Original Article

Osteogenic Differentiation of Human Amniotic Mesenchymal Stem Cells in Chitosan-Carbonate Apatite Scaffold (*In Vivo* Study)

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Abstract

Background: Studies of bone tissue engineering as a viable alternative to autogenous bone graft show promising results, although its mechanism and effectiveness remain only partially understood. **Purpose:** to explain the osteogenic differentiation of scaffold chitosan (Ch)–carbonate apatite (CA) in seeding with human amniotic mesenchymal stem cells (hAMSCs) on the regeneration of calvarial bone defects in rats. **Materials and Methods:** Shitosan-Carbonate Apatite (Ch-CA) scaffold was created by means of a freeze-drying method. Twenty Wistar rats were randomly divided into two groups: control and treatment. Defects were created in the calvarial bone of each treatment group with a scaffold subsequently implanted. After 8 weeks, the rats were terminated for histology and immunohistochemistry examination. **Results:** Expressions of vascular endothelial growth factor, bone morphogenetic protein2, Runt-related transcription factor 2 (RUNX2), and angiogenesis occurred earlier in the tissue-engineered group than that in the control group. An 8-week analysis also showed that the expression of RUNX2, alkaline phosphatase, osteocalcin, and collagen type 1 was at more elevated levels in the treatment group than that in the control group. **Conclusion:** These results showed that the combination of hAMSCs and Ch-CA scaffold may become one of the candidates for bone tissue engineering.

Keywords: *Calvarial bone defect, carbonate apatite, chitosan, human amniotic mesenchymal stem cell*

Introduction

Human amniotic mesenchymal stem cells (hAMSCs) have been known for their pluripotent properties.[1,2] Chitosan (Ch) can stimulate the growth and differentiation of osteoblasts in cell cultures and demonstrates beneficial bacteriostatic and hemostatic characteristics. Chitosan-Carbonate Apatite (Ch-CA) scaffold is reported to have an interconnected, nonfragile three-dimensional (3D) porous structure and the ability to support osteoblast proliferation and differentiation.[3] Combination of Ch-CA scaffold and hAMSCs could be expected to increase the new bone formation. The objective of this study is to examine the effect of hAMSCs seeding in Ch-CA scaffold on initial bone formation *in vivo*.

Materials and Methods

Isolation and human amniotic mesenchymal stem cell culture

The amniotic membrane was removed from a patient in the emergency department. This procedure was performed with the approval of the health research ethics committee (No. 378/Panke. KKE/VII/2015). The fresh amnion was mechanically skinned from the chorion and was washed three times with phosphate-buffered saline (PBS) to remove excess blood before being soaked in Ringer's lactate containing 2.5 μg/mL gentamycin (Gibco™ Gentamicin, New York, USA) and 1000 U/mL amphotericin (Gibco™ Amphotericin B, New York, USA).

The hAMSC isolation and culture procedure was performed at the Stem Cell Research and Development Center. Isolation was achieved by means of a modified Soncini's protocol. The amniotic membrane was rolled with a knife into a very fine piece tissue and then subjected to 0.25% trypsin to remove epithelial cells. The supernatant was discarded after 5 min centrifugation at 2000 rpm. The supernatant was washed with PBS containing 0.75 mg/mL of type IV collagenase (Sigma-Aldrich, St. Louis, MO, AS) and 0.075 mg/mL DNase I (Takara Bio, Shiga, Japan) before being

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incubated at 37°C for 60 min. The cells were obtained after filtration and 5 min centrifugation at 2000 rpm. Single cells were then cultured on collagen-coated discs using Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (1:1) medium (Gibco BRL, Gaithersburg, MD, USA), supplemented by human leukemia inhibitory factor (10 ng/mL) and fetal bovine serum (Gibco BRL). The medium was changed every 3 days, and on reaching 80% confluence, single cell separation was performed using trypsin to enable passage to occur. The isolation procedure used was according to the laboratory of stem cell protocol.

The chitosan–carbonate apatite scaffold preparation

200 mg of medium-molecular weight Ch powder (Sigma-Aldrich, St. Louis, MO, AS) was dissolved in 5 ml acetic acid at room temperature, mixed for 15 min, neutralized with 15 ml of NaOH solution to obtain Ch gel, before 100 mg of CA was added, and the solution was stirred until homogeneous. The gel was centrifuged for 10 min at 1500 rpm. Excess water was extracted, and an impression was made to produce a scaffold. The gel was subsequently frozen at −80°C for 2 h before being transferred to a drying machine.

