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Bone Regenerative Potentiality of Nanostructured Biphasic Hydroxyapatite /Tricalcium Phosphate Prepared from Eggshell as a Bone Graft in Vivo

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الخلاصة

الاهداف: تهدف الدراسة الى تقيم اعادة البناء العظمي باستخدام فوسفات الكالسيوم الثنائي الطور ذات الدقائق النانوية المستخلص قشور البيض. **المواد وطرائق العمل**: تم تجميع قشور البيض بعد تفريغ محتويات البيضة وازالة القشرة البروتينية الداخلية ميكانيكيا بطريقة وكيميائيا باستخدام هايبوكلوريد الصوديوم بعدها تم غسل قشور البيض للتخلص من هايبوكلوريد الصوديوم. تم طحن قشور البيض ووضعها في درجة حرارة ١٠٠٠ درجة مئوية ولمدة ساعة كاملة للتخلص من المركبات العضوية. تم اضافة جزء الناتج الى حامض الفوسفوريك المركز وتم معادلته بالماء ليتكون لدينا الهايدروكسي ابتايت. تم اضافة ما تبقى من قشور البيض المسخنة الى حامض الناتريك المركز وتم اضافة ثنائي فوسفات هايدروجين الامونيوم المذاب في الماء لينتج فوسفات ثلاثي الكالسيوم بعد التكليس لمدة ساعتين في درجة حرارة ١٠٠٠ درجة مئوية. تم خلط الناتجين ميكانيكيا بنسبة ٨٠ % هايدروكسي ابتايت و٢٠% للفوسفات ثلاثي الكالسيوم. اجرت الدراسة على عشرون ارنبا وتم حفر ثقبين في عظم الارنب احداهما ملئت بالمادة المصنعة والاخرى تركت فارغة لتكون سيطرة. **النتائج**: وجدت هنالك زيادة في نمو العظم بفترة زمنية أقصر في الثقب الذي ملئ بالمادة المصنعة وكانت هنالك فروقات معنوية احصائية عند معنوية ٠.٠٥ **الاستنتاجات**: ان فوسفات الكالسيوم الثنائي الطور ذات الدقائق النانوية المصنع من قشور البيض الغير ذات قيمة اقتصادية اظهرت فعالية جيدة في بناء العظم مع توافق حيوي ممتاز.

ABSTRACT

Aims: to evaluate the biological response to nano sized biphasic hydroxyapatite and tricalcium phosphate synthesized from eggshell. **Materials and methods**: Egg shell burned in furnace at 1000 C° for 1 hour. The part of resultant powder was treated chemically with phosphoric acid to form HA, other part of heated egg shell treated with nitric oxide and di-ammonium hydrogen phosphate to form TCP then the two products mixed mechanically at ratio 80%/20% (HA/TCP). The mixture turned to nanoparticles via mechanical attrition and sieving membrane and sent for TEM, the resultant material characterization checked by FTIR spectroscopy. Two defects of 2 mm prepared in the femoral bone of 20 rabbits one filled with prepared material (group A) and the other left empty as negative control (group B). **Results**: greater bone formation was founded in group A where statistically significant. **Conclusions**: biphasic hydroxyapatite and tricalcium phosphate nano particles synthesized from eggshell is effective in bone regeneration and could be used as bone substitute derived from source of no economic value.

Keywords: Nanostructured, Hydroxyapatite, Tricalcium Phosphate, Eggshell

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INTRODUCTION

Nano material defined as materials that have at least one external dimension of 1-100 nm [(1)]. Major trauma and disease like osteoporosis may lead to comminuted fracture which its treatment is a challenging task. Comminuted fracture defect is beyond the ability of bone regeneration capacity to promote healing of bone. Bone graft is used to bridge the defect to act as scaffold to provide support and promote bone healing [(2)]. 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 [(3,4)] Allogenic bone graft and xenograft are still involved in the clinical use, but they are associated with many problems like possibility of cross infection and chance of immune rejection of recipient [(5)]. Recently, various types of synthetic bone substitutes have been introduced for clinical applications [(6)]. Calcium phosphate is mostly used as synthetic bone graft [(7)]. Within group of calcium phosphate, hydroxyapatite (HA) and tricalcium phosphate (TCP) are mostly used as synthetic bone substitute [(8)]. Hydroxyapatites possess excellent biocompatibility, and they exhibit low biodegradation rate brittle nature [(9)]. TCP provides good osteoconductivity and possesses high biodegradation rate (Walsh et al 2008). Therefore, biphasic calcium phosphate (BCP) has been used to take the advantages of HA and TCP providing appropriate dissolution and good bioactivity, cell attachment, proliferation, and differentiation for regeneration of bone [(1)]. Nanotechnology nowadays had a great role in orthopedics because it offers a greater rivaling to nanostructure of natural bone [(10)]. Nanomaterials (which are material with dimensions less than 100nm) can decrease implant infection, increase bone growth, and inhibit inflammation without addition of pharmaceutical materials [(11)].

