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## Effect of a nanocomposite containing ostrich eggshell on calvarium healing in the rabbit: a pathologic study

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**ABSTRACT.** The aim of the current study was to determine effect of a nanocomposite containing ostrich eggshell (NCOE) on the calvarium healing in the rabbit. Fresh ostrich eggshell was ground (300-500 µm), treated in phosphate-containing solutions and sterilized by gamma irradiation. Fifteen New Zealand white adult male rabbits were used. Four full-thickness skull defects were created in the calvarium. The first defect kept unfilled (control). The second defect was filled with autograft bone. The third defect was filled using NCOE. The fourth defect was filled with mixture of the autograft+NCOE bone. At 30, 60 and 90 days after surgery animals were euthanized and tissue specimens were collected and stained with hematoxylin eosin and trichrome staining method. Microsections were examined to assess the extent and intensity of inflammation, calvarium formation status and foreign body reaction. According to the results, filling defect significantly increased in NCOE-treated rabbits compared to the control group at 30 and 60 days post-surgery ( $P<0.05$ ). There a statistically significant difference between experimental groups compared to the control group at 30 and 60 days post-surgery ( $P<0.05$ ) while no statistically significant differences were observed among autograft, NCOE, autograft+NCOE ( $P>0.05$ ). Also, absorb material significantly decreased in NCOE and autograft+NCOE groups compared to the control group at 60 days post-surgery ( $P<0.05$ ). The filling defect significantly increased in autograft, NCOE and NCOE+autograft groups compared to the control group at 90 days post-surgery ( $P<0.05$ ). There was no significant difference on inflammation and absorb material among the groups at 90 days post-surgery ( $P>0.05$ ). These results suggested NCOE+autograft has improved the rate of calvarium healing in rabbits.

**Keywords:** Nano- composite, Ostrich eggshell, Calvarium autograft, Rabbit

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## INTRODUCTION

In the last decade, guided tissue regeneration provided new research area in the bone reconstruction field (Lim et al., 2010). One of the most important factors is biocompatibility and degradation rate of the membranes (Lim et al., 2010). Autogenous bone grafts (ABG) are known as the gold standard and preferred augmentation material (Jones et al., 2010). The ABG has high compatibility with the host tissue and effective in bone graft healing without immune response (Lindhe et al. 2008). High costs donor and prolonged operation times are the disadvantages of the ABG (Jones et al., 2010). Combination of the bone grafts with other treatments enhances the engraftment, bone formation and defect healing (Marx, 2004). Application of the adjuvant bone graft as primary goal of guided bone regeneration (GBR) is an alternative method to promote bone regeneration without additional bone grafting (Yang et al., 2014). Several synthetic allografts have been used for bone regeneration, but there is growing interest to use natural allografts (Toker et al., 2012).

Avian egg shell contains calcium carbonate (97.4%), magnesium phosphate (1.9%) and triphosphate (0.7%) (Durmuş et al., 2008), however, the composition differs among species. The high mineral level can accelerate hydroxyapatite formation in bone regeneration (Leung et al., 2005). Also, eggshell matrix contains proteins (70%), Chondroitin sulphates A and B (35%) and polysaccharides (11%) (Dupoirieux et al., 1995). Dupoirieux et al., (2000) studied role of the pericranium and eggshell as space fillers used in combination with GBR in rat and no resorption or osteoconduction was revealed in eggshell powder of the hen eggshell had safe and inexpensive application in rabbit bone defect model (Dupoirieux et al., 1995). Application of the ostrich eggshell powder had no effect on bone regeneration in rabbits (Durmuş et al., 2003).

Large ostrich grafts are suitable as onlay graft, however a complementary osteosynthesis is suggested to improve osteointegration (Dupoirieux et al., 2001). Eggshell powder increased cell growth and tibia remodeling in dogs (Yadegari et al., 2015). Nanocomposites or nanomaterials contain high level of the collagen and hydroxyapatite and their application has drawn attention compared to bone grafts. There is scarce information on effects of the nanomaterials in bone formation. The small molecular structure of the nanocomposites increases the bioactivity of the

materials used for bone defect healing and regeneration (Dupoirieux et al., 2001). This is due to composition and structural similarity with natural bone as well as larger surface area and superior mechanical strength (Biazar et al., 2015). Because of complexity of the osteoclast and osteoblasts and the other factors the clinicians were compelled to search for alternative bone graft substitutes in regenerating process in human (Dupoirieux et al., 2001). In a recent study, Alemi et al. (2018) showed that nanocomposite containing ostrich eggshell (NCOE) + autograft has positive effects on bone density without adverse effect on white and red blood cells hemoglobin, hematocrit, mean cell volume and platelets levels in rabbit. There is no information on role of the NCOE in bone formation in the rabbit. The aim of the current study was to determine effect of the NCOE on calvarium healing in rabbits.

## MATERIALS AND METHODS

### Animals

Fifteen adult male New Zealand white rabbits (3-3.5 kg) were purchased from the Razi Vaccine and Serum Research Institute (Tehran, Iran) (Durmuş et al., 2008; Paknejad et al., 2015). For acclimatization the animals were kept in individual cages in the laboratory at constant and optimum environmental and nutritional conditions (22 °C, relative humidity 50%) with a 12-hour light/dark cycle. During the study animals provided *ad libitum* commercial chew pellet and tap water. Study procedures were done during the 10:00-17:00 h light phase and executed in accordance with the Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals (Zimmermann, 1983) and National Institutes of Health (USA) and the current laws of the Iranian government. All experiment procedures were approved based on the guidelines for the animal care board of the Islamic Azad University, Faculty of Veterinary Medicine and besides, the current study was approved by the university ethics committee (Ethic code:25876).

### Ostrich eggshell preparation

Fragmented ostrich eggshells were immersed in boiling sterile distilled water and the outer and inner shell membranes of the eggs were removed with forceps. The shells were crushed and sieved until particles (particle diameter, 1mm; porosity, 75%) were obtained (Uraz et al., 2013). Ostrich eggshell ground the into 300-500 µm pieces using an electrical mill, washed 3 times with distilled water, dried and steril-

ized using ethylene oxide (Durmuş et al., 2003). The eggshell powder was immersed in sodium hypochlorite (5%) and then organic components were removed by sodium hydroxide solution (Durmuş et al., 2003). Eggshell powder subsequently washed in deionized water and heat-treated at 300 °C for 24 h. Then treated in phosphate-containing solutions with different hydrothermal conditions (Pastoureau et al., 2010). Soaked in sealed glass bottles containing phosphate buffer saline at 80 °C for 6 days (the solution replaced every 3 days) (Permuy et al., 2014), then soaked in Teflon lined reactor containing 2 wt % di-ammonium phosphate solution at 150 °C for 24 h, then transferred in a phosphate-containing solution (19.5 mM PO<sub>4</sub><sup>3-</sup>, 30 mM Na<sub>2</sub>HPO<sub>4</sub> and 4.3 mM K<sub>2</sub>HPO<sub>4</sub>) at 80 °C for 6 days (Permuy et al., 2014). All ostrich eggshell particles were sterilized by gamma irradiation before use (Permuy et al., 2014).

### Preparation of nanocomposite containing ostrich eggshell

The composite was prepared from monomer loop polymerization in molten state and presence of a tin octoate catalyst. Capro-lactone with a molecular weight of 1000 was accurately weighed and placed in 3 span balloon equipped with Nitrogen gas inlet and outlet and heated by magnetic stirrer equipped. After melting the PEG, the molten catalysts of tin octoate (0.05 wt % of raw materials), added to begin the polymerization reaction and continued with gentle stirring and nitrogen gas flow. At the end of the polymerization, the solution was cooled to room temperature. Solid polymer was dissolved in dichloromethane and poured into a large volume of dry ethyl ether. The polymer was dried and solvent removed by a vacuum attached to the desiccator. Polycaprolactone nanocomposite-ostrich egg shell (PCL-HA) was prepared by particle flush and freeze drying (Park et al., 2008).

