

Comparison of the effect of Nano Ostrich Eggshell and Hydroxyapatite on Bone Defect Healing in Rat Calvaria

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Abstract

Background: Fracture healing is important in medicine; thus seeking new techniques with fewer side effects to improve the speed of the healing is prudent.

Objectives: This experimental study sought to assess the effect of the nano ostrich eggshell (N-OES) and hydroxyapatite (HA) composite on bone defect healing in rat calvaria.

Methods: In this study, a 7-mm bone defect was created in the calvaria of 45 male Sprague–Dawley rats. The animals were randomly divided into three groups, and the defects in each group were filled with N-OES, HA, or as control group. The animals were euthanized and histological and serological assessments were carried out at 14, 28 and 42 days after operation.

Results: At 14 and 28 days after operation, in the edges of the defect, there was a significant difference between the treatment and control groups ($p \leq 0.05$). However, there were no statistically significant differences between N-OES and HA treatment groups ($p > 0.05$). At this time, in the center of the defect, there was a statistically significant difference between the N-OES group with HA and control groups ($p \leq 0.05$). On day 42, no significant difference in the edges of the defect was observed between all groups. However, there was a statistically significant difference in the center of the defect between the N-OES group with HA and control groups ($p \leq 0.05$). Results of the serological assessment indicated that the applied treatments increased the serum levels of alkaline phosphatase.

Conclusions: The results of this study indicate the potential efficacy of nano ostrich eggshell as a bone substitute in rat calvaria defects.

Keywords: Nano Ostrich Eggshell, Calvarium, Hydroxylapatite, Rat.

Introduction

Given the vital functions of bone tissue, any major change in the bone structure following a disease or external damage may substantially affect the balance of the body and the patient's quality of life. Fortunately, most of the bone tissue damages heal by themselves and require minimal treatment. However, there are several conditions such as misaligned bone healing, complete bone loss due to a tumor, and infection of the damaged region, which certainly requires medical treatment. In such cases, various methods, including bone grafting or metal implants can be used for complete bone repair.¹ Although bone autograft is the golden standard of bone graft treatments, limited availability of graft sources makes it ill-suited for many patients, especially those with major bone lesions. Moreover, bone grafting is associated with the risk of infection. An alternative method for the treatment of bone lesions is using metal implants, but these implants have a chance of releasing detrimental ions, which

can accumulate in several organs, which can increase the risk of cancer in those regions. For several decades, researchers have been trying to find novel materials and methods to address these challenges. These efforts have led to the development of a group of materials known as biodegradable biomaterials as well as many advancements in the field of tissue engineering.²

Bird eggshells are very similar to corals in terms of mineral composition and can be viewed as another potential bones substitute for maxillofacial operations. Hence, in recent years, there has been a growing interest in the improvement of functional specifications of hydroxyapatite with alternative materials.³ For example, Kattimani et al, evaluated the efficacy of eggshell derived hydroxyapatite (EHA) in the bone regeneration of human maxillary cystic bone defects secondary to cystic removal/apicoectomy and compared the material properties of EHA in vitro. In the mentioned study, they showed that the osseous regeneration of the bone defect

filled with EHA is significant. EHA showed superior material properties in comparison with synthetic hydroxyapatite.⁴ Baliga et al., have reported the biocompatibility of surface modified eggshell material in cystic cavities of human jawbones with centripetal ossification, which occurred within six weeks.⁵ In Park et al., study on the effect of chicken eggshells and bovine allograft on the repair of rat calvaria, it was found that the great potential of eggshell particles as a bone graft material for rat calvarial defect.⁶ Abdulrahman et al., reported that bird eggshells have a high hydroxyapatite production potential. They also argued that the successful extraction of hydroxyapatite from eggshells could reduce the cost of treating bone lesions, due to its low cost, high availability, and biodegradability of eggshells, which make it a good source of calcium carbonate for bone grafts.⁷

Objectives

In the present study, we investigated the effects of nano-powder of ostrich eggshell and synthetic hydroxyapatite in calvarial bone defect healing in rats.

Materials and Methods

Animals

In the study, forty-five male Wistar rats, weighing 250–300 g were used. The rats were housed for two weeks at the facility for acclimatization. All animals were housed individually in light- and temperature-controlled facilities and maintain with ad libitum access to a standard laboratory diet and water. The Ethics Committee of the School of Veterinary Medicine, Semnan University of Medical Sciences, Iran (No. 43-17/7/96), approved this study.

