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The Effects of Nanocomposite Containing Ostrich Eggshell on Calvaria Bone Formation in Rabbit: Radiographic and Hematological Survey

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Abstract

Objectives: Available methods for treating bone defects occasionally include limitations regarding supplying and applying the biological agents, thus mediators of bone regeneration still the safe method. The current study aimed to determine the possible effects of eggshell as an agricultural waste product in the form of nanocomposite containing ostrich eggshell (NCOE) on calvaria bone formation in rabbits.

Materials and Methods: A number of 15 adult male New Zealand white rabbits were used in this study. Four full-thickness skull defects were created in the calvarial bone. The first defect (control) was kept unfilled. However, the second and third defects were filled with nanomaterial and using an autogenous bone, respectively. In addition, the fourth one was filled with a mixture of NCOE and autogenous bone. On the 30th, 60th, and 90th days after surgery, the blood samples were obtained and red blood cells, white blood cell, hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell HGB, and platelets (PLT) were determined. On the above-mentioned days, the animals were sacrificed and the bone density was determined using radiography images.

Results: Based on the results, a significant difference was observed on bone formation in control (0.18 ± 0.01 mm Al equivalent), autograft (0.1 ± 0.01 mm Al equiv.), NCOE (0.12 ± 0.01 mm Al equiv.), and NCOE + autograft (0.07 ± 0.01 mm Al equiv.) defects at day 30 post-surgery ($P < 0.05$). Further, significant differences were found on bone density in control (0.09 ± 0.01 mm Al equiv.), autograft (0.13 ± 0.01 mm Al equiv.), NCOE (0.14 ± 0.01 mm Al equiv.), and NCOE + autograft (0.20 ± 0.01 mm Al equiv.) defects at day 60 post-surgery ($P < 0.05$). Furthermore, significant differences were detected regarding bone density in control (0.15 ± 0.01 mm Al equiv.), autograft (0.21 ± 0.01 mm Al equiv.), NCOE (0.22 ± 0.02 mm Al equiv.), and NCOE + autograft (0.29 ± 0.01 mm Al equiv.) defects after 90 days ($P < 0.05$). The results revealed that the NCOE + autograft defect had a better bone formation in all stages of the study ($P < 0.05$). However, no significant effect was observed on blood hematology indexes ($P > 0.05$).

Conclusions: Generally, based on the results, NCOE + autograft had positive effects on calvaria healing in the rabbit.

Keywords: Nanocomposite, Ostrich eggshell, Calvaria healing, Rabbit

Introduction

Bone defects happen due to the periodontal disease, tumor resection, skeletal deficiency/disorder, abnormal development, and trauma (1). Numerous approaches were undertaken to treat bone defects aiming at regenerating the lost osseous tissue and thus regaining function (2). Available techniques to treat bone defects are occasionally encountered with limitations in order to supply and transplant rejection/incompatibility along with surgical side effects such as infection, disease transmission, or neurovascular injury (3). These limitations lead to tissue engineering approaches for repairing the skeletal defects (4). Bone grafts are used to augment osseous defects in dental and orthopedic fields (5). Applying the biological agents as mediators of bone regeneration alleviates the safe method (6). Autogenous bone grafts are known as gold standard and preferred augmentation materials (7). An autogenous bone needs donor site morbidity, prolonged operation times, and high costs (7). These bones are effective agents in rapid bone graft healing without triggering an immune response and highest compatibility with the host tissue (8). Clinical research demonstrated that the combination of bone grafts with other treatments can increase the engraftment, the formation of bone tissue, and bone defect healing (9). Moreover, bone graft materials are recommended to be osteoconductive, osteoinductive, and osteogenesis (10,11). Scaffolds, growth factors, and osteoblasts are tissue engineering elements which are used

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to enhance the bone regeneration (12).

