Original Article

In vitro and *in vivo* evaluation of carbonate apatite-collagen scaffolds with some cytokines for bone tissue engineering

Sherman Salim, Maretaningtias Dwi Ariani

Department of Prosthodontics, Faculty of Dentistry, Airlangga University, Surabaya, Indonesia

Abstract

Background: Collagen is regarded as one of the most useful biomaterials. We tried to combine collagen and carbonate apatite (CA) with some cytokines in order to enhance bone formation ability. In this study, we found that CA-collagen sponge (CA-CS) was a possible candidate of newly graft material for bone formation.

Materials and Methods: CA-CS was fabricated by the following procedure. One wt% of pig hide collagen solution (Nippon Meat Packers. Inc., Tokyo, Japan) was neutralized with 0.1 N NaOH, and then mixed immediately 243 mg apatite powder with 0.06 M carbonate contents. After centrifugation at 1500 rpm for 10 min, excess water was removed, and the mixture was packed into Teflon molds (5.0 mm × 2.0 mm). Each 10 μg of basic fibroblast growth factor (bFGF) and recombinant human bone morphogenetic protein-2 (rh-BMP2) were involved in these sponges. Then these scaffolds frozen at −80°C for 2 h and dried in a freeze dry machine for 24 h. CA-CS without cytokines were also prepared as a control. Mouse osteoblast-like cell (MC3T3-E1) proliferations in these scaffolds were investigated by 3-day *in vitro* cell culture using MTT assay examination. Ten New Zealand rabbits (weight: 3–3.5 kg) were used in this *in vivo* study. After 3 weeks of placement, the scaffolds, rabbits were sacrificed, and bone formation in the sockets was evaluated histologically and histomorphometrically.

Results and Conclusion: By histological observation and measurement of bone area ratio, CA-CS with cytokines showed higher bone formation ability (bFGF/CA-CS: 50.7 ± 7.3%, rh-BMP2/CA-CS: 54.2 ± 5.0%) than other groups. From the limited results of this study, it is suggested that CA collagen scaffolds with some cytokines may become an attractive scaffold for bone regeneration.

Key Words: Bone tissue engineering, carbonate apatite, collagen

Address for correspondence:
Dr. Maretaningtias Dwi Ariani, Department of Prosthodontics, Faculty of Dentistry, Airlangga University, Surabaya, Indonesia. E-mail: etaprosto@yahoo.com
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INTRODUCTION

Tissue engineering is required as an emerging technology to reconstruct damaged bone because of tumors, trauma or pathologic disease. The bone formation using bone graft material is a widely accepted technique in the surgical field. In order to repair damaged bone, autogenous bone has been the ideal choice to fill the defect site. However, autografts sometimes have significant limitations coming from a donor site morbidity, a limited donor bone supply, and an inadequate resorption rate during the healing process.[1,2] These limitations have prompted

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increasing interest in alternative bone grafts. Allografts and xenograft are alternative and attractive sources but have several problems such as the risk of disease transmission, inflammation, immunogenicity, loss of biologic and mechanical properties, and religious concerns.[3] Consequently, significant efforts are being made to develop ideal bone graft material to induce the formation of new bone to bridge the defect.

The use of artificial bone graft material to replace bone defect may have many useful benefits, such as the elimination of the risk of disease transmission, fewer surgical procedures, reducing the risk of infection or immunogenicity, and the abundant availability of synthetic scaffold materials.[4] The characteristics of ideal bone graft material would be biocompatible, maintain volume over time, suitable mechanical property, controllable biodegradability, and be replaced entirely by new bone ingrowth.[5]

Many natural and synthetic materials have been developed and characterized, but satisfactory bone-formative materials have not been introduced. Current attempts are focused on bioceramics such as calcium phosphate which is found in the body, demonstrated excellent cellular and tissue responses *in vitro* and *in vivo*, and shows promises of biocompatibility, osteoconductivity, and biodegradability.[6,7] The types of calcium phosphate materials which have developed and investigated as bone graft material are hydroxyapatite (HA,$Ca_{10}(PO_4)_6(OH)_2$), β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$), biphasic calcium phosphate, and multiphasic bio-glasses.[7,8] However, some problems arise such as difficulties to control absorbance time period of HA and it has been reported that HA produced from coral has the inability to control the pore size and chemical composition, thereby resulting in unpredictable outcomes.[9-11] In addition, resorption time of β-TCP is faster 10–20 times than HA, the macroscopic mechanical properties of β-TCP are inadequate for load bearing surface due to the inherent brittleness.[12] Therefore, to overcome these problems, we focused on carbonate apatite (CA) as a biodegradable material. The main reason is because CA resembles bone apatite more closely than any other calcium phosphate. The main inorganic content of bone is CA which contains about 7% carbonate by weight.[13] CA easily dissolves under acidic condition, and thermodynamically under neutral and basic condition. It was reported by the histological observation that CA was gradually replaced by regenerated bone. Considering these properties, CA is expected to become an ideal bone replacement material, which possesses both osseoconductivity and bioresorbability.[14]

