Nano-hydroxyapatite and/or nano-silk fibroin as bone graft materials

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ABSTRACT

PURPOSE: This study involves a comparison between the bone regeneration of nano-hydroxyapatite (nHA), as derived from eggshells either with or without electrospun nano-silk fibroin scaffolds, and the unfilled control in the rabbit calvarial bony defect model.

MATERIALS and METHOD: Sixteen 4-month-old New Zealand white rabbits, each with an average weight of 2.8kg (range 2.5-3.0kg), were used in this experiment. After the formation of bilateral parietal bony defects (diameter: 8.0mm), either a nHA or a nHA+silk fibroin combination (nHA+silk) was grafted. The control was unfilled defect. The bone regeneration was evaluated by micro-computerized tomograms (μCT) at 4 and 8 weeks.

RESULTS: All measured variables of the μCT analysis showed that the grafted groups (nHA and nHA+silk) were significantly higher than the unfilled control groups at both 4 and 8 weeks after operation ($p<0.05$).

CONCLUSION: In conclusion, the nHA from eggshells exerted better bone formation than the unfilled control group. Electrospun nano-silk fibroin also could be considered as a scaffold for nHA.

Keywords: nano-hydroxyapatite, silk fibroin, bone graft, micro-computerized tomogram

1 INTRODUCTION

Restoration of bone defect has been important issue in dentistry. Though autogenous bone graft is a gold standard, it has many disadvantages such as donor site morbidity and limited amount of graft. Bovine hydroxyapatite (HA) has been successfully applied as bone grafts for dental implants [1]. Though it has shown a high success rate, there have been concerns like disease transmission [2]. In addition, the HA graft is not very biodegradable [3, 4]. Ideally, all bone graft materials should be replaced by regenerated bone. When the porous block HA for an alveolar cleft was used, it showed poor results [5]. Contrary to dense HA, a small HA particle can be degraded and remodeled in the host [6].

Recently, nano-HA (nHA) has been introduced as a new bone graft material. It is biodegradable and highly biocompatible compared with micro-HA [7, 8]. nHA can be produced by different methods such as hydrothermal reaction [9], pyrolysis of aerosol [10], sol-gel synthesis [11], and chemical precipitation [12]. The structural characteristics of nHA may be different depending on the production method [13]. We produced nHA from eggshells because it can be collected easily. This production method is also as simple as calcination and milling [14]. However, there has been no in vivo test for its bone formation ability. As many other types of nHA require scaffolds as carriers, we also tested electrospun silk fibroin as a scaffold for nHA.

2 MATERIALS AND METHOD

2.1 Animals, materials, and scanning electron microscopy

Sixteen-4-month-old New Zealand white rabbits with an average weight of 2.8kg (range 2.5-3.0kg) were used in this experiment. This experiment was approved by the Institutional Animal Care and Use Committee of the Bioventure Incubation Center, Hanbat National University, Daejeon, Korea (No. 2009-NCT-007). The nHA was produced from eggshells and the procedure was briefly as follows. Raw eggshells were calcinated at 900°C for 3 hours; then crushed and treated with phosphoric acid powder. The powder was milled for 10 hours in ethanol and then pressed at 220 MPa in dry condition. They were then sintered at 900°C for 2 hours. Detailed procedures can be found in previous publication [14]. The silk fibroin scaffold (molecular weight: 350 kDa) was prepared by the Rural Development Administration (Suwon, Korea) and kindly donated for this experiment. Mixtures of silk fibroin powder and nHA were electrospun and collected. The nHA content was approximately 30% of silk fibroin sponge. All materials were prepared for examination by a scanning electron microscope. After the samples on the plate were immobilized, each sample was coated by gold and examined by the scanning electron microscope (H-800, Hitachi, Japan).

2.2 Surgical method

General anesthesia was induced by intramuscular injection of a combination of 0.4ml of ketamine (100mg/ml) (Ketara; Yuhan, Seoul, Korea) and 0.3ml of xylazine (10mg/kg body