The guided bone regeneration (GBR) is described as a technique which uses a barrier membrane to promote the bone augmentation by applying a barrier membrane. Additionally, the GBR is widely employed for encouraging the formation of new bone in osseous defects by restricting the infiltration of soft tissues (13). Several advantages are introduced for this method such as no need to second surgical procedure to remove absorbable membrane (14). Avian eggshell has a high level of mineral composition which can be used to produce hydroxyapatite, namely, the major inorganic part in bone repair (15). The composition of avian eggshells differs slightly among the species but is mainly a mineral matrix composed of calcium carbonate (97.4%), magnesium phosphate (1.9%), and triphosphate (0.7%) (16). In addition, protein distribution varies based on the structure of the egg. Protein-polysaccharides are the main components of the eggshell matrix which mostly contain 11% polysaccharides and 70% proteins (17). Further, chondroitin sulfates A and B include 35% of the eggshell total polysaccharides (17). Based on the reports, employing the hen eggshell is safe and inexpensive in rabbit bone defect model (17). In a study on the effects of ostrich eggshell combined with eggshell membranes in the healing of the cranial defects in rabbits, Durmus et al (18) emphasized that using the ostrich eggshell powder had no effect on bone regeneration in rabbits. Large ostrich grafts are suitable such as onlay graft, however, a complementary osteosynthesis is suggested to improve the osteointegration (19). Yadegari et al (20) investigating the radiographic effect of eggshell powder on tibia bone defect repair in dog indicated that eggshell powder increased cell growth and bone remodeling. Nanocomposites contain a high level of hydroxyapatite and collagen. These particles gained much attention as bone grafts not only for their composition and structural similarity with natural bone, but also for their unique functional properties such as larger surface area and superior mechanical strength compared to their single phase constituents. Accordingly, the clinicians were recommended to search for alternative bone graft substitutes in the regenerating process in human due to the complexity of osteoclast and osteoblasts, as well as the other factors (19). Therefore, the present study sought to investigate the potential beneficial effects of the nanocomposite containing ostrich eggshell (NCOE) on bone healing in rabbits.

Materials and Methods

Animals

Fifteen adult male New Zealand white rabbits (3-3.5 kg) were purchased from Razi Vaccine and Serum Research Institute (Tehran, Iran). The animals were kept in individual cages in the laboratory at constant and optimum environmental and nutritional conditions (temperature, humidity) with a 12-hour light/dark cycle. Animals had free access to commercial chew pellet and tap water. This experiment was approved by the Institutional Animal Care and the Ethics Committee of the Islamic Azad University of Tehran in order to protect the rights of laboratory animals. Study procedures were implemented during the 10–17:00 hour light phase and in accordance with the *Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals* (*the Guide*) (22) and National Institutes of Health (NIH), as well as the current laws of the Iranian government and the guidelines for the animal care board of the Islamic Azad University, Faculty of Veterinary Medicine.

Ostrich Eggshell Preparation

Fragmented ostrich eggshell was immersed in boiling sterile distilled water (DW) and the outer and inner shell membranes of the eggs were carefully removed with forceps. The shells were then crushed and sieved until particles (particle diameter = 1 mm; porosity = 75%) were obtained (23). An electrical mill was used to ground the eggshell into 300-500 µM pieces, and then the pieces were washed 3 times with DW, dried, and sterilized using ethylene oxide (18). The eggshell powder was immersed in 5% sodium hypochlorite, and then sodium hydroxide solution was applied to remove the organic components (18). Next, the eggshell powder was washed in deionized water and heat-treated at 300ºC for 24 hours and then further treated in phosphate-containing solutions with different hydrothermal conditions (6). Furthermore, the obtained powder was soaked in sealed glass bottles containing phosphate buffer saline at 80ºC for 6 days (the solution was replaced every 3 days) (24), then soaked in Teflon lined reactor containing 2 wt.% of di-ammonium phosphate solution at 150 ºC for 24 hours. It was then transferred to a phosphate-containing solution (19.5 mM PO43, 30 mM Naþ, &4.3 mM Kþ) at 80ºC for 6 days (24). All the ostrich eggshell particles were sterilized by gamma irradiation before using (24).