Preparation of experimental animals

The research reported here received approval from the Health Ethics Committee of the Faculty of Veterinary Medicine No. 49-KE. The animal subjects of the study were twenty male Wistar rats, aged between 8 and 12 weeks and weighing 100–150 g. The rats were divided equally into two groups: the treatment group and the control group, for randomized treatments. Each group was subdivided into further two groups: the first group performed for 1 week and the other performed for 8 weeks.

Chitosan–carbonate apatite scaffold implantation procedure in rat's calvarial

The animal subjects were denied food for 4–6 h before the anesthetic procedure. Ketamine HCl (Ketalar, Ireland) at a dose of 20 mg/kg of body weight and xylazine premedication (Xyla, Ireland) at 3 mg/kg body weight was injected intramuscularly. An aseptic procedure and mid-longitudinal skin incisions on the dorsal surface of the cranium were carried out. A flap was cut until the periosteum was released from the cranium surface. The bone defect site was created using a low-speed contra-angle handpiece (NSK, Japan) of 5 mm diameter with a 2 mm thick round burr. The scaffold implanted into the resulting defect site was then sutured to reattach the wound area.

Termination of experimental animals and collection of research specimens

On conclusion of the experiment, the rats were sacrificed to obtain the required specimens. The area of bone around the implantation was separated from the surrounding soft tissues. Decalcification and embedding in paraffin were completed for the manufacture of microscopic specimens. Hematoxylin and Eosin staining was performed to highlight the angiogenesis and the bone trabecular area. A second staining was performed for further examination, including immunohistochemical imaging using anti-rabbit vascular endothelial growth factor (VEGF) polyclonal antibody (ABIN, USA), anti-rabbit bone morphogenetic protein 2 (BMP2) polyclonal antibody (ABIN, USA), anti-human Runx-2 monoclonal antibody (Cruz Biotech, USA), anti-human osteocalcin monoclonal antibody (Novus Biological, USA), anti-human collagen type I monoclonal antibody (Novus Biological, USA), and anti-human alkaline phosphatase monoclonal antibody (Novus Biological, USA) on the surface of cranium calvarial preparations postscaffold implantation. The raw data were measured using a Remmele scale index. The specimens were then inspected by means of a light microscope (Nikon H600 L, Tokyo, Japan) equipped with a digital camera DS Fi2 300 megapixel and image processing software Nikkon Image System.

Statistical analysis

Data from the experiment described above were expressed as mean values ± deviation standard. Statistical significance was determined by means of ANOVA using SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

Results

The scaffolds were solid 3D structures of 5 mm diameter and 2 mm thickness [Figure 1] implanted into the injury model of the calvarial bone of a rat. After 8 weeks, the expression of VEGF, BMP2, RUNX2, alkaline phosphatase (ALP), collagen type 1, osteocalcin, angiogenesis, and bone trabecular width was observed. Microscopic results of this study are shown in Figure 2.

In all treatment groups, the mean values were higher than that in the control group. The results of statistical analysis are also shown in Table 1.