Surgical procedure

Nano ostrich eggshell composite was prepared according to the method described by Sanosh et al., in 2009.⁸ XRD pattern of a nano-structured eggshell is shown in (Figure-1). The rats were anesthetized intraperitoneally with 50 mg/kg of 10% ketamine hydrochloride (0.05 mL/100 g) and 5 mg/kg of xylazine hydrochloride (0.025 mL/100 g).

The dorsal part of the cranium was shaved and disinfected. Surgical sites were exposed with an incision through the skin and the periosteum at the midline of the calvaria. After the rat calvarium was exposed, a 7 mm circular full-thickness bone defect was created with a trephine bur on the midline, without damaging the underlying dura mater (Figure-2, A-C). Because the dura may play an important role in bone healing and regeneration. The bone is not completely penetrated by the trephine to avoid damage to the underlying tissues. The animals were randomly divided into three groups (15 animals per group) for experimental periods of 14, 28 and 42 days

In the HA group, the defects were filled with hydroxylapatite with granules of 1–5 mm. In the N-OES group, the defects were filled with nano-particles of ostrich egg-shell with granules of <100 nm. The control group, receiving no HA or N-OES therapy.

The soft tissues were sutured with 4-0 mono nylon to achieve primary closure (Figure-2, D). To prevent postoperative infection, cefazoline was given to the animals as intramuscular injections for 3 days (30 mg/kg). Flunixin (Razak Co. Iran) as an analgesic was administrated (2.5 mg/kg) intramuscularly.

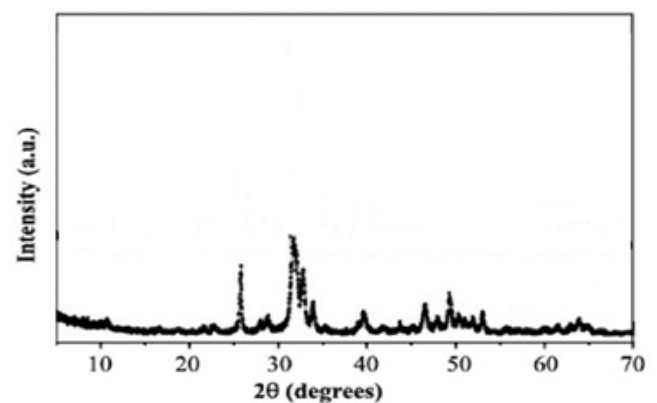


Figure-1. XRD pattern of nano-structured eggshell



Figure-2. A, a 7 mm circular full-thickness bone defect created in rat calvarium, B, defect was filled with hydroxyapatite and C: nano ostrich eggshell, D, soft tissues were sutured

Specimen preparation and histological evaluation

Animals were euthanized with a lethal dose of thiopental (150 mg/kg), at post-operative periods of 14, 28 and 42 days. The skin was dissected and the area of the original surgical defect was removed en bloc with the surrounding tissues from the animals' calvarium bone and then immediately submerged in 10% neutral buffered formalin for 48 h. Afterward, the calvarium bone was rinsed with water and then demineralized in 10% formic acid. After decalcification, each specimen was divided longitudinally into two blocks in the sagittal direction and embedded in paraffin. Serial sections were cut longitudinally, beginning at the center of the surgical defect. The sections were stained with hematoxylin and eosin for analysis. In the histopathologic evaluation, the progression of bone defect healing was investigated in three regions (two edges and center of the defect) in each sample. The levels of granulation tissue formation, fibrosis tissue, immature bone tissue, and adult ossification were evaluated in order to obtain healing rates in different samples.

Measurement of alkaline phosphatase activity

At post-operative periods of 14, 28 and 42 days, and before euthanasia, blood samples were taken from the heart of the animals. After separating serum, ALP activity was determined using a colorimetric ALP activity assay kit (Pars Azmoon, Iran).

Data Analysis

Statistical analysis was performed using SPSS software (version 16.0, SPSS Inc, USA) and Kruskal-Wallis test. Data were expressed as mean±standard deviation (SD). Differences were considered significant at $P < 0.05$.

Results

Table-1 presents the results of histopathological studies. Based on the results, after 40 days, the granulation tissue accompanied by new angiogenesis and edema, and bone matrix formation were observed on the edge of a defect in the control group. In the HA group, the immature bone tissue was observed, even negligible, on the edge of defect, unlike the control group on the same day. In the N-OES group, there was the progression of healing tissue to fibrous tissue on the edge of the defect along with increased thickness and

order of collagen fibers and bone tissue formation in comparison with control and HA groups. Accordingly, there was a significant difference between two treatment groups with the control group on the edge of the defect ($p \leq 0.05$), but the difference between two treatment groups was not significant ($p > 0.05$). It was also found that there was a significant difference between three groups in the center of the defect ($p \leq 0.05$), so that the highest healing rate was seen in the N-OES group (**Figure-3**).