Preparing Nanocomposite Containing Ostrich Eggshell

The composite was prepared from monomer loop polymerization in the molten state and at the presence of a tin octoate catalyst. A certain amount of caprolactone with a molecular weight of 1000 was accurately weighed and placed in 3 span balloon equipped with nitrogen gas inlet and outlet. The balloon was heated by the equipped magnetic stirrer. After melting the polyethylene glycol (PEG), the molten catalysts of the tin octoate (0.05 wt.% of raw materials) was added to begin the polymerization reaction and continued with gentle stirring and nitrogen gas flow. After polymerization, the solution was cooled to room temperature. Moreover, a solid polymer was dissolved in dichloromethane and poured into a large volume of dry ethyl ether. The polymer was then drained by drying the solvent isolated in a vacuum attached to the desiccator. Polycaprolactone nanocomposite -Ostrich eggshell (PCL-HA) was prepared by particle flush and

freeze drying (25).

Surgical Protocol

Six hours before initiating the study, animals were food deprived and 1 hour prevented from drinking before the surgery. The animals were anesthetized with an intramuscular injection of 40 mg/kg ketamine hydrochloride (10%) and 2% xylazine (Alafason, Woeden, Holland, 5 mg/kg). Rabbits were then placed in sternal recumbent position on the operating table. Their heads were shaved and the scalps were prepped with the povidone-iodine solution as a topical microbicide before the surgery (26). A longitudinal (anteroposterior) incision was made along the midline of the skull from the midpoint of the base of ears for 10 cm using No. 15 surgical blade. Before incising the periosteum, the skin was retracted by a surgical mosquito and then the periosteum was separated from the bone surface using a periosteal elevator cranial to caudal. Four bone defects with an internal diameter of 8 mm and width of 0.5 mm were created in the calvaria bone. Then, the defects were created on both sides of the sagittal suture without crossing the midline using an electric 2000 rpm handpiece and milling round surgical trephine (8 mm diameter). Additionally, the defects were frequently irrigated with 0.9% physiologic saline solution in order to prevent overheating until the holes reached the meningeal membrane (i.e., the soft meningeal membrane was palpable) (26). The first defect was maintained unfilled and kept as control. However, the second defect was filled with NCOE. In addition, the third and the fourth defects were filled using an autogenous bone and a mixture of NCOE + autogenous bone, respectively. The materials were filled and placed into the pits in a counterclockwise direction without any pressure in order to ensure that no particles entered the meningeal space (26). After placing the materials in the desired locations, the periosteum and calvarium were sutured with 0-4 simple absorbable and 0-3 nylon stitches, respectively. Further, the skin was inserted a single simple stitch. When coming out of anesthesia, the animal was transferred to a warm place until regaining full consciousness and then returned to its box. Furthermore, tramadol (20 mg/kg; i.m) and cefazolin (20 mg/kg; i.m) were injected in order to relieve the pain and prevent infection a day post-operative. Moreover, the sutures were removed if swelling or inflammation appeared in the region and the presence of infection or possible discharge was evaluated. Skin sutures were removed 10 days after the surgery (26).

Hematology Analysis

At 30, 60, and 90 days after the surgery, blood samples (5 mL) were collected using Vacutainer tubes from the marginal ear vein for hematology (Becton Dickson, Rutherford, NJ). A Coulter S-plus IV (Coulter Electronics Inc., Hialeah, FL) was calibrated with Coulter S-cal. Additionally, Coulter 4-C plus control material was used. The white (10^3/μL) and red (10^6/μL) blood cells, hemoglobin (HGB) (g/L), the hematocrit (HCT) percentage, mean cell volume (MCV) (fL) and HGB (pg), as well as platelets (PLT) (10^3/μL) levels were determined (19).

Radiologic Investigation

The first group of animals was sacrificed 30 days after the surgical procedures, initially by an overdose IV injection of the sodium thiopental. In addition, the second and third groups were euthanized 60 and 90 days after the surgery. The area of the surgical defect and surrounding tissues were dissected out. All the specimens were radiographed on dorsal-ventral position using an aluminum phantom. Further, the radiographs analyzed using ImageJ software and the extent of the defect closure and newly formed radiopaque area were observed (10,11).