To obtain the biomimetic bone graft material, combination of CA with Collagen has been developed. CA and collagen were combined to create new materials CA-collagen sponges (CA-CSs) with unique structural and mechanical properties appropriate for the bone graft material. It is reported that 0.06 M CA-CS has space maintenance ability and show bone formation ability higher than other concentration in bone defect of rabbit's femur.[15-17]

However, osteoinductive proteins play an important role to enhance bone formation ability. There have been a variety of growth factors were identified and isolated for *in vitro* and *in vivo* including bone morphogenetic protein (BMP), fibroblast growth factor (FGF), platelet derived growth factor, and transforming growth factor (TGF). In this study, we focused on BMP and FGF because it has been reported that BMP and basic FGF (bFGF) can induce bone formation and have been used clinically within scaffolding biomaterials carriers. The term "BMP" was introduced to describe the substance(s) in the demineralized bone matrix responsible for the phenomenon. At least 15 BMPs are currently recognized (BMPs 1–15).[18,19] Recently, bone morphogenetic protein-2 (BMP2) is the leading osteoinductive growth factor used clinically in bone-related regenerative medicine. The osteoinductive properties of BMPs have been evaluated extensively both *in vitro* and *in vivo*.[18,20] On the other hand, bFGF is known to be a potent mitogen of bone cells *in vitro* produced by osteoblasts and stored in the bone matrix. It has been demonstrated that a topical application of exogenous bFGF also enhances the healing process of bone fractures.[18] Furthermore, bFGF can accelerate ideal periodontal regeneration without ankylosis or epithelial downgrowth.[19,20]

Based on these recent findings concerning the relationship of CA-CS with BMP2 and bFGF, the combined application of these two factors could be expected to enhance synergistically bone formation. Therefore, we examined the effect of CA-CS with recombinant human BMP2 (rh-BMP2) and bFGF on cell proliferation using MC3T3-E1 *in vitro* and initial bone formation *in vivo*.

MATERIALS AND METHODS

Preparation of sponges

CA-CS was fabricated using lyophilization techniques. To fabricate CA-CS, 10 ml of 1.0 wt% of pig hide collagen solution (Nippon Meat Packers. Inc., Tokyo, Japan) was neutralized with 0.8 ml of 0.1 N NaOH. Adding 243 mg of CA of 70 wt% dry weight and then mixed immediately. After centrifugation at 1500 rpm for 10 min, excess water was removed, and the mixture was packed into 2 types of Teflon molds (5 mm in diameter × 2 mm in height and 3 mm in diameter × 5 mm in height). The molds were frozen at −80°C for 2 h and dried in a freeze dry machine (EYELA Co. Ltd.,

Tokyo, Japan) for 24 h. CA-CS were subjected to UV radiation for 2 h. As a control, we fabricated CS, by not adding 243 mg of CA of 70 wt% dry weight after neutralizing with 0.8 ml of 0.1 N NaOH and then next procedure was the same as described above.

Preparation of recombinant human bone morphogenetic protein-2 and basic fibroblast growth factor solutions

rh-BMP2 and bFGF (PeproTech, Inc., London, UK) were dissolved in phosphate buffered saline and combined with CA-CS in each 20 μg/mL. Then stored at −80°C. Before cell seeding by using MC3T3-E1, CA-CS + rh-BMP2 and CA-CS + bFGF dried in a freeze dry machine (EYELA Co. Ltd., Tokyo, Japan) for 24 h.

Microstructure observation

To ensure that the shape of the sponge was kept after addition of cytokine, we observed the microscopic structure and porosity of CS, CA-CS, CA-CS + rh-BMP2 and CA-CS + bFGF by scanning electron microscopy (SEM) (3D Microscope VE-8800, Keyence, Japan).

