



Osteogenic Regenerative Ability of Hydroxyapatite and Tricalcium Phosphate (Osteon III) in Rabbits

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Abstract

Aims: Bone loss beyond the body's ability to regenerate may occur from various causes. The conventional methods of bone repair commonly used, such as autografts and allografts have their shortcomings and drawbacks. So, this study aims to evaluate the ability of biphasic hydroxyapatite and tricalcium phosphate OsteonIII (60% hydroxyapatite /40% tricalcium phosphate) for osteogenic regeneration in a rabbit model. **Materials and Methods:** Eighteen domestic rabbits were used in the current study. For each rabbit and following intramuscular general anesthesia, the selected site of surgery over the right femur was shaved and cleaned with povidone-iodine. A small incision of about 1.5 cm was created over the femur bone near its head avoiding any trauma to muscle. Two holes of 2 mm in depth and diameter and 15 mm apart were created under copious irrigation with distilled water in the femur. Using a small plastic scoop about 0.0260 g of Osteon III was added directly to fill one hole while the second hole was left empty to be filled with blood. At completion, the wound was closed using sutures. Based on this, the animals were divided into 2 groups and sacrificed at different time intervals at 7 days, 14 days, and 28 days. At the end of the aforementioned time interval, radiographic images of the femoral bones were taken at standard alignment and distance from the X-ray source, and histological assessment was conducted. **Results:** The osteon III bone defects showed high radio-opacity indicating new bone formation and mineralization due to the osteoconduction properties. **Conclusions:** This study supports that Osteon III may have better application prospects for bone repair.

القدرة التجديدية العظمية لهيدروكسيباتيت و فوسفات ثلاثي الكالسيوم (أوستيون الثالث) في الأرانب الملخص

الأهداف: قد يحدث فقدان العظام الذي يتجاوز قدرة الجسم على التجدد لأسباب مختلفة والطرق التقليدية لإصلاح العظام المستخدمة عادة مثل الطعوم الذاتية والطعوم الأخرى، لها عيوبها وإشكالياتها. إذن تمت الدراسة لاجل تقييم قدرة هيدروكسيباتيت ثنائي الطور و فوسفات ثلاثي الكالسيوم OsteonIII (60/40) لتجديد العظام في نموذج الأرانب. **المواد وطرائق العمل:** تم استخدام ثمانية عشر أرانباً منزلياً وتم حقن كل أرنب بالتخدير العام عن طريق الحقن العضلي، ثم حلق المنطقة الموجودة فوق عظم الفخذ الأيمن وتنظيفها باستخدام البوفيدون اليود. يتم إحداث شق صغير بطول 1.5 سم فوق عظم الفخذ بالقرب من رأسه لتجنب أي صدمة للعضلات. تُقَبان بعمق 2 مم وفصل بينهما 15 مم، تم إنشاء قطر تحت الري الغزير بالماء المقطر في عظم الفخذ. بواسطة ملعقة بلاستيكية يضاف حوالي 0.0260 غم من Osteon III مباشرة لملئ حفرة واحدة وترك حفرة ثانية فارغة مملوءة بالدم. تم تقسيم الحيوانات إلى مجموعتين وتم التضحية بها على فترات زمنية مختلفة في 7 أيام و 14 يوماً و 28 يوماً. بعد نهاية كل فترة زمنية تم التضحية بالأرانب في كل مجموعة وأخذت صورة شعاعية لعظم الفخذ في المحاذاة القياسية والمسافة من مصدر الأشعة السينية والتقييم النسيجي الذي تم إجراؤه. **النتائج:** يُظهر osteon III عتامة إشعاعية عالية بسبب تكوين العظام حديثاً وتمعدنها مع تأثير إيجابي لتكوين العظام بسبب خصائص التوصيل العظمي. **الاستنتاجات:** تدعم هذه الدراسة أن Osteon III ربما كان لديها احتمالات تطبيقية أفضل لإصلاح العظام.