The bone matrix creation and immature bone formation continued in the control group on the twenty-eighth day. The bone matrix formation and its distinction from immature bone as well as the progression of ossification were obvious in the HA group. There were increased new immature bone tissue and the progression of fibrosis tissue in the N-OES group. In addition to the formation of immature bone tissue (woven), the formation of a parallel mature bone (Lamellar) was also observed in contrast to the control and HA groups on this day. There was also a large amount of calcium deposition for the continuation of bone formation. Accordingly, there was a significant difference between the two treatment groups with the control group on the edge of the defect ($p \leq 0.05$), while the difference between the two treated groups was not significant ($p > 0.05$). The results revealed that there was a significant difference in the defect place between two groups and the N-OES group ($p \leq 0.05$), while no significant difference was seen between HA and control groups ($p > 0.05$) (**Figure-4**).

On the forty-second day, the progression of defect healing was seen towards dense fibrosis along with thickened collagen fibers in the control group. In addition to immature bone tissue, a small amount of lamellar mature bone was observed on the edge of the defect. In the HA group, the immature and irregular bone was formed on the edge of the defect; and the parallel and blade mature bone formation was higher than the control group at the same time. In the N-OES group, both edges of the defect site were reaching together by new bone tissue. In this group, there was a progressive blade and regular mature bone indicating that the treatment was successful. Moreover, there was no significant difference on the edge of the defect between studied groups ($p > 0.05$). It was also found that there was a significant difference between N-OES group with the other two groups in the center of the

defect ($p \leq 0.05$), while no significant difference was observed between HA and control groups ($p > 0.05$) (Figure-5).

In the field of ALP rate, the results of the present study indicated that applied treatments increased serum levels of Alkaline Phosphatase in studied groups indicating an increase in healing trends in studied groups. In this regard, a

decreasing trend was observed only in the HA group on the 28th day in comparison to the 14th day. This difference was not statistically significant and could be due to a second-degree statistical error and because of the low sample size and higher standard deviation in the results of this group (Table-2).

Table-1. The Mean \pm SD of the histopathological changes in all groups.

Days	Defect site	N-OES	HA	Control	P value
14	margins	9.75 \pm 0.5 ^a	9.8 \pm 0/45 ^a	4.75 \pm 2.87 ^b	0.016
	center	9 \pm 1.15 ^a	6.8 \pm 0.83 ^b	2.66 \pm 1.15 ^c	0.01
28	margins	9.5 \pm 1 ^a	8.75 \pm 0.95 ^a	7.6 \pm 0.54 ^b	0.037
	center	7.75 \pm 0.95 ^a	4.25 \pm 1.26 ^b	4 \pm 2.34 ^b	0/029
42	margins	9 \pm 0.7	9.2 \pm 0.83	9.4 \pm 0.5	0.835
	center	8.4 \pm 0.89 ^a	5.4 \pm 3.13 ^b	6 \pm 0.7 ^b	0.024

N-OES: Nano ostrich eggshell, HA: hydroxyapatite. There was no significant difference between the same capital letters in each column, but significant difference was observed between the non-identical lowercase letters (a, b and c) in each row. Data were represented as mean \pm SD values. The difference was significant ($P \leq 0.05$)

Table-2. The Mean \pm SD of the ALP changes in all groups.

Groups	Days 14	Days 28	Days 42	P value
N-OES	444.42 \pm 57.85	488.05 \pm 27.9	496.89 \pm 42	0.197
HA	545.01 \pm 51.84	467.43 \pm 116.98	515.56 \pm 77.74	0.363
Control	304.74 \pm 10.64	380.23 \pm 57.25	413.42 \pm 103.36	0.093

N-OES: Nano ostrich eggshell, HA: hydroxyapatite. Data were represented as mean \pm SD values. The difference was significant ($P \leq 0.05$)