weight; Rompun; Bayer Korea, Seoul, Korea). The cranium area was shaved and disinfected with povidine-iodine. From the nasal bone to the occipital protuberance, a longitudinal incision was made in the skull. Then, a midline incision was created in the periosteum. Sharp subperiosteal dissection reflected the pericranium from the outer table of the cranial vault, exposing the parietal bones. A dental-trephine bur was used under copious saline irrigation to create a bilateral full-thickness calvarial defect. Two 8-mm-diameter defects were created, one on each side of the midline. The graft – nHA or nHA + silk fibroin- was placed on calvarial defects. Some defects were kept as empty as a control. The assignment of each group for corresponding defects was done randomly. The experimental groups were composed of five at each observation point and the unfilled control was six. Therefore, the total number of prepared defects was 32. None of the animals received the same grafts in both calvarial defects. The experimental designs of previously published studies were referenced in devising the experiment for the present study [15]. Then, the pericranium and skin were closed in layers with 3-0 silk. Postoperatively, the rabbits received gentamicin 1mg/kg (Kookje Inc., Seoul, Korea) intramuscularly 3 times daily for 3 days. Each rabbit was individually caged and received food and water. Previously published studies were referenced to determine the observation point [16]. Eight animals were humanely sacrificed at 4 weeks and 8 at 8 weeks. The dimension of the calvarial specimens was 25 x 12 x 3 mm at the largest including both defects. They were fixed in 10% formalin and underwent a micro-computed tomogram (μCT).

2.3   Micro-computerized tomogram

The prepared specimens underwent μCT using an Explored Locus SP μCT scanner (GE Medical Systems, London, Ontario, Canada). After the calibration, the calvarial specimens were scanned in sections of 0.05mm in thickness. The scanned images were reconstructed by Microview software (GE Medical System). The calibrated 3-dimentional images were shown in the gross profiles of the specimens. Since the initial defect was round in shape with 9.0 mm in diameter, the setting of the region of interest (ROI) was considered the initial defect size and shape. A threshold level of 25% of the bone standard was set as recommended by the manufacturer.

The ROI of each specimen was analyzed for bone mineral content (BMC) and bone mineral density (BMD). The tissue mineral content (TMC) and tissue mineral density (TMD) were calculated by software.

3   RESULTS

3.1   Micro-morphology of graft materials

The particle of nHA showed a rectangular shape and some particles were aggregated with each other (see Fig. 1A). The silk fibroin scaffold with nHA showed irregular surfaced fibers (see Fig. 1B).
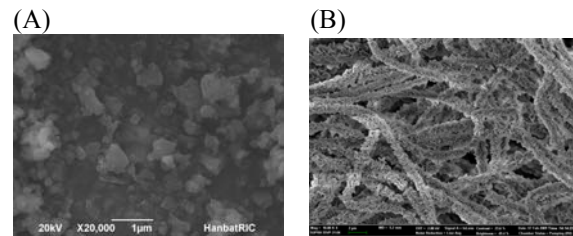
(A)   (B)

Figure 1. Scanning electron microscopic view of nHA (A) and nHA + electrospun silk fibroin (B).

3.2   Micro-computerized tomogram

The results of the μCT analysis were shown in Figure 2. The average value of all measured variables was higher in both experimental groups than in the control group at 4 weeks after the operation (see Fig. 2A, B, C). All variables were statistically significantly different when compared to the control. The BMC in the control group was 43.71 ± 3.28; that in the nHA group showed 190.28 ± 13.67 and the nHA+silk fibroin 138.39 ± 31.18 ($p<0.001$ in nHA and $p=0.002$ in nHA+silk). The BMD in the control group was 303.16 ± 18.82; that in the nHA group showed 635.21 ± 46.48 and the nHA+silk fibroin 856.56 ± 99.68 (both $p<0.001$). The TMC in the control group was 22.43 ± 0.93; that in the nHA group showed 101.39 ± 15.62 and the nHA+silk fibroin 58.52 ± 16.53 ($p=0.002$ in nHA and $p=0.008$ in nHA+silk fibroin). The TMD in the control group was 418.06 ± 25.54; that in the nHA group showed 1158.61 ± 41.34 and the nHA+silk fibroin 1393.55 ± 117.78 (both $p<0.001$). When compared nHA group to nHA+silk fibroin group, there was statistically significant difference ($p=0.018$ in BMC, $p=0.005$ in BMD and TMC, and $p=0.008$ in TMD).

The average value of all measured variables was higher in both experimental groups than in the control group at 8 weeks after the operation (see Fig. 2E, F, G). All variables were statistically significant when compared with the control. The BMC in the control group was 25.66 ± 4.36; that in the nHA group showed 187.77 ± 28.07 and the nHA+silk fibroin 128.02 ± 22.20 (both $p<0.001$). The BMD in the control group was 243.34 ± 10.02; that in the nHA group showed 1245.35 ± 182.95 and the nHA+silk fibroin 808.47 ± 53.85 (both $p<0.001$). The TMC in the control group was 12.32 ± 4.43; that in the nHA group showed 75.59 ± 33.92 and the nHA+silk fibroin 53.62 ± 13.26 ($p=0.014$ in nHA and $p=0.001$ in nHA+silk fibroin). The TMD in the control group was 327.87 ± 8.31; that in the nHA group showed 2100.04 ± 45.24 and the nHA+silk fibroin 1311.18 ± 48.43 (both $p<0.001$). There was statistically significant difference between the nHA and nHA+silk fibroin ($p=0.006$ in BMC, $p=0.004$ in BMD, and