MATERIALS AND METHODS

Chicken eggshell obtained from market and emptied from yolk and albumin. The internal membrane removed mechanically by friction and chemically by using sodium hypochlorite then the eggshell washed with tap water and crushed the eggshell then heated at 1000 c° for 1 h to form calcium oxide.

1. Preparation of Hydroxyapatite:

HA was formed by slowly addition of calcium oxide (heated eggshell) to 10ml of phosphoric acid 98% with continuous admixing until reaction end then 100ml of distilled water added to the solution to elevate the acidic PH to neutralization, PH checked by PH meter. The water was removed and the material left to dry at room temperature.

2. Preparation of Tricalcium Phosphate:

TCP formed by gently addition of 10gm calcium oxide (heated eggshell) to 7ml of nitric acid 100%. Then 10 gm of di-ammonium

hydrogen phosphate dissolved in distilled water and added to solution, then boil for 1/5 h and left for a day at room temperature and filtered, dried and calcinated at 1000c for 2 hours.

3. Preparation of biphasic calcium phosphate (BCP):

BCP is formed by mechanical mixing of hydroxyapatite and tricalcium phosphate by weight to form mixture of HA/TCP 80/20 %.

4. Formation of nano particles:

The resultant material changed to nano form by mechanical attrition. The material placed inside metallic phial contains metal balls when the phial rotates the metallic ball crush the material to change it to nanoparticles. The speed of rotation of phial was 3000 rpm for 1 hour. The resultant material was sieved by using sieving membrane of nano pores for the purpose of equalizing the size of particles. The end material then tested by FTIR spectroscopy and transmission electron microscope.

5. Experimental model and surgical procedure:

Twenty male healthy rabbits weighting 1.3-1.5 Kg and aged 3-4 months were used in this study. Same housing and feeding applied to all rabbits giving them standard diet of vegetables and water. Examination of animals done with veterinary physician to check the health and condition of animals. Each rabbit anesthetized with 0.6mg/kg ketamine and 0.3ml/kg Xylazine injected intramuscularly. After conscious cessation, the rabbit positioned on the side and the area over the femur shaved and cleaned with povidone iodine, small incision created over the femur and the femur bone was exposed by blunt dissection. Two holes of 2 mm in dimensions created under vigorous irrigation with distilled water by using 2mm carbide bur connected to dental engine. About 0.05 mg of nano prepared material mixed with drop of water to form a pasty material for better transportation and stabilization. One hole filled with nano material and the other left empty. The healing evaluated at different time interval at 3 days, 7 days, 14 days, and 28 days.

6. Specimen collection:

At the end of each time interval, the rabbits were sacrificed and the femoral bone was isolated and sectioned into two pieces one contained the control defect and the other contained the treated defect. Each piece radiographed by digital radiograph system Carestreem®. The setting of the machine was 60 kV, 10 mA and 0.30 seconds. The radiographic measurement was conducted by drawing straight line from the cortical bone crossing the defect area by Cs imagining software 7.0.3. Then the specimen sent for histological side preparation and examination.

7. Statistical analysis:

Computer package (SPSS version 26) was used to conduct the histomorphometrical analysis. Data were presented as means ± SE (standard error) of mean and were analyzed using independent t test at significant level set on < 0.05.

RESULTS

Analysis of prepared material

1. IR spectra of nano prepared material

The product then was analyzed by FTIR spectroscopy at room temperature at region (500-4000 cm^{-1}) and the product examined by transmission electron microscope. The spectrum of the synthetic nanostructured biphasic HA/TCP showed in (Figure 1).