Statistical Analysis

All data were recorded and prepared in Microsoft Excel and then analyzed by Student *t*-test, Mann-Whitney (Wilcoxon Rank-sum) test, and one-way or two-way variance analysis (ANOVA) using the statistical package for the social sciences (SPSS) software, version 20. The results are presented as mean ± SD. Furthermore, the Kruskal-Wallis test was applied to compare the group medians for radiological scores. The $P < 0.05$ was considered to demonstrate significant differences between the groups.

Results

Based on the results, significant differences were observed regarding the bone formation in control (0.18 ± 0.01 mm Al equivalent), autograft (0.1 ± 0.01 mm Al equiv.), NCOE (0.12 ± 0.01 mm Al equiv.), and NCOE + autograft (0.07 ± 0.01 mm Al equiv.) defects at day 30 post-surgery ($P < 0.05$). Moreover, statistically significant differences were found on bone density in control (0.09 ± 0.01 mm Al equiv.), autograft (0.13 ± 0.01 mm Al equiv.), NCOE (0.14 ± 0.01 mm Al equiv.), and NCOE + autograft (0.20 ± 0.01 mm Al equiv.) at day 60 post-surgery ($P < 0.05$). Finally, significant variations were detected on bone density in control (0.15 ± 0.01 mm Al equiv.), autograft (0.21 ± 0.01 mm Al equiv.), NCOE (0.22 ± 0.02 mm Al equiv.), and NCOE + autograft (0.29 ± 0.01 mm Al equiv.) defects after 90 days ($P < 0.05$). Generally, the NCOE + autograft demonstrated better bone formation in all stage of the study ($P < 0.05$). The related data are illustrated in Figure 1. Also, the calvarium radiographic images after 30, 60 and 90 days post surgery are provided in Figures 2-4.

Based on Table 1, no significant effect was observed respecting WBC, RBC, HGB, HCT, MCV, MCH, and PLT levels at 30, 60, and 90 days after the surgery and defect filling with nanocomposite ostrich eggshell regarding hematology in rabbit ($P > 0.05$).

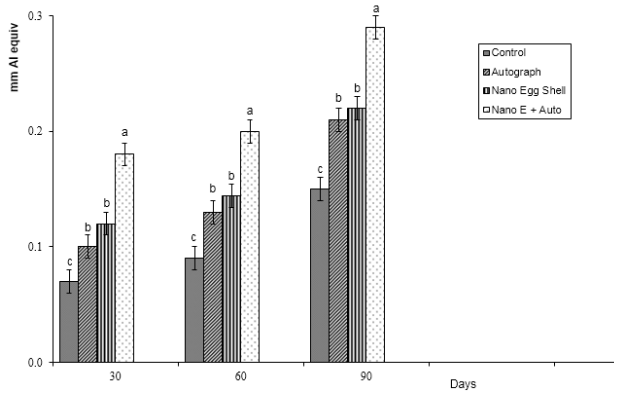

Figure 1. The Densitometry of Bone Density in 30, 60, and 90 Days After the Surgery.

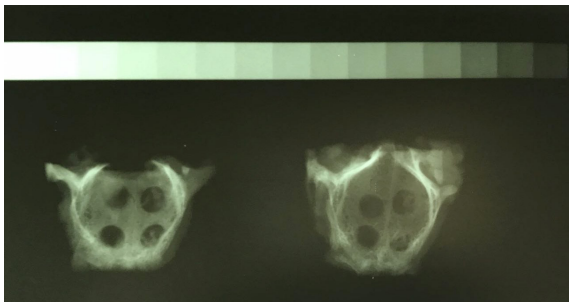

Figure 2. The Calvarium Radiographic Survey 30 Days After the Surgery. The first defect was maintained unfilled and kept as control. However, the second and third defects were filled with autogenous and filled using a nanocomposite ostrich eggshell, respectively. And the last defect was filled with a mixture of nanocomposite containing Ostrich Eggshell (NCOE) and autogenous bone. Significant differences were observed on bone formation in control (0.18 ± 0.01 mm Al equiv), autograft (0.1 ± 0.01 mm Al equiv), NCOE (0.12 ± 0.01 mm Al equiv), and NCOE + autograft (0.07 ± 0.01 mm Al equiv) defects at day 30 post-surgery ($P < 0.05$).