p<0.001 in TMD). When compared TMC in both experimental groups, there was no statistically significant difference.
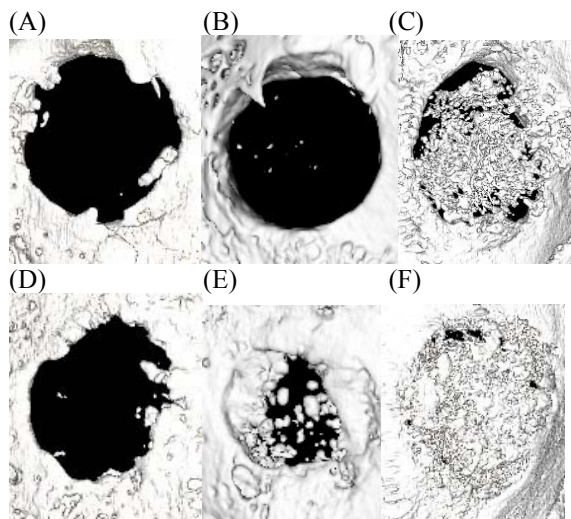

Figure 2. The results of μ CT. The calcified mass was increased at 8 weeks (D, E, F) than those at 4 weeks (A, B, C). It was also increased in nHA (B, E) and nHA+silk fibroin (C, F) than those in unfilled control (A, D).

4   DISCUSSION

In this study, the nHA derived from eggshells showed significantly higher new bone formation than the unfilled control. Interestingly, the nHA also showed significantly higher new bone formation than the nHA+silk fibroin in a μCT analysis at 8 weeks after operation (p=0.006 in BMC, p=0.004 in BMD, and p<0.001 in TMD). However, a wide range of standard deviation was shown in the nHA groups, perhaps owing to the early degradation of graft materials in some of the animals. Therefore, a proper scaffold for nHA should be developed to ensure a more reliable outcome.

Conventional synthetic HA has a large particle size compared with natural bone HA and poor mechanical properties [17]. Bioceramics exert bone regeneration by self degradation, and the space formed by that degradation can be replaced by new bone [18]. Therefore, smaller particles will have more advantages than larger ones. The cellular response to HA particles depends on the particle size, morphology, crystallinity, and chemical composition [19]. In this study, the nHA graft showed much more bone regeneration than the unfilled control in μCT analyses. All measured variables such as BMC, BMD, TMC, and TMD in the μCT analysis were significantly higher in the nHA group than in the unfilled control (p<0.001 except for BMD at 4weeks (p=0.002) and TMC at 8 weeks (p=0.014)). The nHA can increase initial cellular attachment compared with a plastic surface [20]. Calcium ions are important in osteoblast differentiation [21]. The degradation of nHA alters calcium/phosphate metabolism and activates the osteoblast via a specific calcium ion channel [22]. The nHA particles showed increased osteoblast attachment [23] and a higher calcium release than conventional sized HA particles [24].

Silk fibroin also has been used as a scaffold for nHA. There have been positive reports for silk fibroin scaffolds. Wang et al. reported that the HA-chitosan silk composite had similar properties to natural bone [25]. The silk has been reported as biocompatible and low immunogenic [26]. However, the current study showed a less bone formation in the nHA+silk group compared to the nHA group at 8 weeks after operation (see Fig. 2). There was no doubt concerning the slow biodegradation of silk macromolecules [27]. If the silk scaffold is occupied by a bone defect, new bone formation cannot occur until it has undergone biodegradation. Previous reports showed that 6 months to 2 years would be required for the complete digestion of silk [27]. Therefore, the current unmodified silk fibroin might not be a good candidate for the nHA scaffold. The low bone regeneration of the nHA+silk group might be due to slow degradation of silk. Small particles or a low molecular weight protein can be easily degraded. Recently, acid-digested silk fibroin showed high osteogenic properties [28]. Since several domains of the heavy silk fibroin chain were structurally similar to fibronectin, this might provide rapid cellular attachment and contribute to osteoblast activation [29, 30]. For successful bone regeneration, the concentration of osteoinductive materials must be maintained to allow the osteoprogenitor cells to migrate [31]. Therefore, a controlled, localized delivery system is highly important for effective bone regeneration.

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