### Sample characterization

The characteristics of the prepared samples (NCOE) were evaluated by scanning electron microscope (SEM), powder X-ray diffractometry (XRD, X'Pert-APD; Philips, Netherlands) (Park et al., 2008).

### Surgical Protocol

Six hours prior the initiation of the study, animals were food deprived and 1 hour before surgery fasted from drinking. Then animals were anesthetized with an intramuscular (i.m) injection of ketamine hydrochloride 10% (Alafason, Woeden, Holland, 40mg/kg)

and 2% xylazine (Alafason, Woeden, Holland, 5mg/kg) and then were placed in sternal recumbency position on the operating table. The head of the rabbit was shaved and scalp prepared with povidone-iodine solution (Betoni-Junior et al., 2013). A longitudinal anteroposterior incision (10 cm) was made along the midline of the skull from the midpoint of the base of ears using No. 15 surgical blade. Before incising the periosteum, the skin was retracted by a surgical mosquito and then using a periosteal elevator periosteum was separated from the bone surface cranial to caudal. Four bone defects (internal 8 mm diameter) were created in the calvaria (Takauti et al., 2014). Defects were created on both sides of the sagittal suture without crossing the midline using electric 2000 rpm hand piece and 8 mm diameter milling round surgical trephine. To prevent overheating until holes reached the meningeal membrane (the soft meningeal membrane was palpable) 0.9% physiologic saline solution was used (Betoni-Junior et al., 2013). The first defect was maintained unfilled and kept as control. The second defect was filled with autograft bone derived from the site of the defect (Takauti et al., 2014). The third defect was filled using NCOE. The fourth defect was filled with mixture of the autograft + NCOE bone. The filling and placement of the material into the pits was done in a counter clockwise direction and without pressure to ensure the particles did not enter the meningeal space (Betoni-Junior et al., 2013). After placing the materials, the periosteum was sutured with 4/0 simple absorbable sutures (Polyglycolate coated, SUPA, Iran). The calvarium was sutured with 3/0 nylon sutures and skin was sutured with a single simple suture (Monofilament Polyamide, SUPA, Iran). After animal was coming out of anesthesia, they were transferred to a warm place until regaining full consciousness and then into cage. To prevent infection and relieve pain, day post-operative, cefazolin 1g, Exir, Iran (20 mg/kg; i.m) and tramadol 50 mg, Exir, Iran (20 mg/kg; i.m) were injected. If swelling or inflammation appeared in the surgery area, the sutures were removed and the presence of infection or discharge was evaluated but there were no complications in current study. Skin sutures were removed 10 days after surgery (Betoni-Junior et al., 2013).

### Assessment of bone regrowth

At 30, 60 and 90 days after surgery, 5 animals were euthanized with pentobarbital (88mg/kg IV) and tissue specimens were collected in 10% for neutral buffered formalin solution to evaluate regrowth. Af-

ter fixation, tissues were decalcified in 5% nitric acid. Then 5-micron sections were cut using a microtome (Leica RM 2145 Rotary, Germany) for processing and embedding of tissue. Then samples were stained with hematoxylin eosin [H&E] and trichrome staining method (Jörundsson et al. 1999). Microsections were examined with light microscope (Olympus, CX21i, Germany) and MOTIC camera and image analyser software version 1.6.0 to assess the extent and intensity of inflammation, formation status and foreign body reaction. For each section 10 microscopic fields were evaluated.

### Evaluation of the formation

Inflammation was graded using a five-tiered grading system as follows: grade (0): no inflammatory mononuclear cells, (grade I): mild inflammation with mononuclear inflammatory cells (<25%), grade (II): presence of the focal mononuclear inflammatory cells (25-50%), grade (III): focal inflammation with presence of the mononuclear inflammatory cells (50-75%) and grade (IV): focal inflammation with presence of the high level of the mononuclear inflammatory cells (>75%). The filling of the defect determined by investigation of the newly formed bone trabecula inside the cannula using a nine-tiered grading system as follows: (0): not filling, (I): just fibrous and low cartilage, (II) same percent of fibrous and cartilage, (III) low fibrous and high cartilage, (IV) just cartilage, (V) high cartilage and immature bone, (VI): same percent of cartilage and immature bone, (VII): low cartilage and high level of immature bone, (VIII): healed with immature bone and (VIII): healed with mature bone. The remodeling was determined using a five-tiered score as follows: (0): not remodeling, (I): <25% remodeling, (II): 25-50% remodeling, (III): 50-75% remodeling and (IV): >75% remodeling. The material absorbance was determined using a 4 grading system as follows: (grade 0): not absorbed, (grade I): 25-50%, (grade II): 50-75% and (grade III) fully absorbed (Huo et al. 1991).

### Statistical analysis

The parametric data analyzed with one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean values  $\pm$  standard error of mean (SEM). Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test. The KruskalWallis test was used to compare group medians for histopathological scores.  $P < 0.05$  was

considered to denote significant differences between groups.

### RESULTS

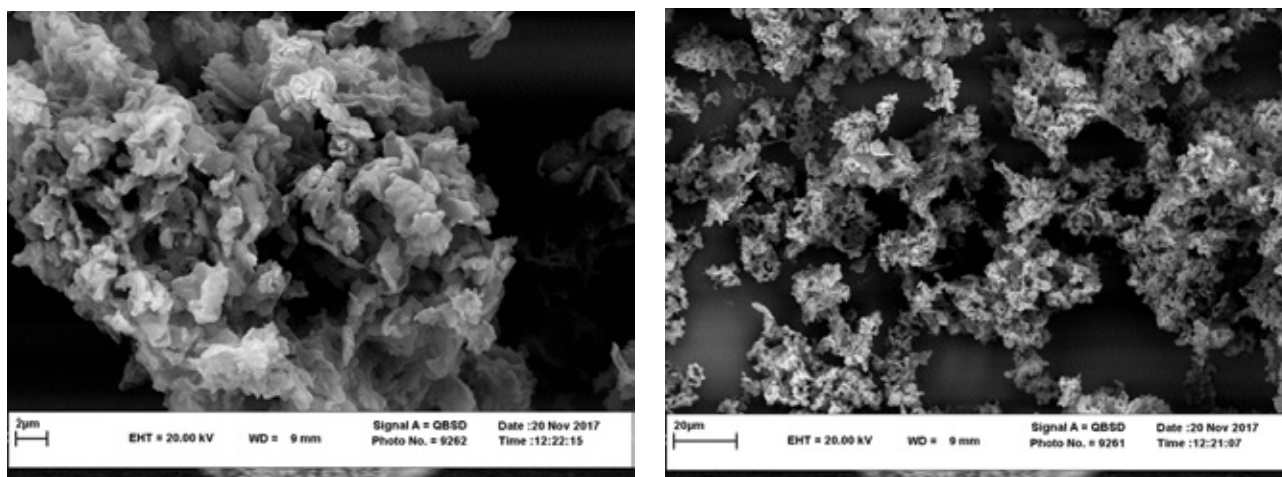
The SEM image of the NCOE is presented in figure 1. The X-ray diffraction pattern of the NCOE is presented in figure 2. The NOCE showed different surface morphology platelet-like, needle-like, or rod-like microstructure.

Effects of NCOE on filling defect, inflammation, remodeling and absorb material on calvaria at 30 days post-surgery is presented in figure 3. Filling defect statistically increased in NCOE-treated rabbits compared to the control group at 30 days post-surgery ( $P < 0.05$ ). There were significant differences between experimental groups compared to the control group at 30 days post-surgery ( $P < 0.05$ ) while no statistical differences observed among autograft, NCOE, autograft + NCOE ( $P > 0.05$ ). The absorbed material significantly decreased in experimental groups compared to control group. No statistically significant differences observed between NCOE and autograft + NCOE ( $P > 0.05$ ).

Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria at 60 days post-surgery is presented in figure 4. Significant increase was observed on filling defect in NCOE compared to the control group ( $P < 0.05$ ). Significant increase observed on inflammation in experimental groups compared to the control group ( $P < 0.05$ ) while no statistical differences observed among autograft, NCOE, autograft + NCOE ( $P > 0.05$ ). Also, absorb material significantly decreased in NCOE and autograft + NCOE groups compared to the to the control group at 60 days post-surgery ( $P < 0.05$ ).

The filling defect significantly increased in autograft, NCOE and NCOE + autograft groups compared to the control group at 90 days post-surgery ( $P < 0.05$ ). There was no statistical significant difference on inflammation and absorb material among the groups at 90 days post-surgery (figure 5) ( $P > 0.05$ ). The histological images of the tissue at 30, 60 and 90 days post-surgery are presented in figures 6-17.



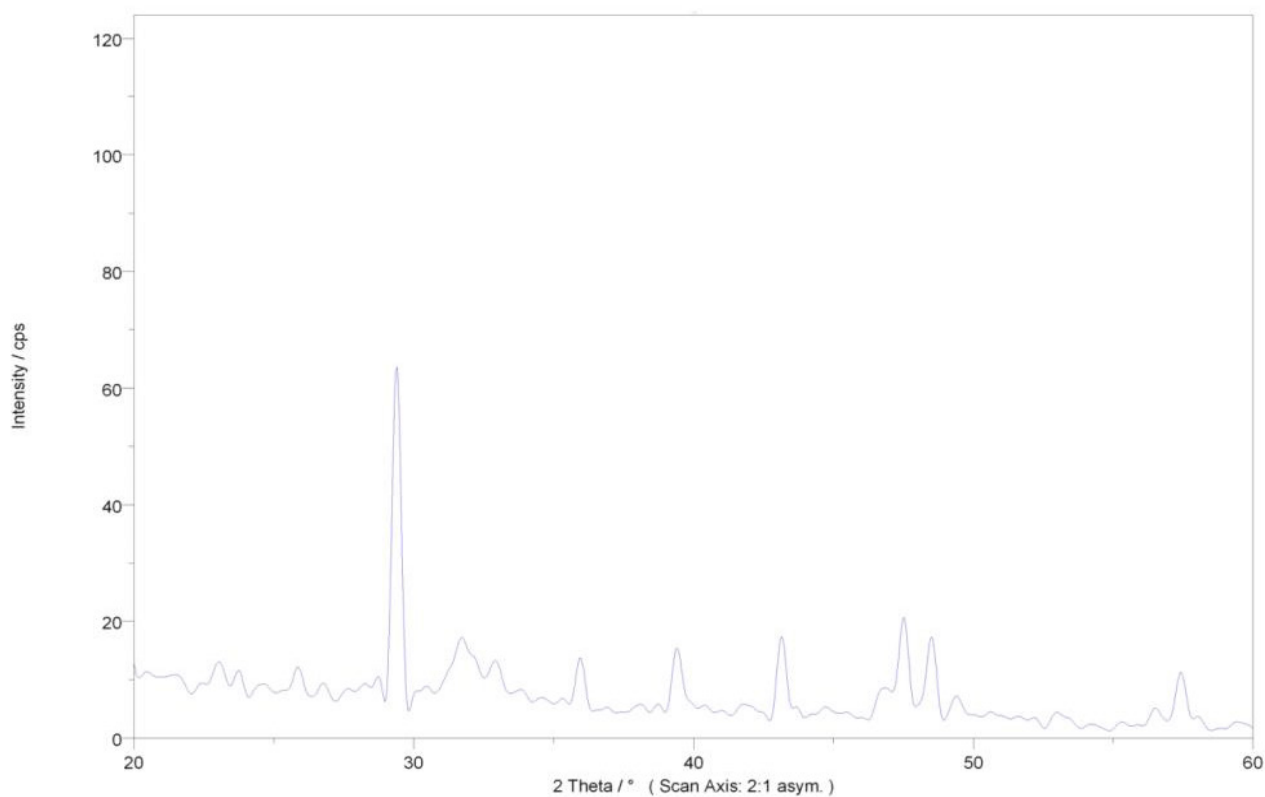


**Figure 1.** The electron microscope image of the ostrich Eggshell nanocomposite

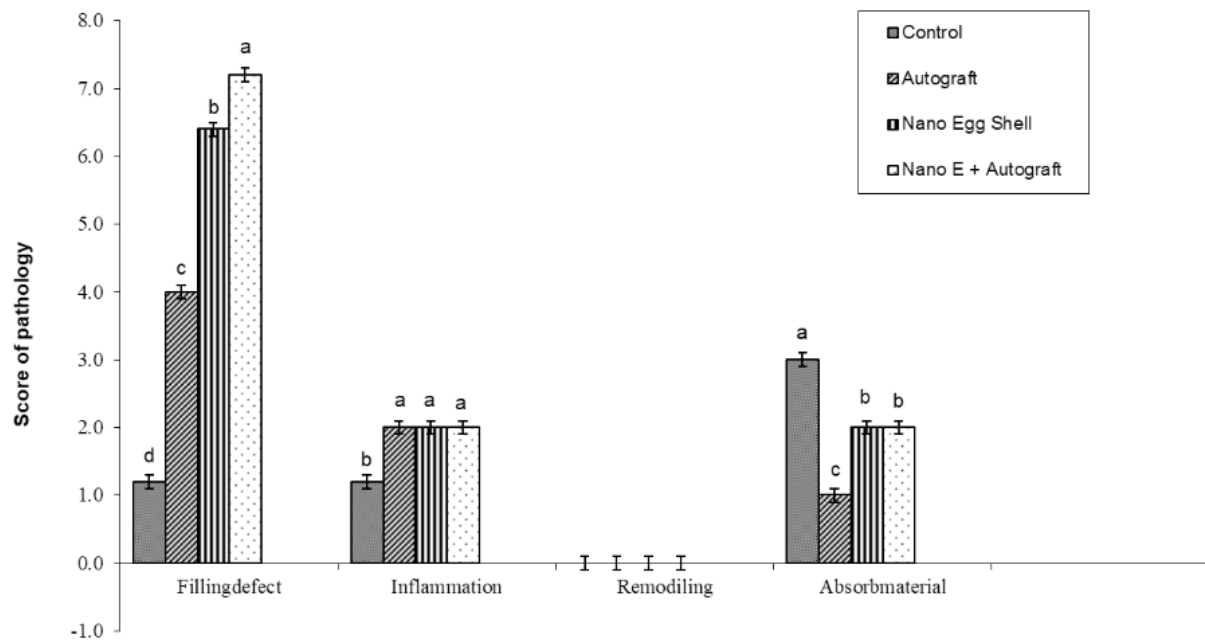
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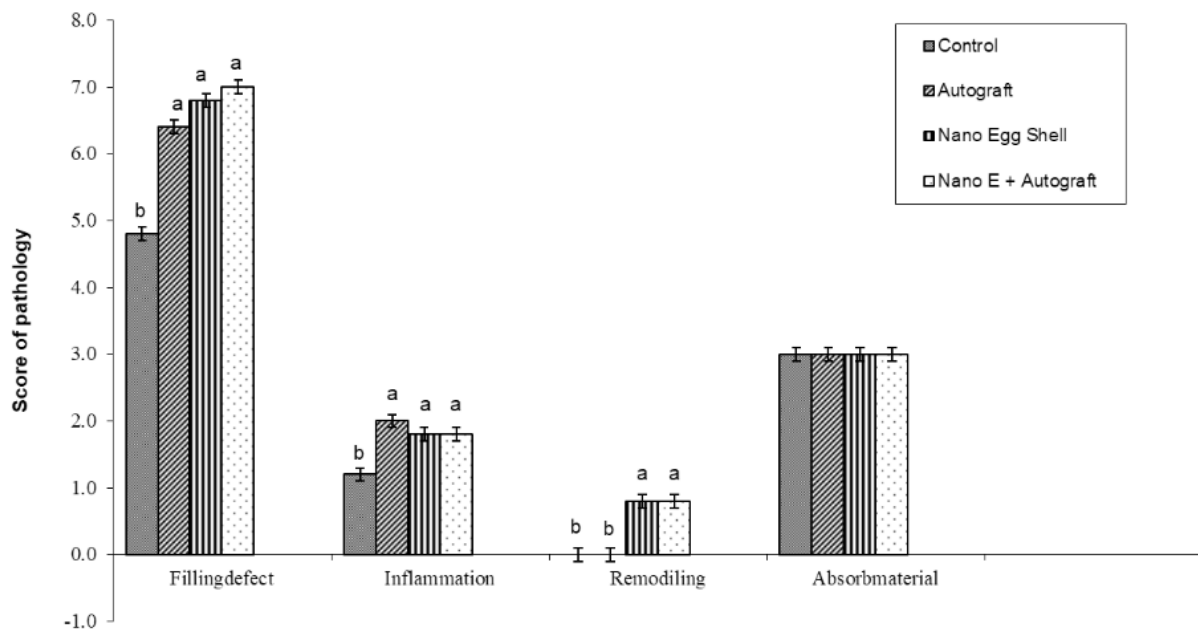
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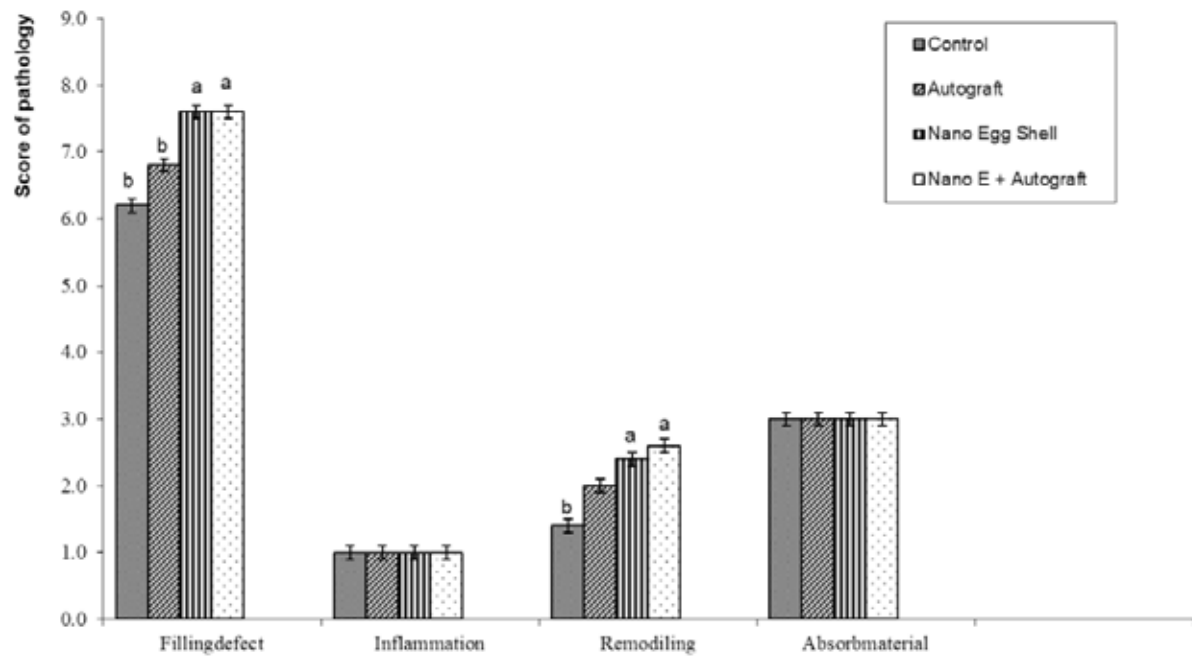
**Figure 2.** X-ray diffraction patterns of the NCOE. Arrows indicate the peaks of hydroxyapatite



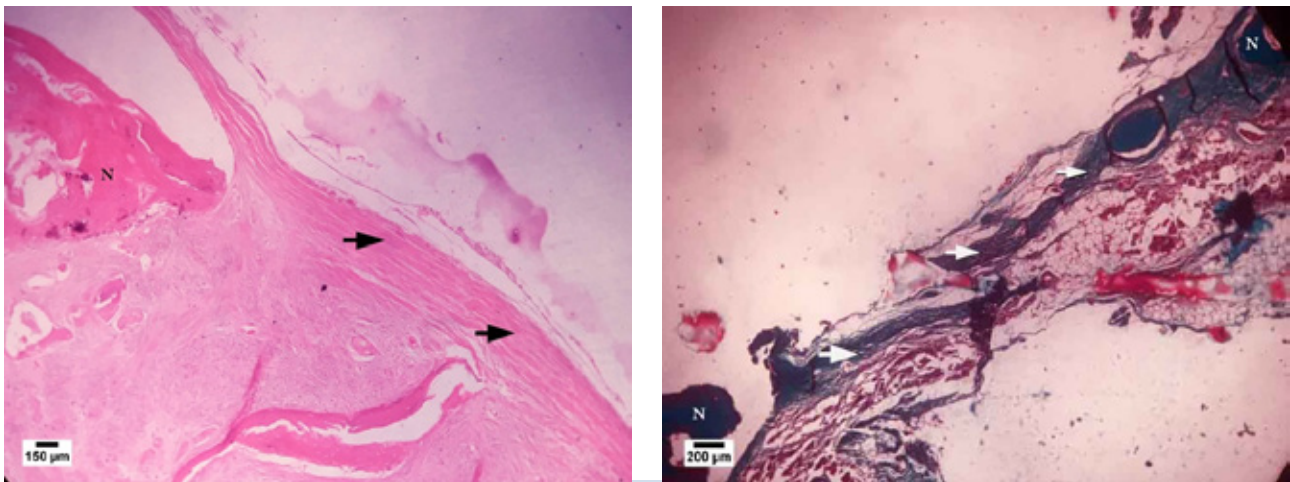
**Figure 3.** Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria bone at 30 days post-surgery ( $P < 0.05$ ). NCOE: nanocomposite containing ostrich eggshell



**Figure 4.** Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria bone at 60 days post-surgery ( $P < 0.05$ ). NCOE: nanocomposite containing ostrich eggshell

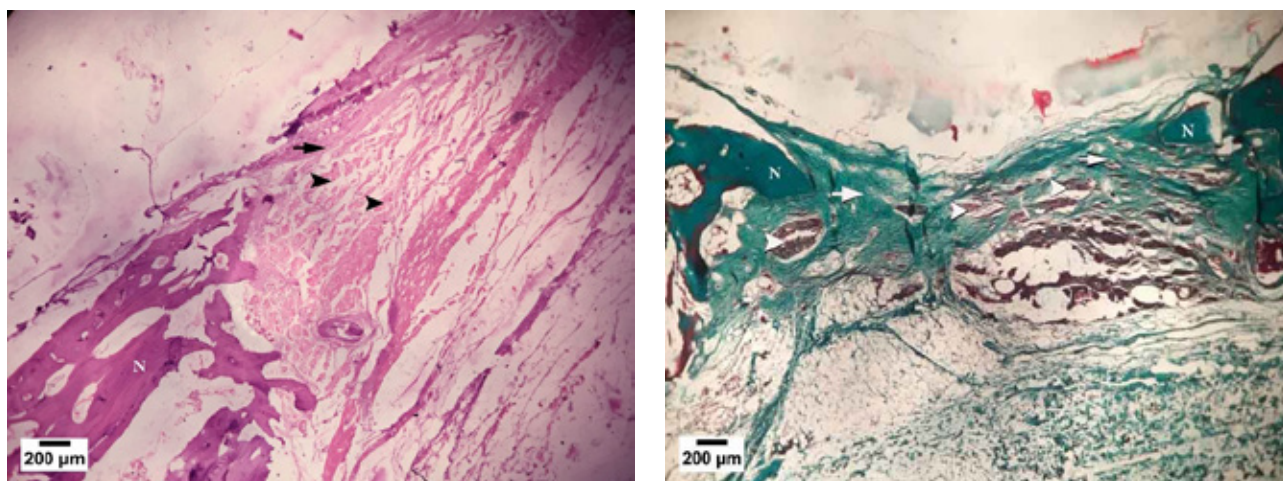


**Figure 5.** Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria bone at 90 days post-surgery ( $P < 0.05$ ). NCOE: nanocomposite containing ostrich eggshell