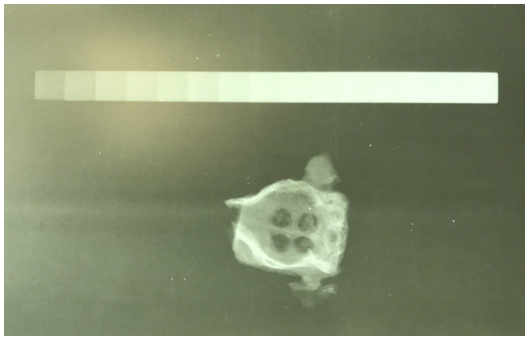

Figure 3. The Calvarium Radiographic Survey 60 Days After the Surgery. The first defect was maintained unfilled and kept as control while the second defect was filled with autogenous. In addition, the third and fourth defects were filled using a nanocomposite ostrich eggshell and a mixture of nanocomposite containing Ostrich Eggshell and autogenous bone, respectively. Further, significant differences were found on bone density in control (0.09 ± 0.01 mm Al equiv), autograft (0.13 ± 0.01 mm Al equiv), NCOE (0.14 ± 0.01 mm Al equiv), and NCOE + autograft (0.2 0 ± 0.01 mm Al equiv) defects at day 60 post-surgery ($P < 0.05$).

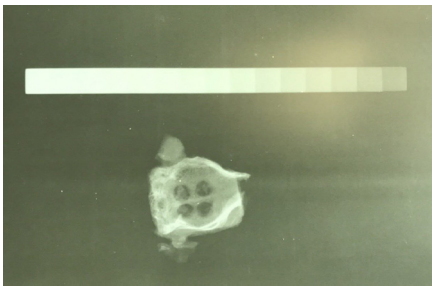

Figure 4. The Calvarium Radiographic Survey 90 Days After the Surgery. The first defect was maintained unfilled and kept as control while the second and third defects were filled with autogenous and using a nanocomposite ostrich eggshell. Furthermore, the last defect was filled with a mixture of nanocomposite containing Ostrich Eggshell and autogenous bone. Significant differences were detected on bone density in control (0.15 ± 0.01 mm Al equiv), autograft (0.21 ± 0.01 mm Al equiv), NCOE (0.22 ± 0.02 mm Al equiv), and NCOE + autograft (0.29 ± 0.01 mm Al equiv) defects after 90 days ($P < 0.05$).

Table 1. The Effect of Calvaria Defect Filling with Nanocomposite Ostrich Eggshell on Hematology in Rabbit

Hematological Values	Days Post-surgery		
	30	60	90
WBC (10^3/μL)	8.89	8.79	9.01
RBC (10^6/μL)	4.9	5.7	5.4
HGB (g/L)	123	124	122
HCT (%)	27	35	36
MCV (fL)	53.8	54.7	59.9
MCH (pg)	21.5	23.4	24.6
PLT (10^3/μL)	450	486	467

Abbreviations: WBC, White blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, Hematocrit; MCV, mean cell volume; MCH, mean cell HGB; PLT, platelets.