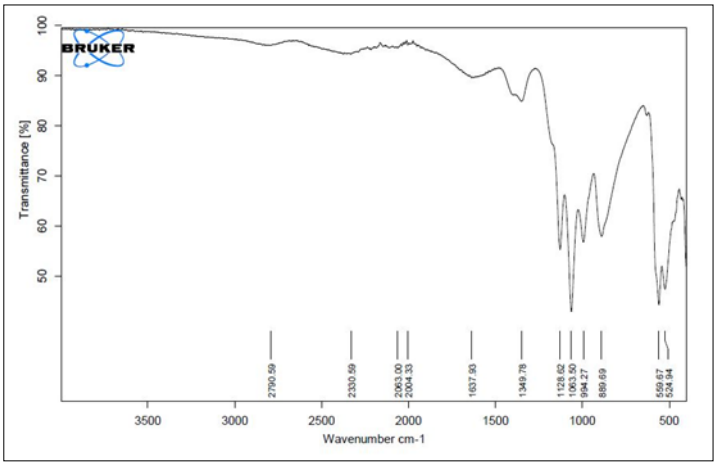

Figure (1): The infra-red spectra of nano-structured biphasic HA/TCP80/20.

2. Transmission electron microscope

The transmission electron microscope examination showed that the particles size of prepared biphasic HA/TCP ranged from 27-37.5 nm. As shown in (Figures 2 and 3)

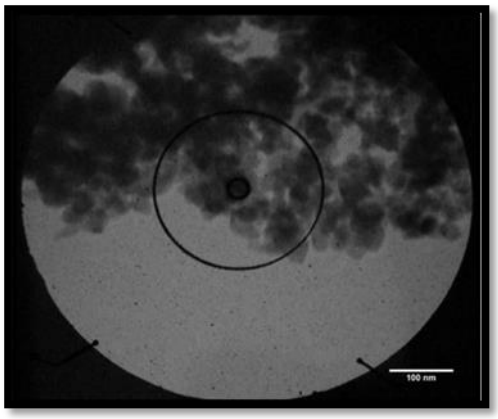

Figure (2): TEM of magnification of 180000x and the size of particle is 27nm.

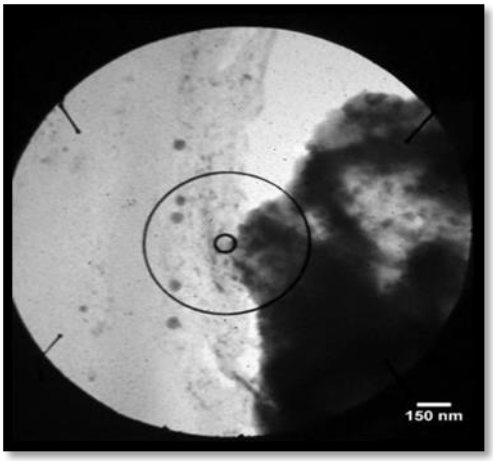

Figure (3): TEM of magnification of 130000x and the particle size is 37.5 nm.

3. Densitometric analysis

1. Three days after surgery:

All three defects were easily detected. They appeared like round radiolucencies, the control group showed no bone formation, while the group of prepared material shows higher radio-opacities as shown in (Figure 4).

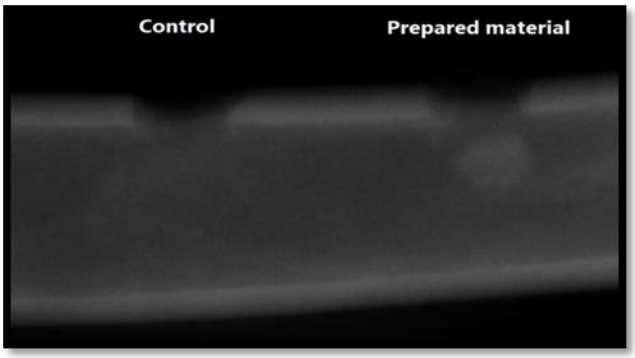

Figure (4): Densitometric analysis of control and prepared groups at 3 days

2. Seven days after surgery:

The control group was again easily detected with slight increase in radio-opacity. The other group showed better signs of healing and greater radio-opacity. As shown in (Figures 5).

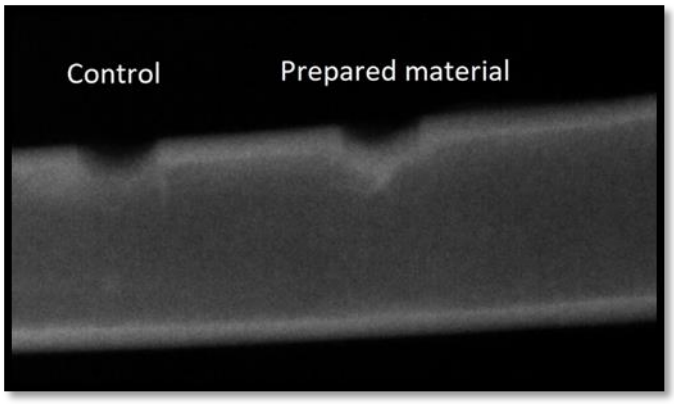

Figure (5): Densitometric analysis of control and prepared material groups at 7 days'

3. Fourteen days after surgery:

The control groups were still detected with clear margins with higher radio-opacity, the margins of prepared material group detected and obliterated with the surrounding bone indicated obvious bone formation as shown in (Figures 6).