Cell viability assay

Mouse osteoblast cells line MC3T3-E1 (RIKEN BioResource Center, Tsukuba, Japan) were cultivated on cell-culture plate in Dulbecco's Modified Eagle's Medium (DMEM) medium (Sigma, USA) supplemented with 2% penicillin/streptomycin, 10% fetal bovine serum and passage every 3–4 days. MC3T3-E1 cells between 2×10^4 cells in DMEM medium were statistically seeded onto CS, CA-CS, CA-CS + rh-BMP2, and CA-CS + bFGF. Cells were left in DMEM medium for 3 h, and then 980 μl of the culture medium was added to each well of 24-well culture plates. Cells were cultivated in 5% CO_2 at 37°C in 95% relative atmospheric humidity for 1, 3, 5, and 7 days. The culture medium was changed every 3 days.

Cell viability in the porous structure was examined by MTT assay (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc., Rockville, Maryland, USA). Following incubation with 550 μl/well for 2 h in the incubator (in 5% CO_2 at 37°C). The solution was then transferred to 96 wells and the absorbance of the solution in each well was measured by using a microplate reader (BiotrakTM II, Amersham/Bioscience, Australia) at wavelength 450 nm.

Measurement of alkaline phosphatase activity

Cells were seeded on 24-well tissue culture treated plates at a density of 2×10^4 cells/well. At each time points, cells were washed 3 times with saline solution. The cell suspension was then transferred to a centrifuge tube, treated by ultrasonication and centrifuged (7200 g for 10 min, 4°C). The alkaline phosphatase (ALP) activities of the samples were determined by a colorimetric assay using an ALP reagent containing p-nitrophenyl phosphate (p-NPP) as substrate (BioAssay, Hayward, CA, USA). The absorbance of p-nitrophenol formed by the hydrolysis of p-NPP, catalyzed by ALP, was measured using microplate reader at 405 nm wavelength.

Animal experiments

In vivo study of this experiment was approved by Research Facilities Committee for Laboratory Animal Science, Hiroshima University School of Medicine. Sixteen New Zealand rabbits (weight: 3–3.5 kg) were used. Four bone sockets (3 mm in diameter, 5 mm in height) were prepared in right femur of a rabbit [Figure 1]. Four types of scaffolds (CS, CA-CS, CA-CS + rh-BMP2 and CA-CS + bFGF) were randomly implanted into the bone socket, and one socket was kept with blood clot as a control. 2 and 3 weeks after implantation, rabbits were sacrificed. Tissue block, including the sample were cut and observed on X-ray radiography. Then fixed in 10% neutral formalin for 1 week and embedded with paraffin through dehydration with ethanol. Specimen were cut into 5 μm thickness and stained with hematoxylin and eosin.

Images of new bone formation were examined by light microscopy and counting number with an image analyzing software (Image J, National Institutes of Health, Bethesda, USA). The percentage ratio of the newly formed bone area against the total compact bone area was analyzed statistically by ANOVA.

RESULTS

Microstructure observation

Microstructures of the scaffolds were examined by SEM Figure 2 showed that all of the scaffolds had three-dimensional porous structures together with good interconnections between

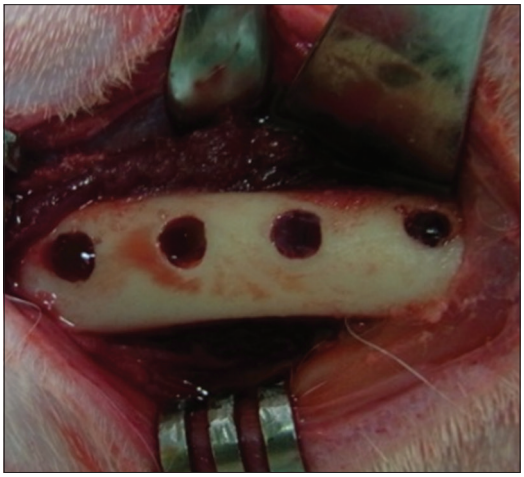

Figure 1: Photo showing four bone sockets (3 mm in diameter, 5 mm in height) were prepared in right femur of a rabbit using drills for Brånemark® implant system