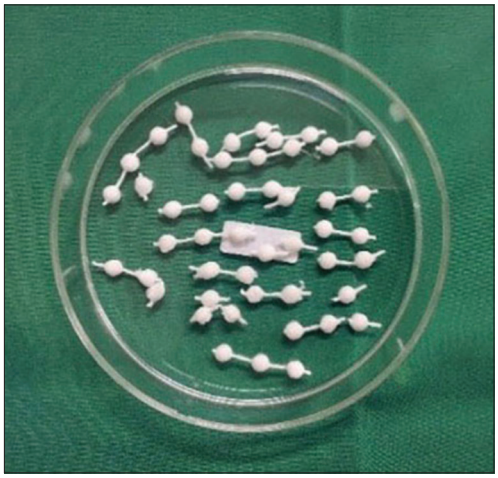

Figure 1: Carbonate Apatite-Chitosan Scaffold structure

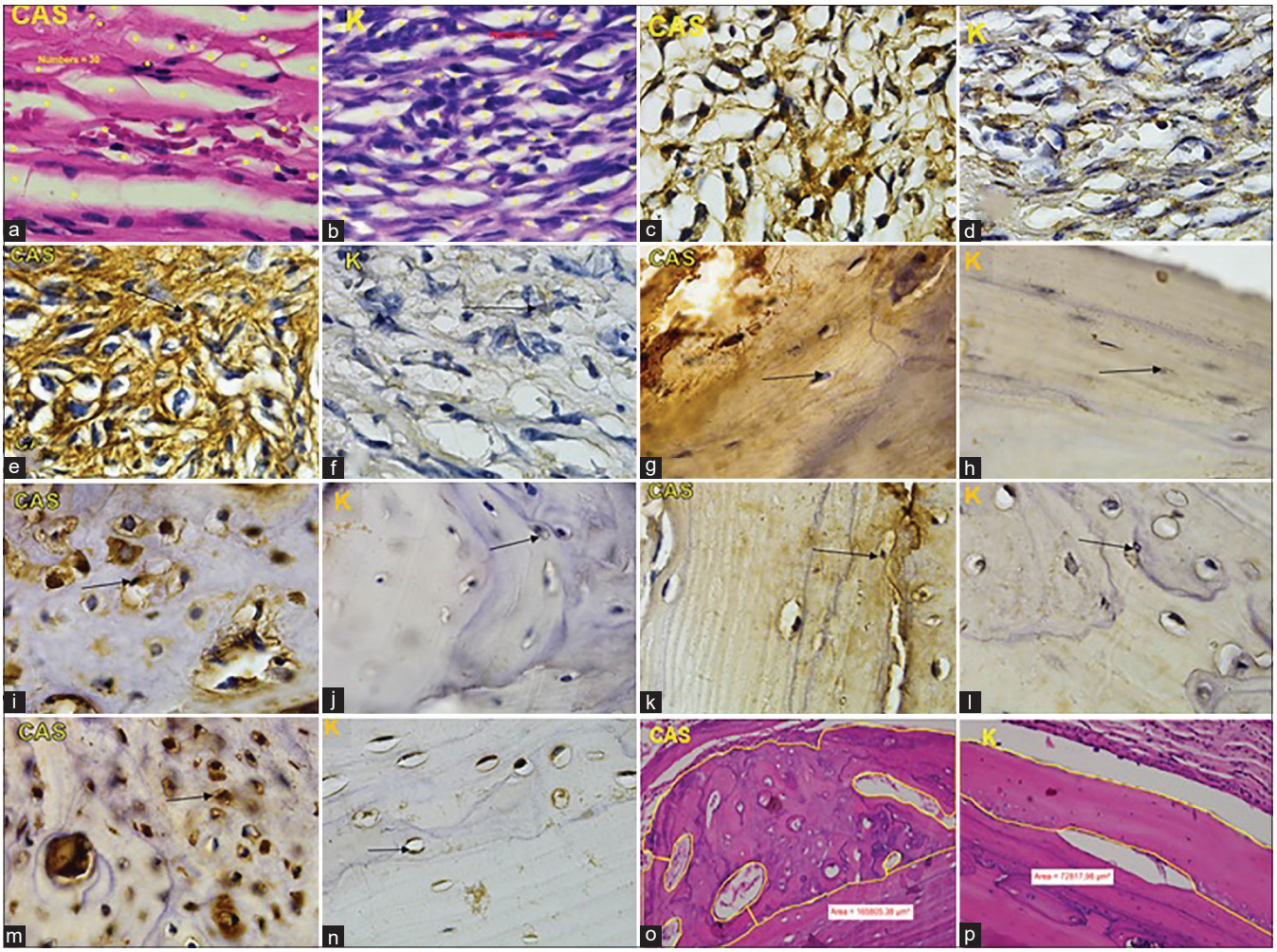

Figure 2: Microscopic picture of sample at × 1000 magnification for angiogenesis (a and b), expression of vascular endothelial growth factor (c and d), bone morphogenetic protein 2 (e and f), RUNX2 (g and h), alkaline phosphatase (i and j), type 1 collagen (k and l), osteocalcin (m and n), and trabecular bone area (o and p). CAS is the carbonate apatite-chitosan scaffold treatment group and k is the control group

Table 1: The mean value and standard deviation of experiments on various parameters

Parameter	*K*	CAS	*P*
Angiogenesis	190.20±36.67	281.40±148.58	0.000[a]
VEGF	5.44±3.79	7.92±1.15	0.443
BMP2	3.44±0.74	8.08±1.19	0.051
Runx2	2.88±1.69	8.38±3.62	0.062
ALP	1.32±0.23	5.84±2.29	0.038[b]
Type 1 collagen	2.92±1.50	6.52±3.62	0.012[c]
Osteocalcin	4.52±3.19	10.88±1.01	0.026[d]
Trabecular bone area	58,279.99±5769.33	116,119.42±27,525.49	0.002[e]

The $P<0.05$ is considered statistically significant[(a,b,c,d)].
CAS: CAS: Ch-CA scaffold group; VEGF: Vascular endothelial growth factor; BMP2: Bone morphogenetic protein 2; ALP: Alkaline phosphatase

Discussion

Autologous bone marrow-derived MSCs (BM-MSCs) have the disadvantage of morbidity due to invasive procedures necessary to perform a bone marrow aspiration in patients. Moreover, the quality of MSCs derived from BM-MSCs is influenced by the age and physical condition of the patient (Ilancheran, Moodley, and Manuelpillai, 2009).[4] Against this background, thoughts of identifying other sources of MSCs unaffected by the age and physical condition of the patient are gaining wider currency. Therefore, the potential of hAMSC as a form of xenogeneic MSCs in bone tissue engineering procedures is being increasingly investigated. Several *in vivo* studies utilizing xenogeneic hAMSCs transplantation in the repair of heart muscle, liver, and pancreatic cell damage in rats revealed no significant immunologic response that could affect tissue healing processes.[5-7] The xenogeneic MSCs transplant procedure does not potentially cause rejection that can affect the bone healing process.