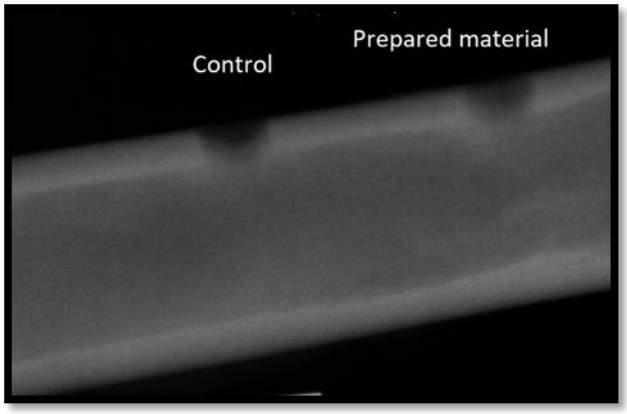

Figure (6): Densitometric analysis of control and prepared material groups at 14 days

4. Twenty-eight days after surgery: The control group margins were still detectable; the group prepared material is difficult to detect as shown in (Figure 7).

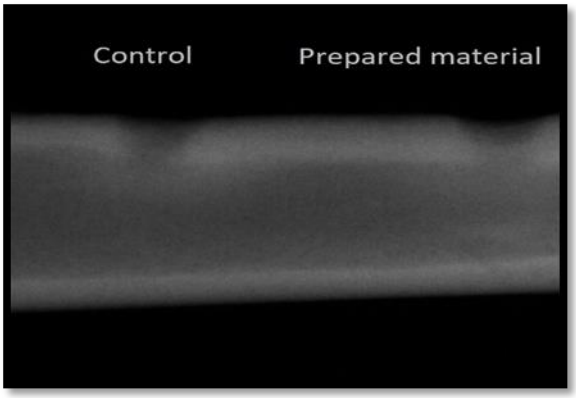

Figure (7): Densitometric analysis of control prepared material groups at 28 days

4. Histological results

1. Three days after surgery

A. the control group: The bone defect was still detected with sever infiltration of huge number of inflammatory cells (++++). Scanty granulation tissue formed at the periphery of the defect area. No bone spicules founded. No osteoblast and osteoclast found at defect area. As shown in (Figure 8).

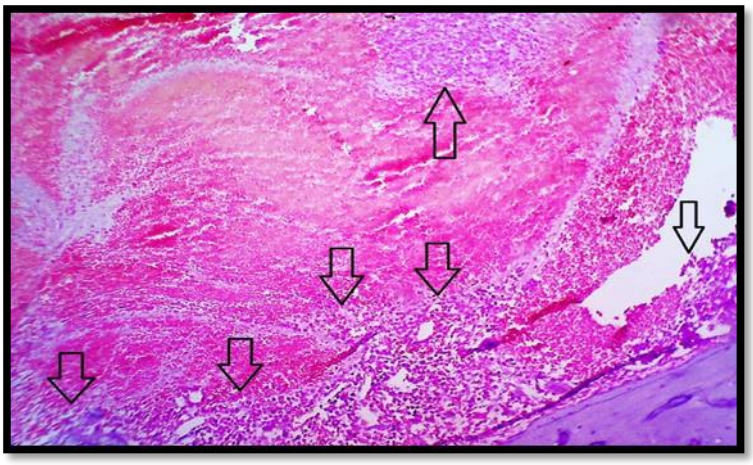

Figure (8): Histological section of control group 3 days after surgery at 10 X magnification. Arrows show inflammatory areas.

B. The prepared nano-structured material group: Biomaterial still founded at the defect area and few granulation tissues fill the area with good vascularization. There is mild infiltration of inflammatory cells (+). Tinny bony spicules detected near biomaterial in defect area. As shown in (Figure 9).