Discussion

Bone defects occur due to several causes and are responsible for medical, social, and economic problems in human (18). Additionally, bone regeneration includes different intra and extracellular signaling pathways which lead to osteoinduction and osteoconduction in order to increase the bone regeneration in human (18). New materials and products are introduced for use in GBR but they are relatively expensive (27). Human clinical research revealed that the combination of bone grafts with other materials can improve the engraftment, bone tissue formation, and bone defect healing (9). Several studies suggested that bone graft materials should be osteoconductive, osteoinductive, and osteogenesis (10, 11). Recently, there is growing interest in developing new biocompatible materials from animal products (27). Avian eggshell membrane powder has unique physical requisites for GBR as space-filling material (28). Based on the reports, physical characteristics of the membrane ostrich eggshell was found to have a more satisfactory effect on successful bone regeneration (27). In addition, the findings of the

current study indicated that the NCOE + autograft had better bone formation compared to the other defects at 30, 60, and 90 days post-surgery. However, there are few and controversial reports on osteoconductive effects of the eggshell as filling material for bone regeneration (25). New bone formation was found to be higher in hen eggshell compared to the bovine bone (Bio-Oss, BO) calvarial defects at 4 and 8 weeks post-surgery in rats (25). This is in line with the result of the present study. Conversely, Durmus et al (18) revealed that there was no effect on bone regeneration using the ostrich eggshell powder in rabbits. Large ostrich grafts are suitable including onlay graft, however, a complementary osteosynthesis is suggested to improve the osteointegration (19). Further, based on the radiographic results of another study, eggshell powder increased cell growth and bone remodeling on tibial bone defect repaired at 30 and 60 days after placement in dog (20). Poor mechanical possessions, small degradation time, and the lack of integrated biological components lead to the inability to form, maintain, and actively support tissue remodeling. Furthermore, ostrich shell membranes degrade more slowly than the collagen membranes (25). Based on previous studies, numerous bone graft and tissue engineering materials were introduced using the defects created in the rabbit cranium aiming at confirming their biologic stability and osteoinductive properties in clinical applications. However, to the best of our knowledge, this is the first report on the role of the NCOE in calvaria healing in the rabbit. The particle size and foreign body reaction were the main factors for an osteoconductive material. Based on the radiologic findings of the current study, no foreign body reaction was observed using the NCOE. In a previous study, 50 and 75 μM chicken eggshell particles were not detected by radiology after 60 days whereas 150–300 μM particles were resorbed during 4 months (19). However, no larger ostrich eggshell particles were resorbed completely at the same time period (19). Moreover, non-collagenous proteins of the bone matrix regulate bone formation and remodeling (20) and soluble eggshell matrix proteins may play a key role in increasing the calcium transport (20). Additionally, the eggshell organic matrix proteins can modify calcite crystal morphology during the chicken eggshell formation. In addition, transforming growth factor β1, lectin-like proteins, and calbindin (calcium binding protein) were isolated from the eggshells stimulating the bone formation (20). Eggshell specific proteins, ovocleidin, and ococalyxin are responsible for bio-mineralization during the eggshell development in the uterus of the hen and release in the eggshell degradation procedure during the chicken embryonic development (29). Further, eggshell particles in the defect edges promote the vascularization and wound procedure (29). Eggshell is an ideal source for hydroxyapatite and calcium carbonate is the main component in the eggshells (30). The hydroxyapatite synthesized from eggshell demonstrated superior sinter ability (30). Furthermore, hydroxyapatite favors adhering to the osteoblast cells and calcium carbonate improves the properties of the hydroxyapatite (30). Uraz et al (23) revealed that eggshell-derived graft substitutes enhance the new bone formation and higher levels of osteoid formation in the eggshell grafted defects. Therefore, enhanced bone regeneration is suggested in the defect margins (31). This is in conformity with the result of the present study.

Conclusions

The increased bone proliferation rate which is induced by the eggshell powder is known (29). Generally speaking, the findings of this study revealed that NCOE was an effective inhibitor of bone resorption. However, NCOE was highlighted to be effective and tolerant against postmenopausal osteoporosis (19). The mechanisms through which NCOE prevents bone loss have not been fully elucidated. Accordingly, further research is needed to determine the direct cellular and molecular mechanisms of action for further use of NCOE in clinical trials.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

The current study was approved by the Ethics Committee of Islamic Azad University of Tehran under the Ethical code of 25876.

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