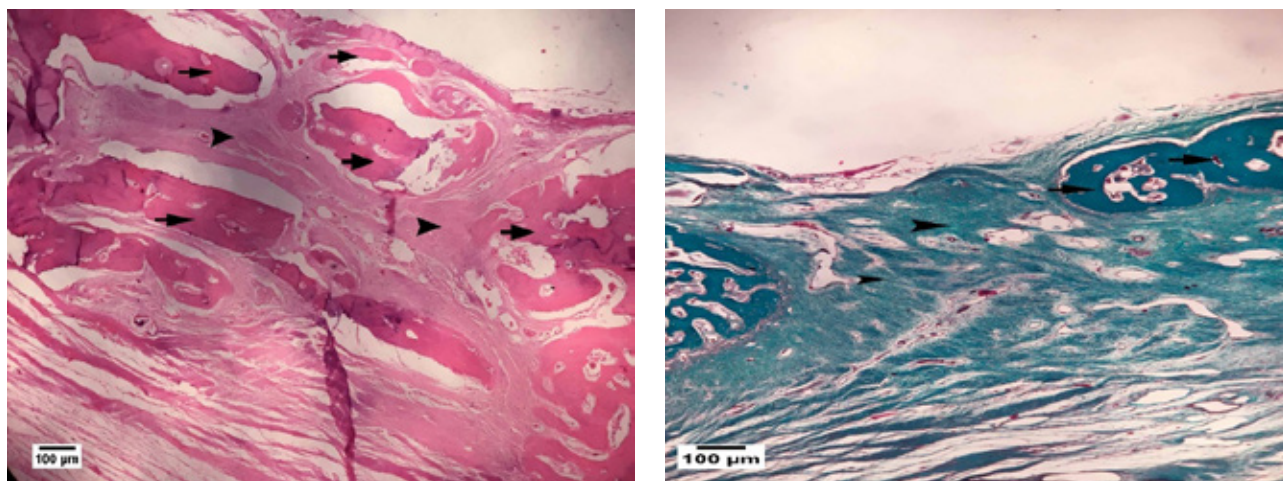


**Figure 6.** Control group (30 day) that is filled with a large amount of fibrous tissue (arrows). A part of the normal calvarium bone (N) is seen. Control group (30 day) that is filled with a large amount of fibrous tissue (arrows). A part of the normal calvarium bone (N) is seen (left: H&E, Right: Masson trichrome, 40×)

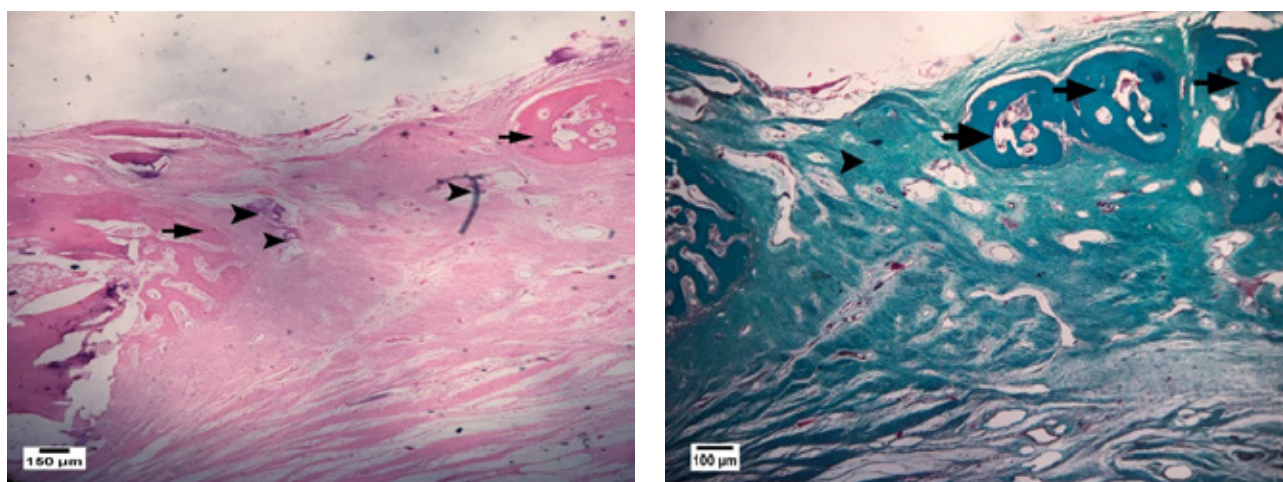




**Figure 7.** Autograft group (30-day) with a large amount of cartilage (arrow head) and a few fibrous tissue (arrows) that filled defect. A part of the normal bone (N) is seen. Autograft group (30-day) with a large amount of cartilage (arrow head) and a few fibrous tissue (arrows) that filled defect. A part of the normal bone (N) is seen (left: H&E, Right: Masson trichrome, 40×)

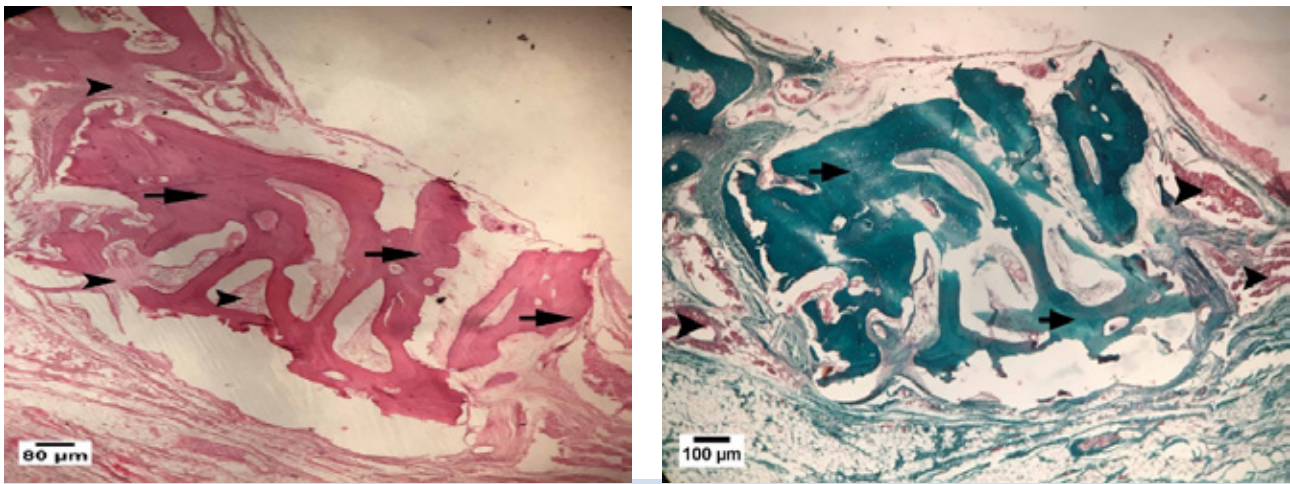


**Figure 8.** Nano group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow). Nano group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow) (left: H&E, Right: Masson trichrome, 100×)