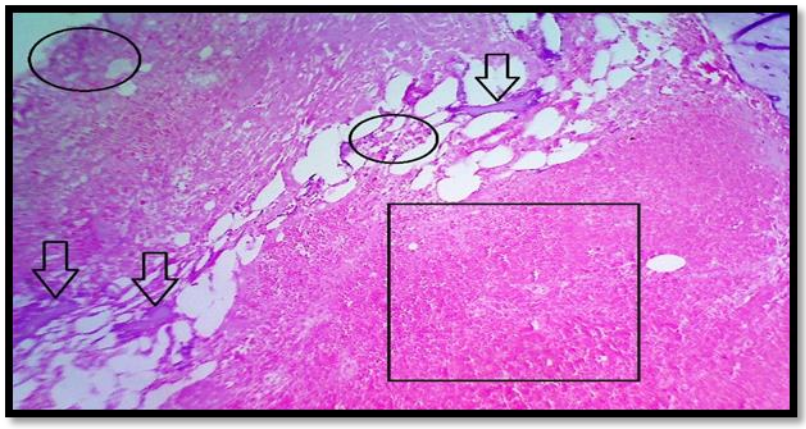

Figure (9): Histological section the nano-prepared material group 3 days after surgery at 10X magnification. Arrows show newly formed bony spicules, the circles show the areas of inflammation while the square is the biomaterials.

2. Histological findings seven days after surgery:

A. The control group: There was an increased granulation tissue formation with well-defined blood vessels. Infiltration of inflammatory cells (++), part of defect area was occupied by fat cells. Tinny bony spicules formed but not well organized. There is cellular activity where numbers of osteoblast started to form bone spicules as shown in (Figure 10).

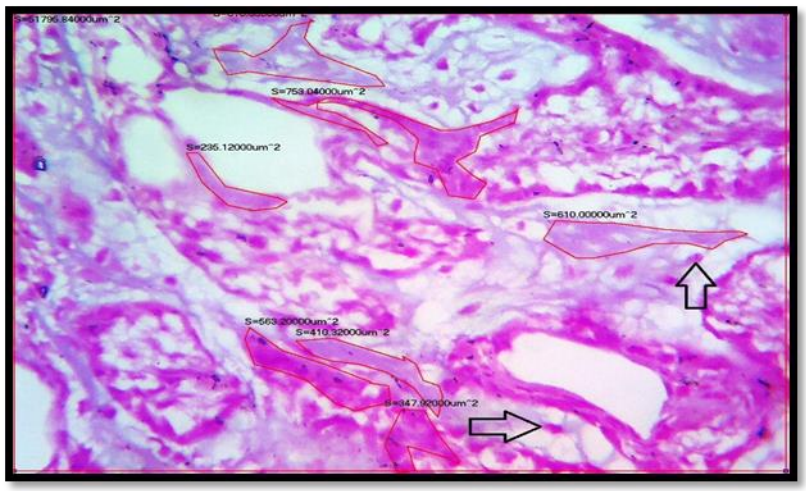

Figure (10): Histological section of control group at 7 days after surgery of 40X magnification. Selected area indicates newly formed bony spicules. Arrows show osteoblast

B. The prepared nano-structured material: The defect area filled with granulation tissue as well as trabecular bone with good vascularization. No inflammatory cells found in the area(-ve). As shown in (Figure 11)

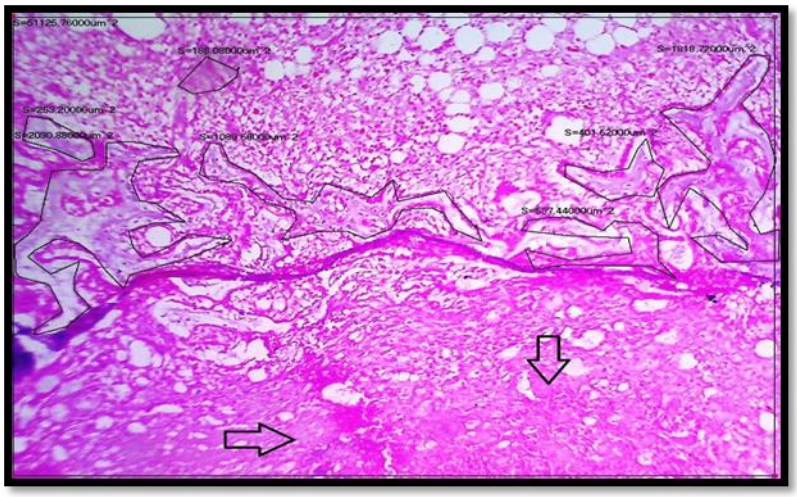

Figure (11): Histological section of the prepared nano-structured material of 10X magnification at 7 days after surgery. The selected areas indicate the newly formed bone trabeculae. the arrows show the osteoblast. No inflammatory cell founded. The arrow shows the biomaterial.