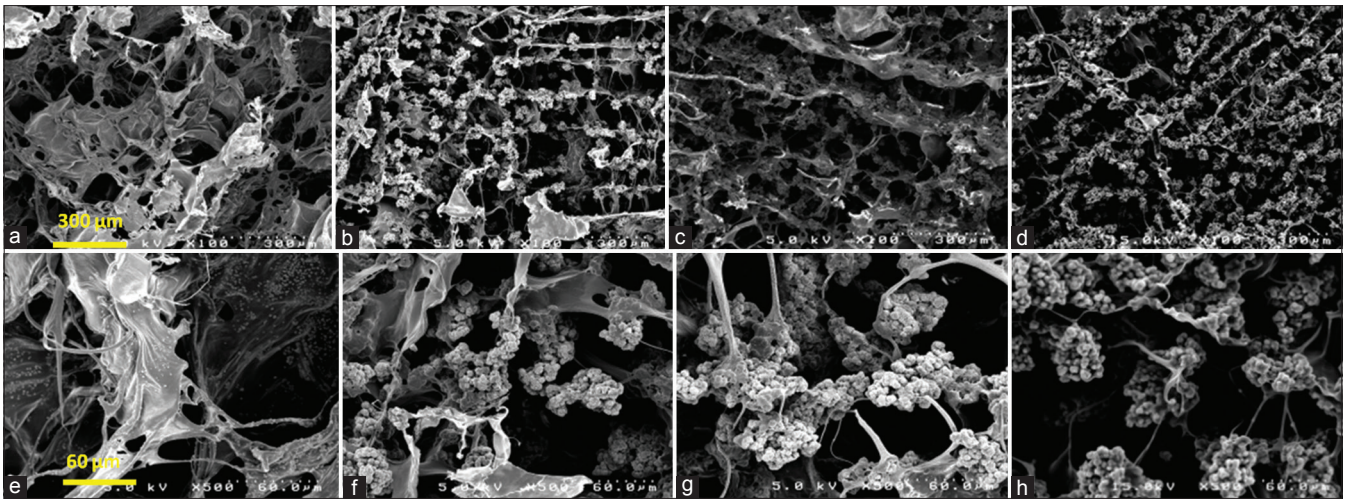

Figure 2: Scanning electron microscope images of collagen sponge (a and e), carbonate apatite-collagen sponge (b and f), carbonate apatite-collagen sponge + recombinant human bone morphogenetic protein-2 (c and g), carbonate apatite-collagen sponge + basic fibroblast growth factor (d and h) at ×100 (a-d) and ×500 (e-h)

the pores. This structural characteristic was preserved after cytokine incorporation.

Cell viability assay

The significant increase of cell numbers was seen in both of bFGF/CA-CS and rh-BMP2/CA-CS than in CA-CS at 3 days ($P < 0.05$) [Figure 3]. There was no significant difference between bFGF/CA-CS and rh-BMP2/CA-CS.

X-ray observation

X-ray examination was done after 2 weeks and 3 weeks implantation at femur of rabbits. X-ray observation of CA-CS + rh-BMP2, CA-CS + bFGF, CA-CS, CS, and open defect are shown in Figure 4. There are some high-density developments in the bone defect area. It meant that the new bone had formed at week 3 [Figure 4b].

Histological observation

The results of histological observation of CS, CA-CS, CA-CS + rh-BMP2, CA-CS + bFGF, and open defect after 2 and 3 weeks implantation at femur of rabbits with hematoxylin and eosin staining are shown in Figures 5 and 6. It could be observed that more new bone formation had developed. This is supported by the results of the calculation from the percentage ratio of the newly formed bone area at 3 weeks implantation [Figure 7].

DISCUSSION

In this study, we demonstrated a novel method to fabricate CA-CS based on lyophilization technique. CA was combined with collagen to improve the biological stability and strength of CA-CS to reach the demand of an application in the bone graft material. It has been known that the function of scaffolds not only as a delivery vehicle for growth factors and living cells but also support and regulate bone formation.[21]

To allow ingrowth of cells and migration of vascular tissue, porous structure is needed.[22] From the SEM images of this study revealed that CA-CS had much porous and three-dimensional structure and also attachment of CA powders on CA-CS were observed. We created CA-CS with pore size approximately 50–300 µm. These pore sizes had sufficient space for cells to invade. Highly porous and interconnected pore structures were required to ensure that the biological environment is conductive to cell attachment, proliferation, tissue growth and adequate nutrient flow.