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INTRODUCTION

Bone defects may develop in various systemic and dental disorders. The conventional methods of bone repair commonly used, such as autografts and allografts have their own shortcomings and drawbacks. Auto grafts are limited in terms of the availability of materials and may result in donor site morbidity. Using allografts may be more desirable in some cases, but the possible immune reaction and infection transmission limit their application.^(1,2,3,4) To overcome these limitations, various natural and synthetic biomaterials such as bone substitutes made of various composite structures have been introduced to accelerate and improve the process of bone regeneration.^(5,6) Owing to the increasing rate of invasive surgical procedures especially in the fields of orthopedics and dentistry, bone repair techniques using new materials are getting more popular. The new materials used should help to reduce the operation time, scar size, and post-operation pain, and also improve patient recovery.^(6,7) Recently, special attention has been made to many types of alloplastic biomaterials that have been introduced, and among these biomaterials is biphasic calcium phosphate (BCP) which shows promising results. Hydroxyapatite and tricalcium phosphate are the most commonly used calcium ceramic bone grafts. This BCP is composed of hydroxyapatite and tricalcium phosphate of different ratios. The sapience of this

the combination is to produce a material with optimum dissolution rates obtained from the resorption rate of TCP to be replaced by new bone and the slow degradability of HA providing mechanical support under load and volume^(4, 5, 8 - 10). This study aimed to evaluate the efficiency of Osteon III in accelerating bone healing in a rabbit animal model.

MATERIALS AND METHODS

This study was carried out at 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.

A total of 18 rabbits weighing 1.3-1.5 Kg and aged 3-4 months were chosen. A synthetic osteoconductive bone substitute (Osteon III). Osteon II is composed of 60% hydroxyapatite and 40% beta-tricalcium phosphate with a particle of size 1-2 mm and 0.5 cc volume (Dentium USA Made in Korea) porosity of 80% as determined by Fourier-transform infrared spectroscopy FTIR (to determine the function groups) and *Scanning electron microscope* SEM (to determine the surface morphology) and as shown in (Figures 1,2,3).

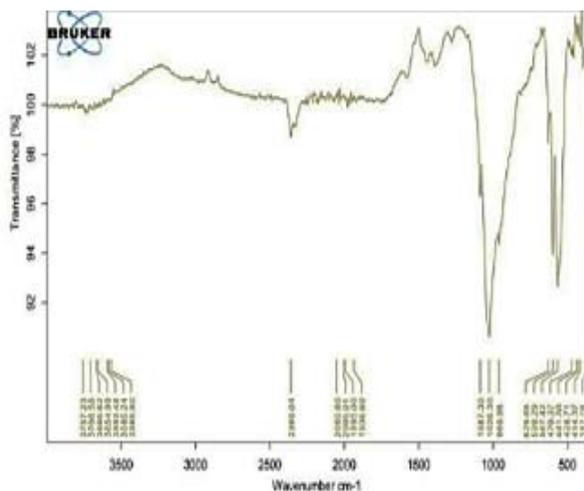


Figure (1): The infra-red spectra (FTIR) of OSTEONIII biphasic HA/TCP (60/40).

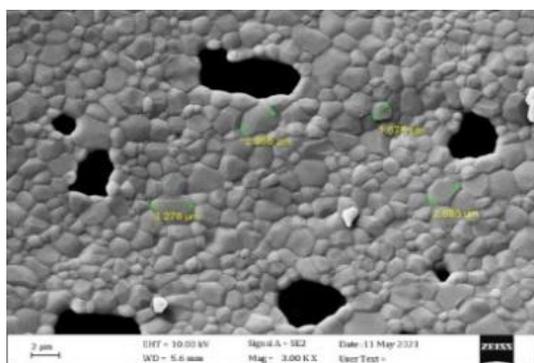


Figure (2): scan electronmicroscope of OSTEONIII biphasic HA / TCP (60/40) magnification power 3.00 KX.

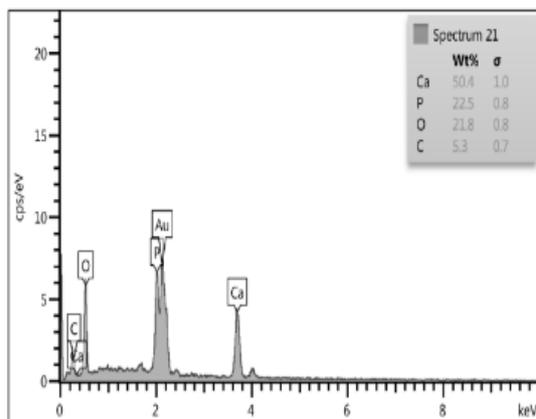


Figure (3): Energy dispersive spectra (EDS) of OSTEONIII was used to identify the chemical composition of the BCP HA/TCP (60/40).