3. Histological findings fourteen days after surgery:

A. Control group: there were well defined bone trabeculae. The defect filled with granulation tissue with no inflammatory reaction presented as shown in (Figure 12).

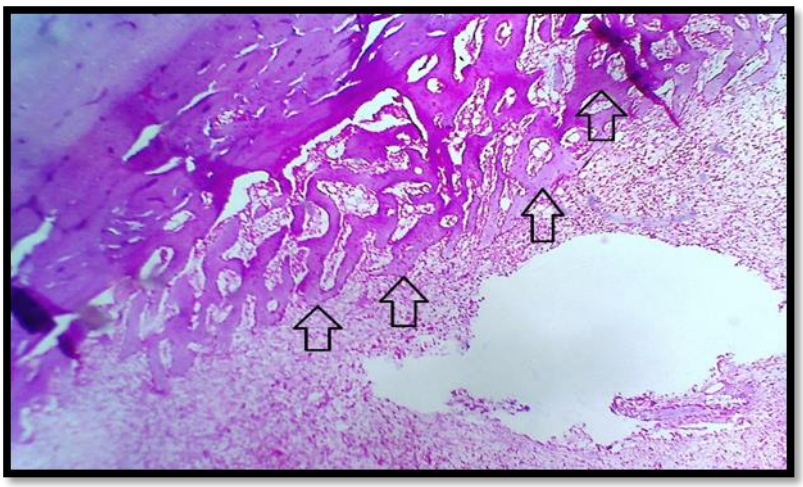

Figure (12): Histological section of 10x magnification of control group at 14 days after surgery. Arrows show the newly formed bone.

B. The prepared nano-structured group: The trabecular bone filled the defect with few compact bones at the periphery of the defect. Few connective tissues found. The spaces between bone trabeculae were filled with bone marrow. Barely residual biomaterial found with no inflammatory cells as shown in (Figure 13).

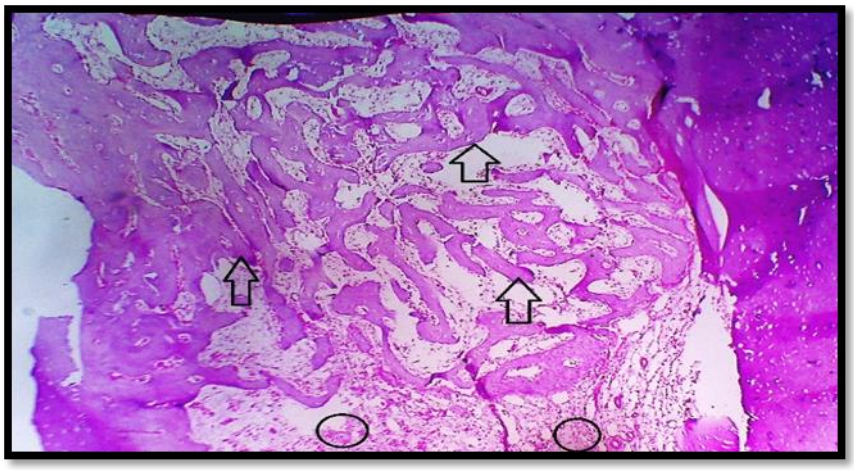

Figure (13): Histological section 4X of prepared nanostructured material group at 14 days after surgery. The arrows show the newly formed bony trabeculae. The circles show the remaining biomaterial.

4. Histological findings twenty-eight days after surgery after surgery:

A. Control group: There was formation of new compact bone to close the defect area. Most cells found in the area are osteocyte as shown in (Figure 14), the selected areas showed formation of new compact bone closing the defect.

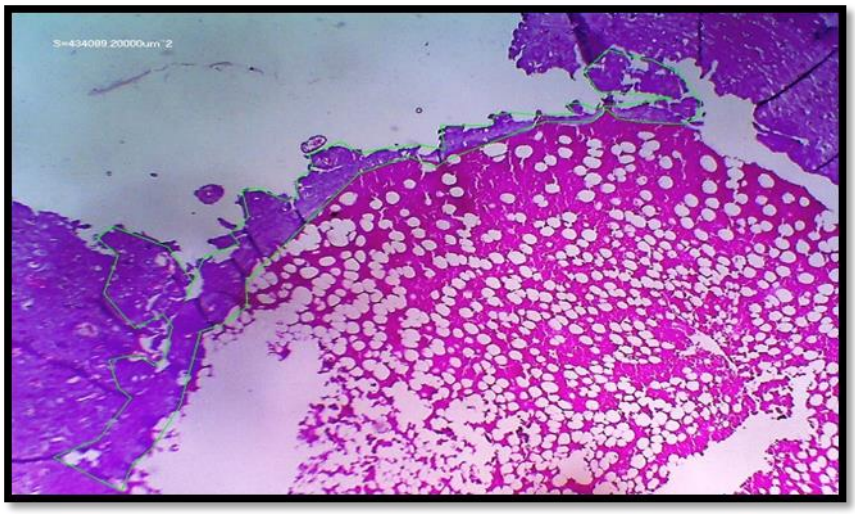

Figure (14): Histological section of 4X magnification of control group at 28 days after surgery. The selected areas show formation of new compact bone closing the defect.