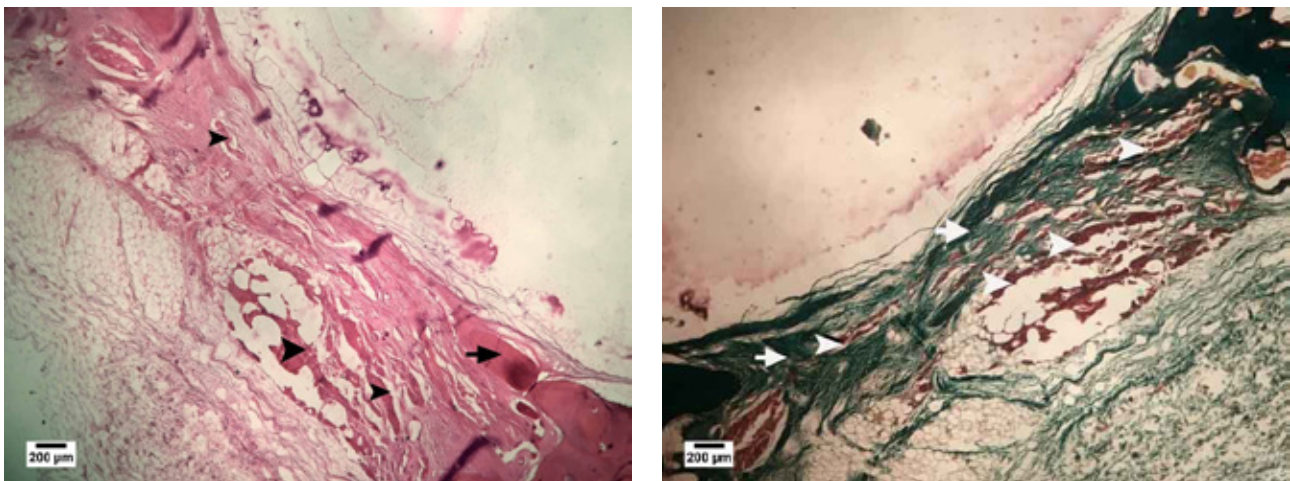


**Figure 9.** Nano autograft group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow). Nano autograft group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow) (left: H&E, Right: Masson trichrome, 100×)

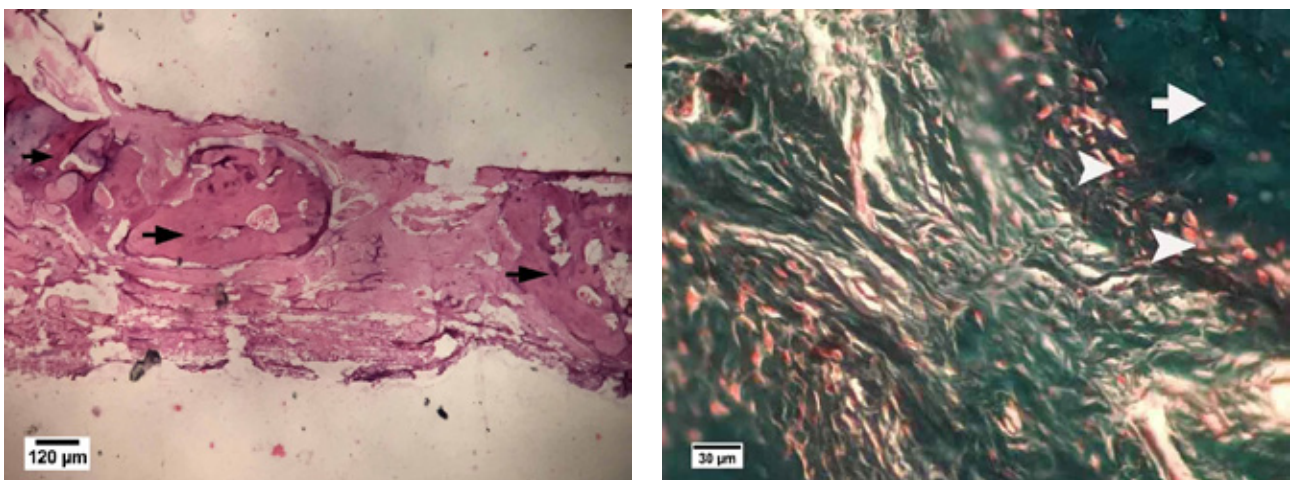




**Figure 10.** Control group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and immature bone tissue (arrow). Control group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and immature bone tissue (arrow) (left: H&E, Right: Masson trichrome, 100×)

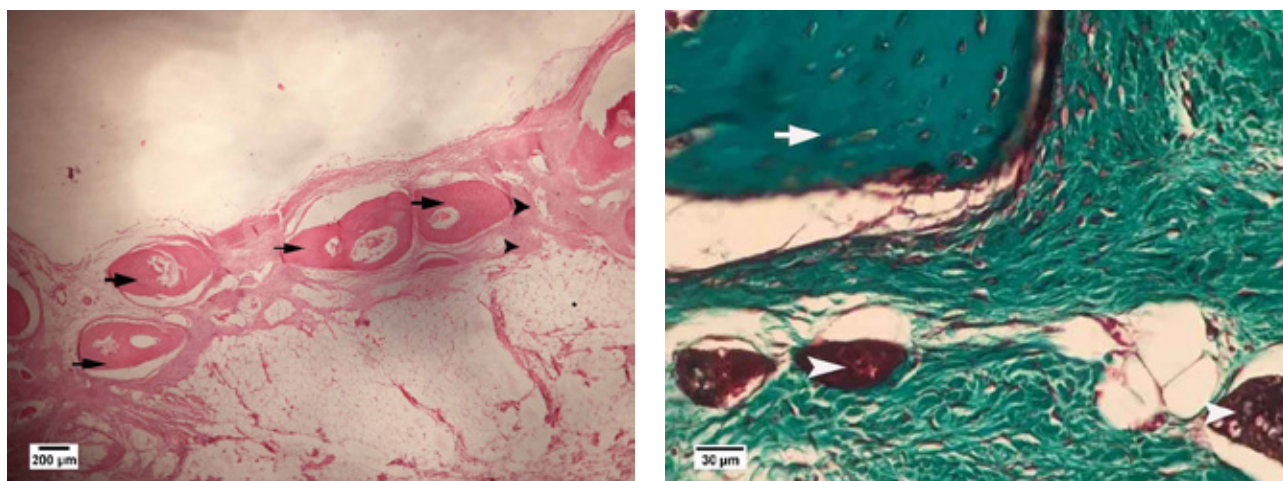


**Figure 11.** Autograft group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and few immature bone tissues (arrow). Autograft group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and few immature bone tissues (arrow) (left: H&E, Right: Masson trichrome, 40×)

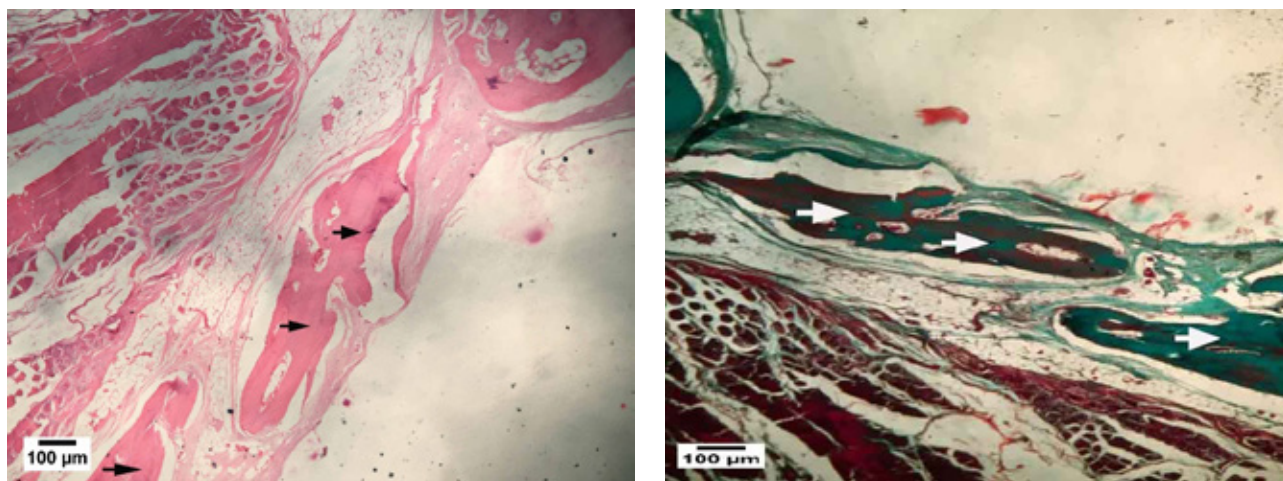


**Figure 12.** Nano group (60-day) that the defect filled with a large amount of immature bone tissue (arrow). Nano group (60-day) that the defect filled with a large amount of immature bone tissue (arrow) (left: H&E, 40×, Right: Masson trichrome, 400×)