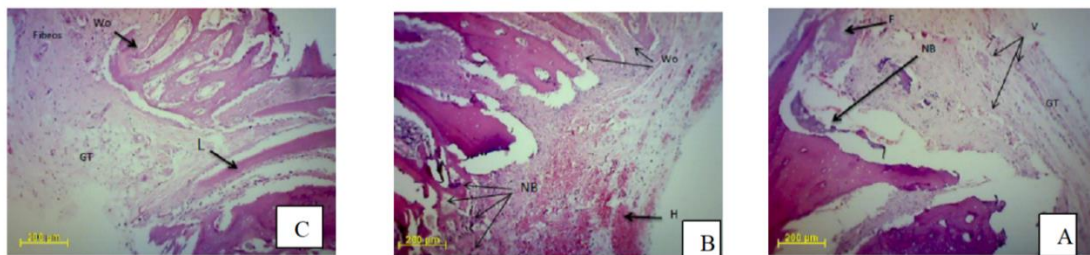


Figure-3. Photomicrograph of all groups 14 days after operation. A: in the control group, the granulation tissue accompanied by new angiogenesis and edema, and bone matrix formation were observed on the edge of defect. B: in the HA group, the immature bone tissue was seen. C: In the N-OES group, there was the progression of healing tissue to fibrous tissue on the edge of defect along with increased thickness and order of collagen fibers and bone tissue formation. N-OES: Nano ostrich eggshell, HA: Hydroxyapatite, Co: Control, Wo: Woven, L: Lamellar, H: Hematoma, NB: new bone ossification, V: Vessel, GT: granular tissue, F: Fibrous (H&E, $\times 100$).

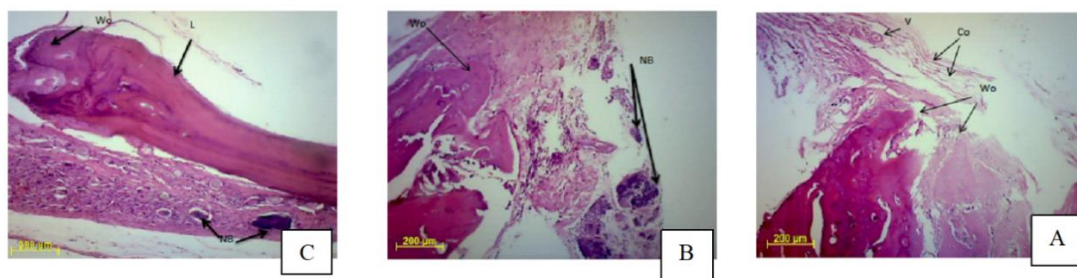


Figure-4. Photomicrograph of all groups 28 days after operation. A: The bone matrix creation and immature bone formation continued in the control group. B: The bone matrix formation and its distinction from immature bone as well as the progression of ossification were obvious in the HA group. C: There were increased new immature bone tissue and the progression of fibrosis tissue in the N-OES group.

N-OES: Nano ostrich eggshell, HA: Hydroxyapatite, Co: Control, Wo: Woven, L: Lamellar, H: Hematoma, NB: new bone ossification, V: Vessel, GT: granular tissue, F: Fibrous (H&E, $\times 100$).

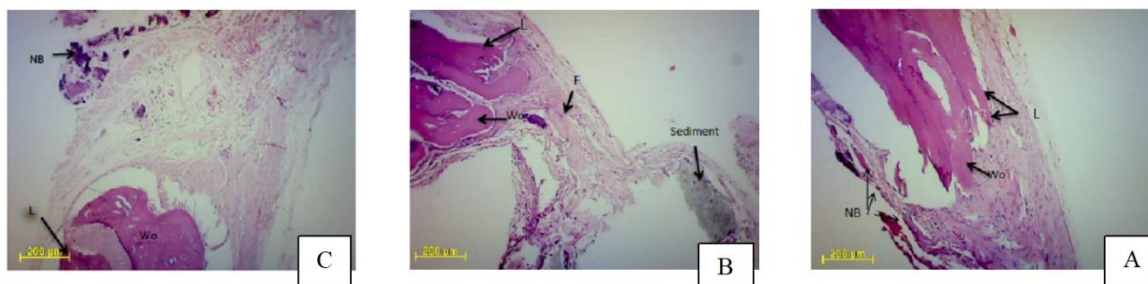


Figure-5. Photomicrograph of all groups 42 days after operation. A: The progression of defect healing was seen towards dense fibrosis along with thickened collagen fibers and a small amount of lamellar mature bone in the control group. B: In the HA group, the immature and irregular bone was formed on the edge of defect; and the parallel and blade mature bone formation was seen. C: In the N-OES group, both edges of defect site were reaching together by a new bone tissue. N-OES: Nano ostrich eggshell, HA: Hydroxyapatite, Co: Control, Wo: Woven, L: Lamellar, H: Hematoma, NB: new bone ossification, V: Vessel, GT: granular tissue, F: Fibrous (H&E, $\times 100$).