B. The prepared nanostructured material group:

The defect was filled with compact bone with no spongy bone and the osteocyte represented the most cells as shown in (Figure 15).

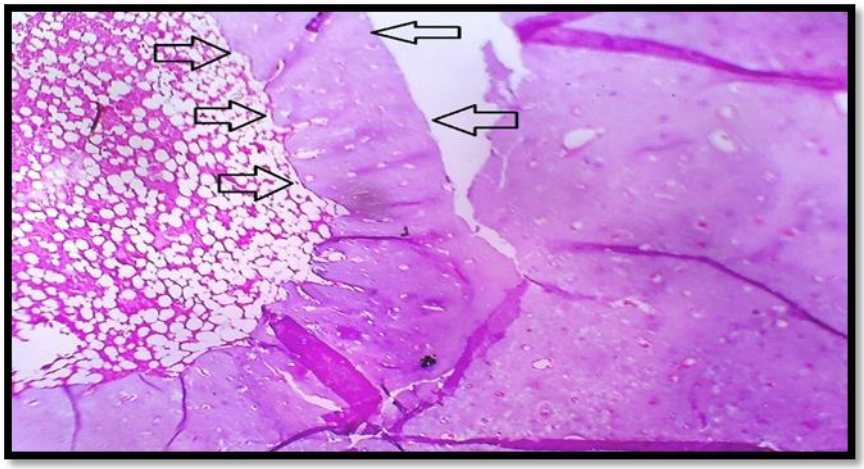

Figure (15): Histological section of 4X magnification of prepared nano-structured material at 28 days after surgery. Arrows show the compact bone closing the defect area.

4. Inflammatory response:

At three days after surgery the control group showed sever inflammatory response, while the synthetic nano material showed only mild inflammatory response

At one week after surgery the control group showed moderate inflammatory response, while prepared nano material showed no inflammatory response

At two weeks and four weeks' period, no inflammatory response saw at the two groups.

5. Statistical analysis:

In all statistical analysis, small letters reflect comparison within group at different periods (comparison in rows) where change in the small letter means there is statistically significant difference at $p \leq 0.05$. While capital litters reflect comparison between groups at each time intervals (comparison in columns) where change in the capital letter mean there is statistically significant deference at $p \leq 0.05$.

Statistical analysis of radiological results

The nano prepared material shows greater radioopacity as compared with control and there was statistically significant difference till the 28 days, as shown in (Table 1).

Table (1): Show the statistical analysis of radiographical findings expressed as mean ± stander error.

	3D Mean ± SE	**7 day Mean ± SE**	**14 day Mean ± SE**	**28 day Mean ± SE**
CONTROL	0.00 a A	28559.38±1679.3 b A	111486.82±1818.6 c A	231346.76±4356.6 d A
SYNTHETIC	8985.3±758.9 a B	54000.94±1407.7 b B	395794.82±11651.8 c B	533256.7±6930.8 d B

Small letters refer to comparison within group, their changes reflect statistically significant difference.Capital letters refer to comparison between groups their change reflect statistically significant difference.

Statistical analysis of histological findings

1.Statistical analysis of newly bone

Statistical analysis showed that there was an increased significant difference within each group with period. The group A which treated with nanostructured material showed greater bone formation than control group throughout the periods of study with significant difference statistically, as shown in (Table 2).

Table (2): Show the statistical analysis of newly bone area formed, mean ± stander error.

	3 day Mean ± SE	**7 day Mean ± SE**	**14 day Mean ± SE**	**28 day Mean ± SE**
CONTROL	90.8±3.2 a A	137±6.5 b A	179.6±12.9 c A	181.6±9.8 c A
SYNTHETIC	109.6±6.3 a B	156±6.8 b B	198.2±3.8 c B	199±11.5 c A

Small letters refer to comparison within group, their changes reflect statistically significant difference.
Capital letters refer to comparison between groups their change reflect statistically significant difference.