To improve bone formation ability, growth factors are needed. A variety of growth factors participates in the regulation of cell proliferation, differentiation, and bone metabolism. Osteoblasts differentiation and matrix mineralization are regulated by the actions of systemic and local signaling factors. Currently, many cytokines have been evaluated and investigated including rh-BMP2 and bFGF and it was identified that rh-BMP2 and bFGF can accelerate bone formation.[16]

BMP2 belong to the TGF superfamily, has a powerful inducer of osteoblast differentiation *in vitro* and bone formation *in vivo*. BMP2 are required for bone regeneration. On the other hand, FGFwas originally identified from extracts of the pituitary gland and brain and has been demonstrated to stimulate the proliferation of fibroblasts. It has been established that bFGF is a potent mitogen not only for fibroblasts, but also for other mesoderm-derived cells including osteoblasts and vascular endothelial cells.[21-23] Most *in vitro* studies have reported that bFGF can influence the differentiation of bone marrow stromal cells (BMSCs), and enhance chondrogenic and osteogenic differentiation of BMSCs.

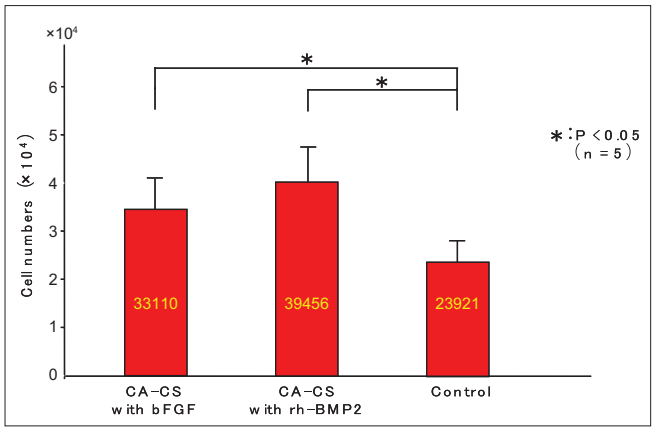

Figure 3: Cell viability assay of the scaffolds

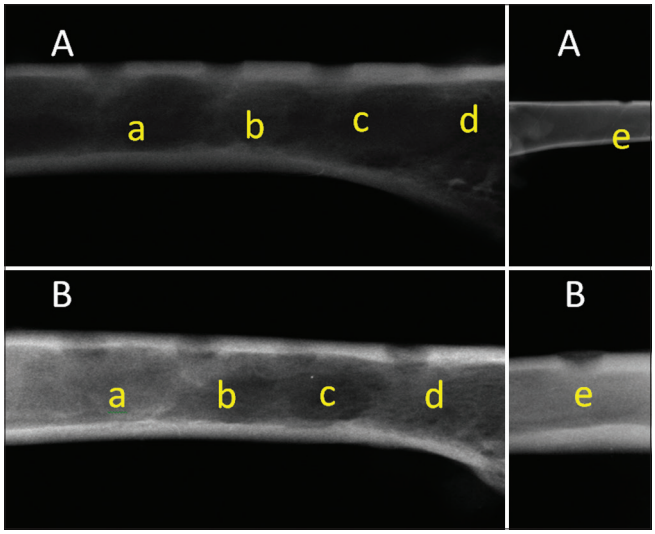

Figure 4: X-ray observation of carbonate apatite-collagen sponge + recombinant human bone morphogenetic protein-2 (a), carbonate apatite-collagen sponge + basic fibroblast growth factor (b), carbonate apatite-collagen sponge (c), collagen sponge (d), and open defect (e) after 2 weeks (A) and 3 weeks (B) implantation at femur of rabbits

BMP2 and bFGF are growth factors that act as prototypical mitogen and morphogen, respectively.[24] The combination of growth factors has been shown to result in synergistic effects that might further stimulate the complex cellular events and interactions that lead to new bone formation.[25]

Cell viability of MC3T3-E1 in CA-CS was measured by MTT assay. The significant increase of cell numbers were seen in both of bFGF/CA-CS and rh-BMP2/CA-CS than in CA-CS at 3 days ($P < 0.05$). There was no significant difference between bFGF/CA-CS and rh-BMP2/CA-CS. All sponges supported the growth of cells on three-dimensional structures in a similar manner. Moreover, rh-BMP2/CA-CS had a tendency to promote the growth of cells to a greater extent than other.