Housing and feeding for all rabbits were the same and all rabbits were examined by a licensed veterinary

physician to check the animal's health condition. Each rabbit was given general anesthesia using a mixture of ketamine 0.6 mg/kg and Xylazine 0.3ml / kg injected intramuscularly. Following anesthesia, the rabbit was positioned on his left side and the area over the right was femur shaved and cleaned with povidone iodine. A small incision of about 1.5 cm was created over the femur bone near its head using surgical blade no.15 and avoid any trauma to muscle followed by blunt exposure of bone. Two holes of 2 mm depth and width being 15 mm apart, were created under copious irrigation with distilled water in the femur using a 2 mm carbide bur connected to a slow-motion dental engine. Using a small plastic scoop, 0.0260 g of Osteon III was added directly to fill one hole with the second hole left empty to be filled with blood. At completion, the wound was closed using a 3/0 silk black suture simple interrupted technique. The rabbits were monitored carefully after surgery till the time of sacrifice. In both groups, the sacrifice of animals was to be scheduled at 7 days, 14 days, and 28 days. Following the sacrifice and retrieval of the required specimen, a radiographic image of the femoral bone was taken at standard alignment and distance and angulation from the X-ray source using a radiographic digital system (Care stream®). The radiographic image was taken for each femur (as one piece which contains the 2 defects) All specimens were examined radiographically to evaluate the amount of

bone formation by digital radiograph system Carestream (that measure bone and bone marital density radio opacity by depending on No. of gray scale that range between 0-255). The setting of the machine was 60 kV, 10 mA and 0.30 seconds. The radio-graphical measurement done by drawing a line from the cortical bone crossing the defect by Cs imaging software 7.0.3. straight line from the cortical bone crossing the defect area Then femur specimen was then decalcified in nitric acid 15% for three days, after decalcification the specimens dehydrated through graded series of ethanol and xylene (70%-99%) then enclosed in paraffin wax and sectioned by microtome, each section of 4µm and stained by hematoxylin and eosin stain. Each slide was examined under light microscope by the examiner and supervisor

The histological parameters include:

1. Bone trabecular surface area.
 2. Number of osteoblasts.
 3. Number of osteoclasts.
 4. Degree of inflammation.
- The inflammatory response degree shown in (Table 1).

Table (1): The inflammatory response degree ⁽²¹⁾

Grade	Histopathological response observed
0	- Nil No inflammatory cells seen in the field of operation 100X
1	- mild /scanty When inflammatory cells present in few numbers, less than half of the field 100X
2	- Moderate Inflammatory cells could be seen in more than half of the field 100X
3	- Sever/marked Inflammatory cells present in large numbers more than 3/4 of the field 100X

All parameters were measured using special computerized image analysis soft ware named OMAX Toupview that

designed to be integrated with OMAX digital camera which is equipped to the light microscope used for study the specimen's visualization.

SPSS program version 25. was used to conduct the radiographically and histo-morphometrically analysis. Data were presented as means and standard deviation and analyzed by independent T-test. The significance was set at $p \leq 0.05$.

RESULTS

Radiographic Assessment:

At day 7 after surgery, the defect filled with Osteon III showed a higher radio-opacity compared with the control defect. The margins of the defect in the control group were clearly round compared with the Osteon III defect group in which the borders appeared obstructed and as shown in (Figure 4 A). At day 14 after surgery, the borders in the control group were still detected, while the borders in the Osteon III group were barely detected and material still detected and as shown in (Figure 4 B). At day 28 after surgery, the borders of both groups were not detected, and Osteon III group showed a higher radio-opacity and as shown in (Figure 4 C).