2.Statistical analysis of number of osteoblasts found:

The statistical analysis showed that there was significant difference within each group with period. At three days, one week and two weeks' period there was significant increase of number of osteoblasts founded with synthetic material group in comparison with control group. At four weeks, period the mean of osteoblast number found in the nanostructured material microenvironment was greater than control group but statistically not significant. At four weeks the lower the number of osteoblasts mean the healing close to end as shown in (Table 3).

Table (3): Statistical analysis of number of osteoblasts founded, mean ± stander error.

	3 day Mean ± SE	**7 day Mean ± SE**	**14 day Mean ± SE**	**28 day Mean ± SE**
CONTROL	0.00 a A	11±0.7 b A	23.4±1.2 c A	16.8±0.9 d A
SYNTHETIC	4.8±0.6 a B	22.8±1.2 b B	31±1.7 c B	14.8±0.7 d A

Small letters refer to comparison within group, their changes reflect statistically significant difference.
Capital letters refer to comparison between groups their change reflect statistically significant difference.

3.Statistical analysis of numbers of osteoclast founded

The statistical analysis showed that there was significant difference within each group with period. Throughout the periods of study there was significant increase of number of osteoclasts founded with synthetic material group in comparison with control group. At four weeks, the lower number of osteoblasts mean the healing close to end as shown in (Table 4)

Table (4)**:** Statistical analysis of numbers of osteoclast founded, Mean ± stander error.

	3D Mean ± SE	**1W Mean ± SE**	**2W Mean ± SE**	**4W Mean ± SE**
CONTROL	0.00 a A	2.2±0.3 b A	3.4±0.4 c A	1.8±0.1 b A
SYNTHETIC	1.8±0.1 a B	3.6±0.4 b B	5.8±0.8 c B	0.8±0.1 a B

Small letters refer to comparison within group, their changes reflect statistically significant difference.
Capital letters refer to comparison between groups their change reflect statistically significant difference.

DISCUSSION

The nano prepared biphasic hydroxyapatite and tricalcium phosphate estimated Fourier-transform infrared spectroscopy (*FTIR*) at room temperature at region (500-4000 cm^{-1}).

FTIR spectra expressed many peaks at the range between (500-4000 cm^{-1}). The absorption peaks detected at the regions of 1063, 994 and 559 cm^{-1} related to the phosphate ions. The characteristics TCP peak noticed at 1128 cm^{-1} region. The weak barely appeared absorption bands at 2790, 1637 cm^{-1} corresponding to organic matter functional group as protein. The absorption bands peaks at 880 cm^{-1} and 1349 cm^{-1} related to $CO3^{-2}$ ion. The incorporation of $CO3^{-2}$ ion turns the HA to carbonated HA and it substitutes either OH group or $PO4^{-3}$ or both.

The absorption peaks at 1637 cm^{-1} and 1349 cm^{-1} related to presence of $CO3^{-2}$ at crystal surface. This show good agreement with (Zhang et al. 2019: Zhu et al 2017) [12,13]

Preparation of bone graft with excellent biocompatibility, osteogenic capacity without immune rejection is a big challenge for researchers [14]. The prepared nano material showed enhancement of bone regeneration as compared to control group as well as lesser inflammatory response detected at defect treated with nano prepared material. The greater bone regeneration capacity of nano material is related to their osteoconductive properties when the biomaterial resorbs calcium and phosphate ions released stimulating proliferation and differentiation of osteoblast i.e. osteogenic activity and consequently bone regeneration. Khoshniat *et al*. (2011) [15] found that changes of ions concentration in the microenvironment lead to the expression of many genes include proliferation, differentiation, mineralization, and apoptosis of skeletal cells.

The results of our study agreed with Rezaei *et al* (201) [16]. The lesser inflammatory response at the treated defect may be related to anti-inflammatory action of hydroxyapatite and tricalcium phosphate. also difference in topography and the higher surface area of nano-structured material leading to increase ionic and protein depletion which delay macrophage and cellular proliferation these results are in agreement with Sadowska *et al*. (2019) [17] and Mestres *et al*. (2016) [18]

The more abundant osteoblast and osteoclast in the microenvironment of treated defect related to the presence of porosities that permit neovascularization to the core of material allowing migration of cells and transportation of nutrients. These results agree with Ebrahimi *et al* (2014) [19].

CONCLUSIONS

The prepared nano structured biphasic hydroxyapatite and tricalcium phosphate synthesized from eggshell showed a good bone regeneration capacity and excellent biocompatibility with very little inflammatory response and it could be used as bone substitute material prepared from eggshell of no economic value.

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