The early stage of the healing process in bone defects begins with the inflammatory phase, occurring within the first 3 days postimplantation, which gradually decreases and precedes tissue repair. In the inflammatory phase, the occurrence of platelet degranulation in the hematoma and hypoxic conditions within the Ch-CA scaffold triggers an

increase in VEGF expression that induces angiogenesis. The occurrence of angiogenesis is essential to the early healing process because functional capillary tissue will ensure adequate oxygen tension, nutritional intake, and bioactive molecules.[8] In this study, it was found that an increase in VEGF expression and capillary numbers occurred in the treatment group compared to those of the control group. Angiogenesis plays an important role in cell survival in the Ch-CA scaffold as a bridge to the healing process in bone defects.[9]

The high expression of VEGF and angiogenesis in the early phases of healing in the treatment group was thought to be due to the role of Ch, which can stimulate osteoblastic growth and differentiation through cell paracrine signals on Ch-CA scaffolds. Knowing the hypoxic condition of bone defects, hAMSCs grown on Ch-CA scaffolds increase the expression of angiogenic growth factors, particularly VEGF. Previous research has also shown that if MSC is in a hypoxic microenvironment, it will increase the production of angiogenic factors, especially VEGF.[10]

In the early stages of the regeneration process, MSC proliferation occurs followed by an osteoblastic differentiation process, which is influenced by external signals, particularly BMP2 proteins produced by MSC and osteoblasts, as well as extracellular matrices. In the later stages, BMP2 leads to activation of the transcription factor RUNX2 to regulate MSC differentiation toward osteoprogenitor and preosteoblast, which serves to form a collagen and noncollagen bone matrix.[11]

The immature collagen fibers type I produced by osteoblasts form an osteoid matrix, which, in later stages, will undergo mineralization as part of the bone matrix maturation process. Examination of the expression of type I collagen fibers is performed to assess the maturation level of bone matrix; the lower thickness of I-type collagen fibers indicates the higher maturation level of bone matrix and vice versa.

Osteocalcin is a noncollagen protein in bone matrix specifically expressed by osteoblasts, which, in this case, is used as a matured osteoblast marker.[12,13] The result of calvarial bone defect is the formation of new trabecular bone. At the end of the 8th week, the area of trabecular bone in the treatment group was significantly greater than that in the control group. This finding suggests that the rate of new bone formation in the tissue-engineered group is higher than that in the control group.

The analysis results of Run × 2, ALP, type 1 collagen, and osteocalcin expression, which represents an osteogenesis process, confirmed a higher increase in the treatment group compared to the control group. This indicates that, at the end of the 8th week, the maturation level of bone matrix in the control group was lower than in the treatment group. MSCs can differentiate into osteoblasts, given the appropriate environment or stimulus. While engaging in osteogenic differentiation, MSCs will express several genes such as ALP, osteocalcin, and type 1 collagen. The increase in those expressions indicates the occurrence of osteogenic differentiation. When the osteoprogenitor forms a new bone, ALP activity will decrease momentarily. The bone matrix will increase again when there is differentiation and maturation. Once the osteoblast turns into osteocytes, ALP activity will decrease. Osteocalcin is thought to be the ultimate marker of mature osteoblasts that appear on osteocytes. Therefore, only few bones express osteocalcin in the initial bone formation.[14]

There are some limitations in this study, including the risk of complication systemically. This research only focused on the osteogenic differentiation of hAMSCs and CA-CS scaffold. These materials should be investigated further in some aspects, including the mechanical testing, the inflammatory response, and systemic toxicity and also need further study to be applied as future mandible augmentation.

Conclusion

Tissue healing occurred earlier and more effectively in the tissue-engineered group compared to the control group. The extent of bone matrix (trabecular) in the later stages of healing of the calvarial defects in the rats was greater in the tissue-engineered group than the control group. The combined application of CA-CS scaffold and hAMSCs may be suggested as a novel bone tissue engineering for provoking bone formation in clinical use.

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Conflicts of interest

There are no conflicts of interest.

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