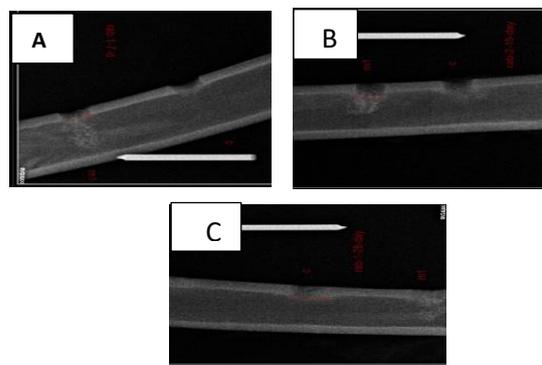


Figure (4): M and control defect at A=7 days, B=14 days, C=28 days after surgery.

Statistical analysis of radiographic observations showed that there was a statistically no significant difference between the defect filled with osteon III material and control defect at days7, there was significant difference between the defect filled with osteon III material and control defect at14, there was highly significant difference between the defect filled with osteon III material and control defect at 28 and as shown in (Table 2). The mean of radio opacity as fellow M>Cr in all period of study.

Table (2): Statistical analysis of radio graphical results, represented as mean and standard deviation of mean at significant level ≤ 0.05 .

Group Statistics				
	Factors	Mean	Std. Deviation	Sig. level ≤ 0.05
7 days	con	58.8333	13.28428	.160
	M	71.0000	15.83667	
14 days	con	56.8333	17.98533	.003
	M	87.0000	7.61577	
28 days	con	71.0000	4.79583	.000
	M	90.6667	2.94392	

Histopathology Assessment:

At day 7, histological findings in the control group showed granulation tissue formation with inflammation (score 3) and scanty amount of new bone formation (bony spicules) and as shown in (Figure 5).

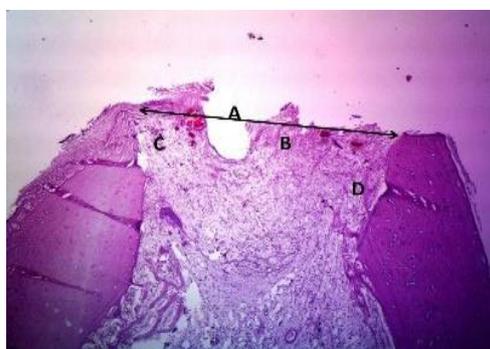


Figure (5): Histological section of control group7 days after surgery at 40 X magnification. H&E stain. (A= wound site, B= granulation tissue, C= inflammation tissue, D= bony spicules)

In the study group defects, histological observations showed the material in red color and granulation tissue characterized with inflammation (score 1) and a scanty amount of new bone formation (bony spicules) as shown in (Figure 6).

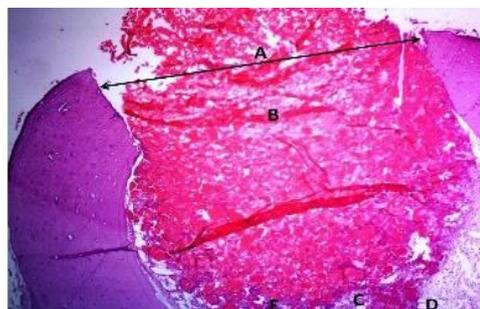


Figure (6): Histological section of M group7 days after surgery at 40 X magnification. H&E stain

At day 14, histological findings in the control group showed granulation tissue formation with inflammation (score 1) and moderate amount of new bone formation (bony spicules) and as shown in (Figure 7).

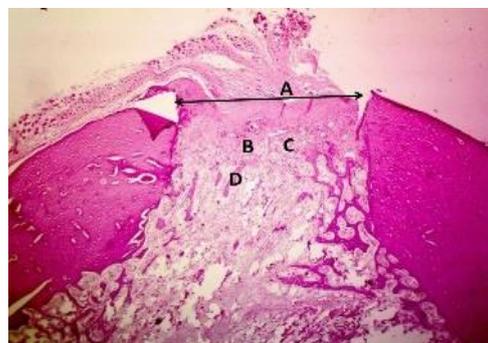


Figure (7): Histological section of control group14 days after surgery at 40 X magnification. H&E stain.

On the other hand, in the study group defects, histological observations showed the material in red color and granulation tissue characterized without inflammation (score 0) and moderate amount of new bone formation (bony spicules) and as shown in (Figure 8).

