone in rabbits”, In *AIP Conference Proceedings*, vol. 2547, no. 1, pp. 020006, 2022.

[19] B.N. Nagaraja, C.L. Srinivas, S.M Jayadevappa, B.N. Ranganath, S.K Vijayasarathy, “Biochemical and histopathological changes in dogs with femoral fractures immobilized with plastic rods*”, Indian Journal of Veterinary Surgery,* vol. 24, no. 2, pp.111-112, 2003.

[20] A.H. Naji, G.A. Taqa, andW.T. Al water. “The Effect of Xylitol on Osteoclastogenesis in Experimentally Induced Bone Defect in Rabbits”, *Journal of Applied Veterinary Sciences*, vol. 7, no. 1, pp. 58-63, 2022.

[21] A. Nanci, A, Ten *Cate's Oral Histology-E-Book: Development, Structure, and Function*, 2017. Elsevier Health Sciences. [E-book].

[22] T. Nyary, and B.E. Scammell, “Principles of bone and joint injuries and their healing” *Surgery,* vol. 36, no. 1, pp. 7-14, 2018.

[23] Z. Orman, S. Yucel, Y. M. Sahin, O. Gunduz, and F.N Oktar, “Bioactivity of Hydroxyapatite Produced from Sea Snail Turritella Terebra*”, Acta Physica Polonica,* vol. 135, no. 5 pp.77-84, 2019.

[24] E. Prondvai, K.H.W. Stein, A. de Ricqlès, J. Cubo, “Development-based revision of bone tissue classification: the importance of semantics for science”, Biological *Journal of the Linnean Society,* vol. 1, no. 12, pp.799- 816, 2014.

[25] S.U. Rahman, Origin and history of hydroxyapatite, vol. 16, no.1, p. 383. 2019. Handbook of Ionic Substituted Hydroxyapatites. *Elsevier,* 2019.

[26] A. Ripszky Totan, M. M. Imre, S. Parvu, D. Meghea, R. Radulescu, D. S. A. Enasescu, & S.M. Pituru, "Autophagy Plays Multiple Roles in the Soft-Tissue Healing and Osseointegration in Dental Implant Surgery", *A Narrative Review. Materials,* vol. 15, no. 17, pp. 6041, 2022.

[27] Y. Shapovalova, D. Lytkina, L. Rasskazova, I. Zhuk, A. Gudima, A. Filimoshkin, and J. Kzhyshkowska, "Preparation of biocompatible composites based on poly-L-lactide/hydroxyapatite and investigation of their anti-inflammatory activity", In *Key Engineering Materials,* vol. 6, no. 83, pp, 475-480, 2016.

[28] D. Sharifi, H.R. Khoushkerdar, G. Abedi, A. Asghari, and S. Hesaraki, "Mechanical properties of radial bone defects treated with autogenous graft covered with hydroxyapatite in rabbit", *Acta cirurgica brasileira*, vol. 27, no. 3, pp. 256-259, 2012.

[29] L. Sundarabharathi, D. Ponnamma, H. Parangusan, M. Chinnaswamy, and M. A. An Al-Maadeed, "Effect of anions on the structural, morphological and dielectric properties of hydrothermally synthesized hydroxyapatite nanoparticles", *SN Applied Sciences*, vol. 2, no. 1, pp. 94, 2020.

[30] G.T. Tihan, V. Sereanu, A. Meghea, G. Voicu, M.G. Albu, V. Mitran, and R.G. Zgârian, "Innovative methodology for developing a bone grafting composite biomaterial starting from the seashell of Rapana thomasiana ", *Comptes Rendus Chimie,* vol. 20, no. 4, pp. 440-445, 2017.

[31] R. Usha, and S. Sudhaparimala, "Nano Scale Hydroxyapatite from Crassostreao Virginica (Oyster Seashells) and its Modification with Azadirachta Indica (Neem) Extract", *Material Science Research India*, vol. 15, no. 3, pp. 296-306, 2018.

[32] S. Verrier, M. Alini, E. Alsberg, S.R. Buchman, D. Kelly, M.W. Laschke, and T. Miclau, "Tissue engineering and regenerative approaches to improving the healing of large bone defects", *Eur Cell Mater*, vol. 3, no. 2, pp. 87-110, 2016.