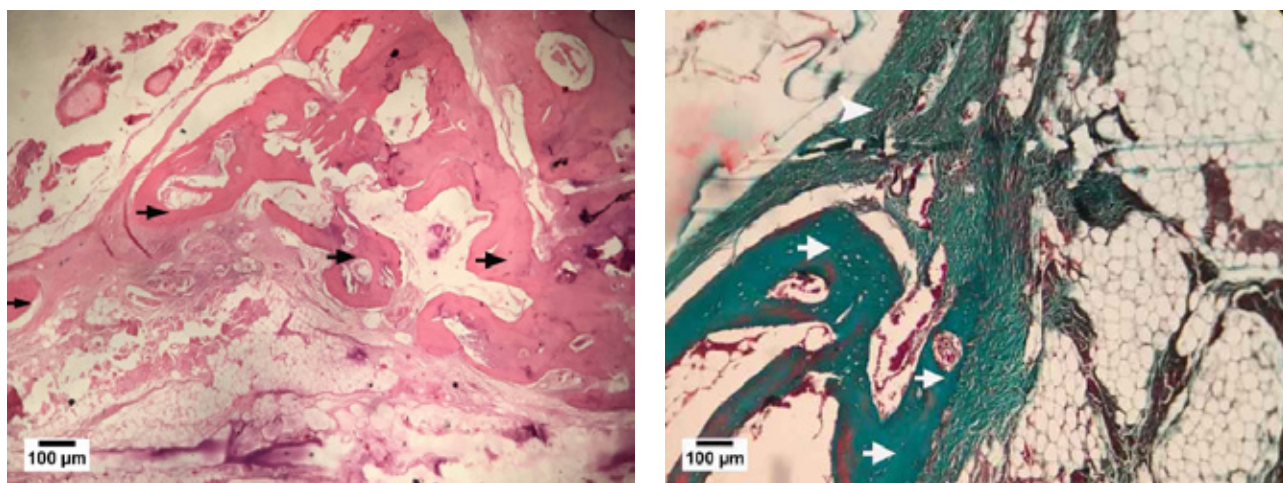




**Figure 13.** Nano autograft group (60-day) that the defect filled with a large amount of immature bone tissue (arrow head) and few cartilage tissues (arrow). Nano autograft group (60-day) that the defect filled with a large amount of immature bone tissue (arrow head) and little cartilage tissue (arrow) (left: H&E, 40×, Right: Masson trichrome, 400×)

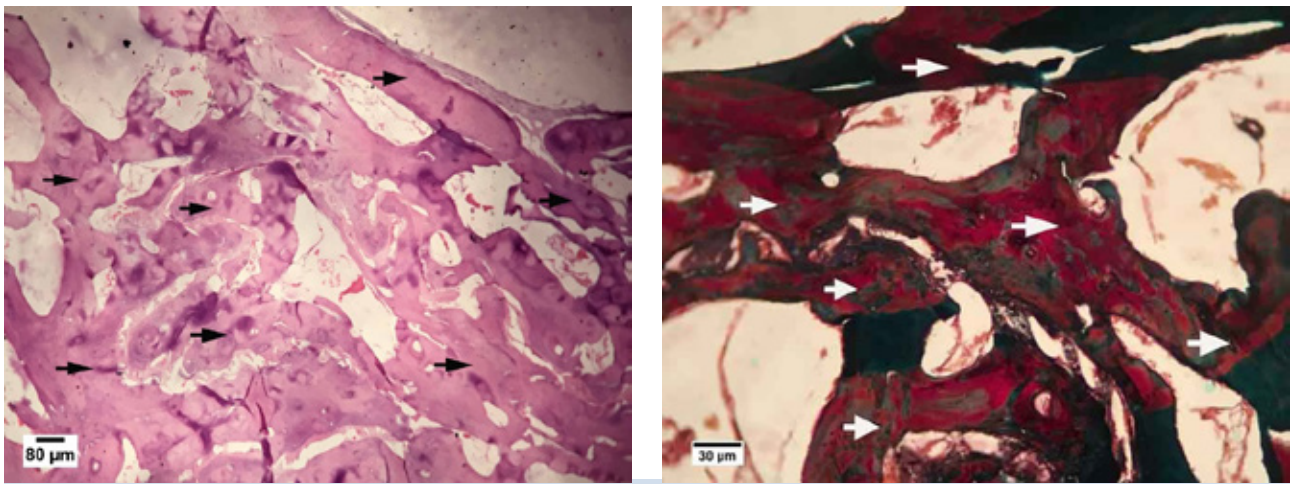


**Figure 14.** Control group (90-day) that the defect filled with a large amount of immature bone tissue (arrow). Control group (90-day) that the defect filled with a large amount of immature bone tissue (arrow) (left: H&E, Right: Masson trichrome, 40×)

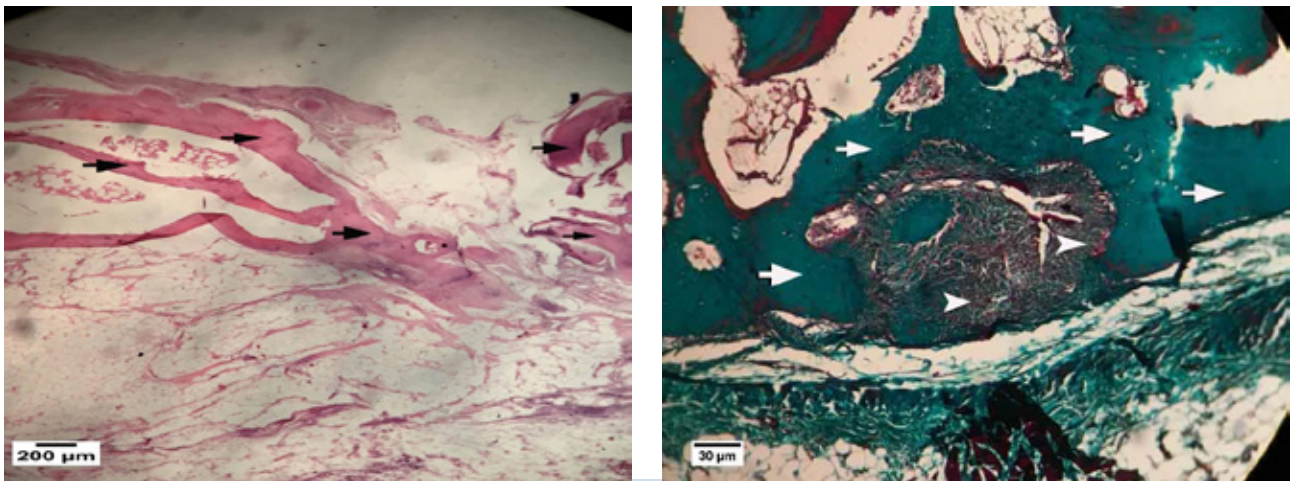


**Figure 15.** Autograft group (90-day) that the defect filled with a large amount of immature bone tissue (arrow). Autograft group (90-day) that the defect filled with a large amount of immature bone tissue (arrow) and few cartilage tissues (arrow head) (left: H&E, 40×, Right: Masson trichrome, 100×)