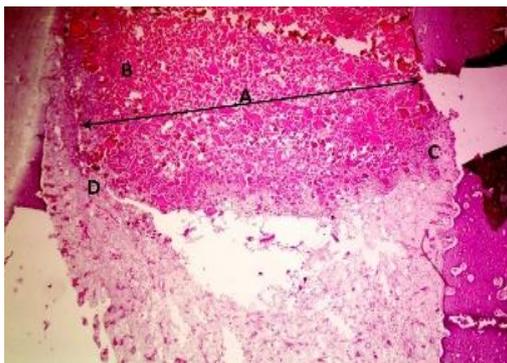


Figure (8): Histological section of M group 14 days after surgery at 40 X magnification. H&E stain.

At day 28, histological findings in the control group showed no granulation tissue formation and inflammation (score 0) and a thin layer of new bone formation (bony spicules) seen almost closing the defect and as shown in (Figure 9).

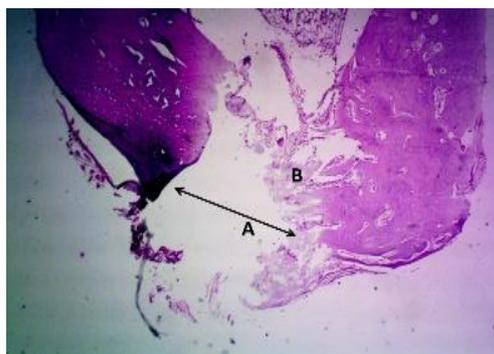


Figure (9): Histological section of control group 28 days after surgery at 40 X magnification. H&E stain.

In the Osteon III group, histological observations showed no granulation tissue and inflammation (score 0) with clear evidence of biomaterial degradation and presence of profound amount of new bone formation filling the defect and as shown in (Figure 10).

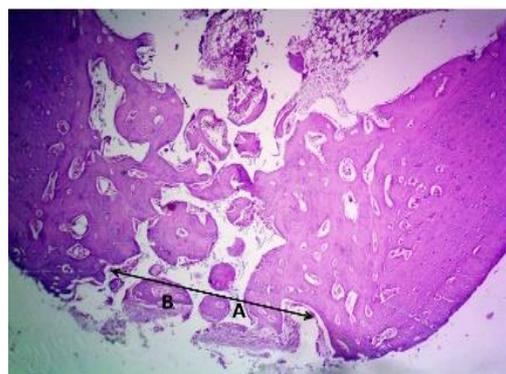


Figure (10): Histological section of M group 28 days after surgery at 40 X magnification. H&E stain.

In regard to bone surface area and throughout the periods of study, the osteon III material showed greater bone surface area formation with a statistically significant difference and as shown in (Table 3).

Table (3): Statistical analysis of new formed bone area represented as mean and standard deviation of mean at significant level ≤ 0.05 .

		Group Statistics		
Factors		Mean	Std. Deviation	Sig. level ≤ 0.05
7day	Con	2940.8200	319.95640	.002
	M	3942.6000	535.06533	
14day	Con	12623.4229	733.56853	.000
	M	18514.2833	2321.28414	
28day	Con	19404.6671	1233.42489	.000
	M	31278.5000	2932.06477	

A higher number of osteoblasts was found in the Osteon III defect compared to the control defect and a statistically no significant difference between both groups observed throughout the period of study as shown in (Table 4).

Table (4): Statistical analysis of osteoblast numbers represented as mean and standard deviation of mean at significant level ≤ 0.05 .

Group Statistics				
	Factors	Mean	Std. Deviation	Sig.level ≤ 0.05
Day 7	CON	7.0000	1.63299	.697
	M	7.3329	1.49071	
Day14	CON	18.1714	1.57238	.545
	M	17.3329	3.19722	
Day28	CON	23.0000	3.21455	.601
	M	22.1671	2.54406	

In addition, a higher number of Osteoclasts was observed in the Osteon III defect when compared with the control defect and a statistically highly significant difference disclosed between both groups at days 14 and 28 and shown in (Table 5).

Table (5): Statistical analysis of osteoclast numbers represented as mean and standard deviation of mean at significant level ≤ 0.05 .