Discussion

In the present study, histopathologic examinations of the center and margins of the lesion were performed on days 14, 28 and 42 after the operation. On the 14th and 28th days, both N-OES and HA groups showed significantly better healing rate in the margin of the lesion compared to the control group. In these instances, the healing rates in the center of the lesion was significantly better in the N-OES group than in both the HA group and control group. On the 42nd day after surgery, the three groups had no significant difference in terms of healing at the edge of the lesion, but the N-OES group showed significantly better results in the center of the lesion than both the HA and control groups. Since the process of healing and reconstruction of a fractured bone begins from the edges of the lesion and gradually progresses towards the center, the differences observed in the extent of bone regeneration in the edges and center of the lesion highlight the positive effect of nano-powder of ostrich eggshells on healing. This positive effect is reflected in the fact that at all stages of examination, healing progressed faster in the N-OES group than in the other two groups. The results implied that nano-hydroxyapatite exhibits not only bone conduction property but also bone induction feature.⁹ According to Coelho et al., study, nano-hydroxyapatite enhanced bone regeneration by stimulating osteoblasts and facilitating their separation and differentiation. In other words, nano-hydroxyapatite stimulates bone regeneration in the early stages of healing by forming a scaffold for bone growth and stimulating osteogenic progenitors.¹⁰

Webster et al., showed that nano-hydroxyapatite enhanced the formation of new bone tissues by increasing the binding

of osteoblasts to the scaffold, to each other, and to calcium-containing minerals on the scaffold surface.¹¹

In this study, the difference between the two treatment groups can be attributed to the origin of their compounds and the size of their particles. Many clinical studies have shown that hydroxyapatite powders extracted from bird eggshells enhanced tissue compatibility and osteogenic stimulation with minimum inflammatory responses. Meanwhile, synthetic hydroxyapatites that are extracted from marine products and corals, exhibit limited healing effects compared to animal products.^{12, 13}

In Lee et al., study on the effect of hydroxyapatite extracted from eggshell in comparison with hydroxyapatite extracted from marine products for healing parietal bone lesions in rats, it was found that both treatment groups showed better performance than the control group. However, a comparison between the two groups revealed that the hydroxyapatite extracted from the eggshell performed better in terms of bone regeneration.¹⁴ These results are consistent with the histopathological findings of the present study. This study also showed that the hydroxyapatite extracted from eggshell contains higher levels of magnesium than the one extracted from marine products, which may be due to the higher rate of healing in the nano-eggshell group. Because the important role of magnesium in bone metabolism and bone regeneration is widely accepted.^{15, 16} Crespi et al., investigated the effects of magnesium-enriched hydroxyapatite implants on lesions in pig tibia and reported that due to the high levels of magnesium contained in the implant, it was much more effective in bone regeneration than other solutions.¹⁷

It has been reported that the size of the particles used in bone grafts is a key determinant of their degradation rate.¹⁸

According to Dupoirieux et al., ultrafine particles with a diameter of about 50 nanometers result in faster bone recovery than those of 150 and 300 nm in size.¹⁹ In the present study, eggshell particles were in nanoscale, but the size of hydroxyapatite particles ranged from 1 to 5 nm. Therefore, the difference in terms of healing rate observed between the treatment groups can be attributed to their different sizes.

Regarding the ALP level, our results showed that the treatments increased the serum level of alkaline phosphatase in the groups, indicating an enhanced healing rate. The only decreasing trend was seen in the hydroxyapatite group between the 14th day and 28th day, but this decrease was not statistically significant and could be due to type II statistical error, the small sample size, and high standard deviation in the data of this group.

The findings suggested that the bone formation and reconstruction process coincided with the release of a number of enzymes and proteins in the blood, which reflected the activity of osteoblasts and the stability and consolidation condition of calluses formed during bone regeneration.^{20,21} After a fracture, the level of alkaline phosphatase is significantly increased, and thereby stimulates the hydrolysis of phosphomonoesters. The release of mineral phosphate plays a role in bone mineralization. Therefore, alkaline phosphatase is one of the most accurate serum markers of bone regeneration.^{22, 23}

Conclusions

Overall, our results suggest that using the nano-powder of ostrich eggshell instead of hydroxyapatite leads to faster osteogenesis in the 42-day period and better healing of calvarial bone defect in rats. Hence, the nano-powder of ostrich eggshell can be used as a suitable option for improving bone healing in orthopedic operations as well as tissue engineering applications. Since ostrich eggshells can be recycled from food waste, they can be used as a cheap, available and eco-friendly choice for a calvarial bone defect.

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None.

Authors' Contribution

All authors pass the four criteria for authorship contribution based on the International Committee of Medical Journal Editors (ICMJE) recommendations.

Conflict of Interests

The authors declared no potential conflict of interests with respect to the research, authorship, and/or publication of this article.

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