**Figure 16.** Nano group (90-day) that the defect filled with only large amount of immature bone tissue (arrow). Nano group (90-day) that the defect filled with large amount of immature bone tissue (arrow) (left: H&E, Right: Masson trichrome, 400×)



**Figure 17.** Nano-autograft group (90-day) that the defect filled with large amount of immature bone tissue (arrow). Nano-autograft group (90-day) that the defect filled with large amount of immature bone tissue (arrow) and little cartilage tissue (arrow head) (left: H&E, 40×, Right: Masson trichrome, 400×)

**Table 1.** Criteria for scoring histological sections

Score	Parameter	Criteria
0	Newly formed vessels	None
	Numbers of fibroblasts	None to very minimal
	Osteoid (bone matrix)	None
	Bone	None
1	Newly formed vessels	Few blood vessels
	Numbers of fibroblasts	Few fibroblasts
	Osteoid (bone matrix)	Evidence of matrix osteoid
	Bone	Evidence of bone formation
2	Newly formed vessels	Moderate blood vessels number
	Numbers of fibroblasts	Predominantly fibroblasts
	Osteoid (bone matrix)	Moderate bone matrix deposited
	Bone	Moderate bone cells
3	Newly formed vessels	Extensive blood vessels
	Numbers of fibroblasts	Fewer number of fibroblasts
	Osteoid (bone matrix)	Dense highly organized bone matrix
	Bone	Extensive bone cells

## DISCUSSION

There is growing interest on producing new biocompatible materials from animal products in regeneration (Kattimani et al., 2014). Avian eggshell has similar physical characteristics to the GBR for space filling (Krishna et al., 2007). Additionally, ostrich eggshell has better effect in regeneration physical characteristics (Kattimani et al., 2014). To determine accuracy of the new biocompatible materials for bone healing, bilateral calvarial defect is the common method because of easy handling and low morbidity by minimize damage to the superior sagittal sinus (Park et al., 2008). In the defects we used bilateral calvarial defect to determine effect of the NCOE on calvaria healing in rabbit. According to the results, filling defect significantly increased in NCOE-treated rabbits compared to the control group at 30- and 60-days post-surgery ( $P < 0.05$ ). There was significant difference between experimental groups compared to the control group at 30- and 60-days post-surgery while no differences observed among autograft, NCOE, autograft + NCOE ( $P > 0.05$ ). Also, absorb material significantly decreased in NCOE and autograft + NCOE groups compared to the control group at 60 days post-surgery. The filling defect significantly increased in autograft, NCOE and NCOE + autograft groups compared to the control group at 90 days post-surgery ( $P < 0.05$ ). There was no significant difference on inflammation and material absorption among the groups at 90 days post-surgery ( $P > 0.05$ ). The biocompatibility of the filling defect is based on reaction of the surrounding tissue (Yadao et al., 2004). The biocompatibility of the eggshell was expected because the calcium carbonate, as main component of the bone (Yadao et al., 2004). Dense alloplastic materials have higher incidence of extrusion compared to bone grafts (Zingg et al., 1991). The lack of porosity in eggshell implant inhibits the invasion of fibro-vascular network that could help anchor the implant to the underlying bone (Zingg et al., 1991). No change was observed in bone resorption and eggshell graft placed in the craniofacial region of the rabbits 5-20 weeks after implantation (Yadao et al., 2004).

On evaluation of bone healing with eggshell-derived bone graft substitutes in rat calvaria, Park et al. (2008), reported eggshell-treated animal had greater new bone formation and mineralized bone-to-graft contact of surface-modified. Biodegradability and microporous surface structure of bone has key role in bone healing (Park et al., 2008). Biphasic calcium phosphate ceramics has higher osteoconduction than

stable hydroxyapatite because of its biodegradability in body fluids and surface microstructures (Furlaneto et al., 2007). Microporosity affects the dissolution rate of bone substitutes in biological fluids (Daculsi et al., 2003). Surface microstructure improves adsorption of proteins and fibronectin which affects cell adhesion, cell proliferation and differentiation. So, based in the above, nano-composite form of the ostrich eggshell was used. Hydrothermal phosphate solutions achieved partial conversion of the particulated hen eggshell to calcium-deficient hydroxyapatite (Rouahi et al., 2006). Calcium-deficient hydroxyapatite has higher biodegradability and rapid surface apatite layer formation and bone bonding (Barrere et al., 2003). These surface properties might contribute to the increased osteoconduction of hydrothermally treated eggshell in the healing of rat calvarial defects (Barrere et al., 2003). The formulations of eggshell used as mineral and trace element supplying agent (Kattimani et al., 2014). In role of the eggshell-derived bone graft substitutes on bone healing in rats, it is reported eggshell-derived bone graft enhances the new bone formation (Uraz et al., 2013).

Small degradation time, poor mechanical possessions and low integrated biological components lead in inability to form, maintain and actively support tissue remodeling. Ostrich shell membranes has gradual degradation than collagen membranes (Park et al., 2008). The main factor for an osteoconductive material is the particle size and foreign body reaction. Based on findings of the current study, no foreign body reaction was observed using NCOE. Based on radiologic report, 50 and 75  $\mu\text{m}$  chick eggshell particles fully absorbed after 60 days and 150-300  $\mu\text{m}$  particles resorbed after 4 months (Dupoirieux et al., 2001). Soluble eggshell matrix proteins are critical for calcium transport (Yadegari et al., 2015). Bone formation and remodelling are controlled by non-collagenous proteins of the bone matrix. Non-collagenous proteins of the bone matrix regulate formation and remodeling (Yadegari et al., 2015). During the chicken eggshell formation, these matrix proteins have effect on calcite crystal morphology. Transforming growth factor  $\beta 1$ , lectin-like proteins and Calbindin (calcium binding protein) is isolated from eggshell and stimulate bone formation (Yadegari et al., 2015). Durmus, et al., (2003), reported application of the eggshell powder, the outer and inner ostrich eggshell membranes produced little adjunctive effect. Ovocleixin and ovocleidin are eggshell specific proteins and has the eggshell formation in hen's uterus during the chick-



en embryonic development (Neunzehn et al., 2015). Eggshell is ideal source for hydroxyapatite and calcium carbonate (Neunzehn et al., 2015) which promotes the vascularization and wound procedure in the defect edges (Abdulrahman et al., 2014). Eggshell-derived graft substitutes enhance the new bone formation and higher levels of osteoid formation in the eggshell grafted defects (Uraz et al., 2013). It is suggested enhanced bone regeneration in the defect margins (Baliga et al., 1998) which our result was similar to their report. No inflammation, encapsulation and foreign body reaction reported 3 months after treatment, with larger eggshell particles in dog tibial (Yadegari et al., 2015) which our finding was similar to this report. Using standardized defects (8mm diameters) in the parietal bones of rabbit calvaria allowed large increases in their interface with bone graft materials without any effect on the other defects (Takauti et al., 2014).

In conclusion, bone defects occur because of the

medical operations, social and economic problems in human (Durmuş et al., 2003). These results suggested NCOE + autograft has positive effects on calvaria healing in rabbit. It seems NCOE has potential efficacy of osteoconductive bone substitute in a rat calvarial defect model. Bone regeneration includes several intra cellular and extra cellular signaling pathways which lead to osteoinduction and osteoconduction to increase bone regeneration in human. It seems, merit researches needed to determine direct cellular and molecular mechanisms of action for further application of the NCOE in clinical trials.

## ETHICAL ISSUES

All protocol of the study was approved by ethic committee of Islamic Azad University, Science and Research Branch, Tehran, Iran.

## CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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