Group Statistics				
	FACTOR	Mean	Std. Deviation	Sig.level ≤ 0.05
Day 7	CON	.5000	.50000	1.000
	M	.5000	.50000	
Day 14	CON	3.5000	.95743	.015
	M	5.8329	1.95078	
Day 28	CON	5.0000	1.29099	.056
	M	6.1671	.68719	

DISCUSSION

In the current study, the radiographic results revealed a higher radio-opacity in Osteon III filled bone defects versus control defects due to new bone formation and a higher mineralization ration achieved with a slow degradation of Osteon III which coincided with results disclosed in some studies. (5,7) It was noticed that the degradation of ceramics (i.e. by its solubility properties and resorption activity of osteoclast) which depends on the HA/ β

-TCP ratio within its composition, they observed that solubility influences the pattern of osteoblastic resorption activity in terms of shape and distribution of resorption lacunae. For example, in pure β -TCP, lacunae appear discontinuous like a chain of small islands whereas they are large and continuous on BCP (resorption activity β -TCP>BCP>HA) resembling those on bone. In addition, the shift in functional phases of osteoclast from resorption to migration seems to occur earlier on β -TCP than on BCP with different HA/ β -TCP ratio.(11)

By controlling the ratio of HA- β TCP of BCPs, it should be possible to control not only the resorption rate of BCPs but also the release of Ca⁺², P in the vicinity of bone cells, and consequently modulate the biological properties of BCPs. Some free ions Ca⁺² and inorganic P released could thus trigger the osteogenic differentiation. The results of the current study displayed an enhancement of bone regeneration when using Osteon III material as the biomaterial resorbs free calcium and phosphate ions released and the change in the ions concentration stimulate formation and differentiation of osteoblast and eventually bone formation either by directly response or by the expression of Ca⁺² binding-proteins and Ca²⁺ incorporation into the extracellular matrix.(12,13, 14) Moreover, the increase in inorganic phosphate (Pi) has also been shown to act as a specific signal, affecting the expression of various genes implicated

in the proliferation, differentiation, mineralization and apoptosis of skeletal cells. In addition, by hydrolyzing inorganic pyrophosphate ions (PPi) into Pi, ALP promotes type I collagen mineralization by preventing the inhibitory action of PPi on mineralization. A sustained release of Pi was shown to upregulate the mineralization process of collagen by overriding the inhibitory effect of PPi.^(13,15) The results of the current study came into agreement with Puttini et al. (2019).⁽¹²⁾ Biphasic calcium phosphate is made from hydroxyapatite (HA) $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ and tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ and are definitely considered the gold standard of bone substitutes in bone reconstructive surgeries.⁽¹⁶⁾ Its extent of dissolution depends on the β -TCP/HA ratio, the higher the ratio, the higher the extent of dissolution.⁽⁵⁾ Biphasic calcium phosphate has the advantages of providing better bioactivity and controlling resorption and solubilization of the scaffold and this in turn enhances stability leading to better bone ingrowth.⁽¹⁷⁾ The HA in this scaffold provides better mechanical properties while the TCP provides a better biodegradation rate. The properties of the BCP manipulated by the composition ratio of HA/TCP may be the reason behind the increase in the number of osteoclast along the period of study.⁽⁴⁾ while the of scaffold provided by Osteon III (80% porosity and a particle size of 1.0-2.0mm) may be the reason in the increase in number of osteoblasts along the period of study.⁽¹⁸⁾

The porosities act as a channel for cell migration and protein adhesion, diffusion of cells, angiogenesis and nutrients transportation. These porosities act as a nidus for formation of new blood vessels. These events coincided with the results of Ebrahimi et al (2014)⁽¹⁹⁾ whom found that BCP stimulates cellular accumulation. The inflammatory reaction in the defects filled with Osteon III was less than that seen in the control defect groups which showed a severe inflammatory response at 7days. This may be due to the anti-inflammatory action of both hydroxyapatite and tricalcium phosphate. These results agreed with Sadowska *et al.* 2019.⁽²⁰⁾ Different studies have been conducted to form a BCP with different HA/TCP ratios in order to enhance its properties yet no agreement has been suggested regarding the optimum HA / TCP ratios. Ratios of 60 / 40 and 50/50 had been used in human studies with encouraging results.⁽¹⁶⁾

CONCLUSION

In conclusion, Osteon III material showed an acceleration in bone formation and a high potential capacity of 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.

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