The histological observations show that the combination of CA-CS with rh-BMP2 and bFGF promotes bone formation. There were trabecular bone included many osteocytes and regularly lined with many osteoblasts, indicating the bone-forming activity was observed. There was no evidence of inflammation or foreign body reaction in the host tissue adjacent to the new bone. After 3 weeks implantation, the percentages of newly formed bone at femur of rabbits were 23.7% in bone defect, 25.8% in CS, 38.1% in CA-CS, 50.7% in CA-CS + bFGF and 54.2% in CA-CS + rh-BMP2, respectively, indicating that new bone formation ratio of CA-CS with rh-BMP2 was significant higher than those of other group. This demonstrated that there were significant differences in the amount of new bone formed in response to the different cells.

CONCLUSION

We have shown that the combination of CA-CS with rh-BMP2

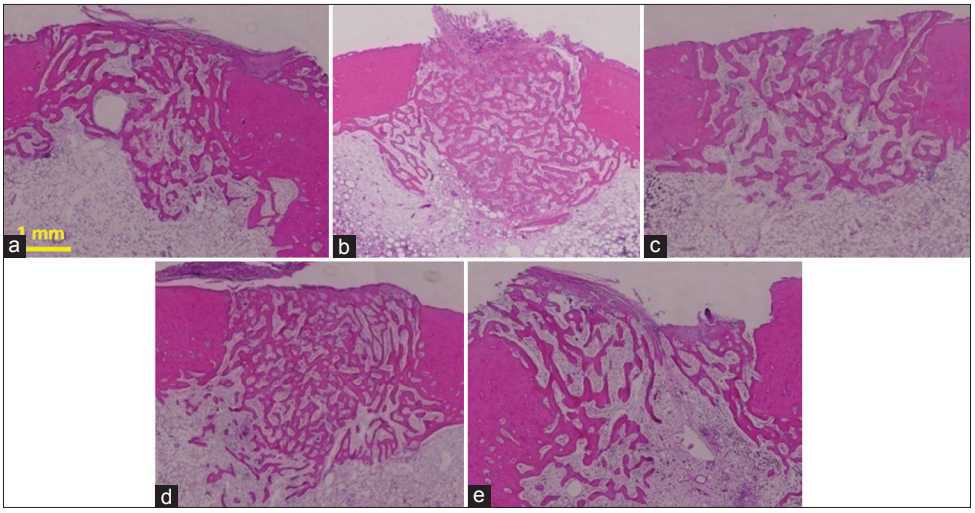

Figure 5: Histological observation of collagen sponge (a), carbonate apatite-collagen sponge (b), carbonate apatite-collagen sponge + recombinant human bone morphogenetic protein-2 (c), carbonate apatite-collagen sponge + basic fibroblast growth factor (d), and open defect (e) after 2 weeks implantation at femur of rabbits with H and E staining

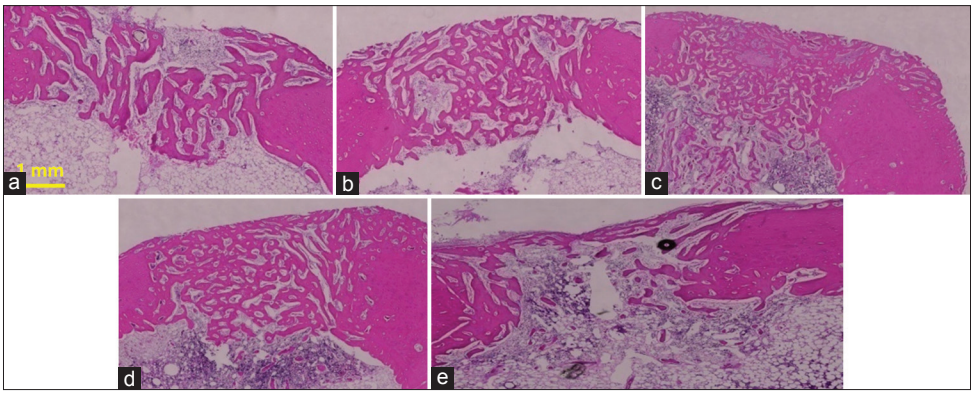

Figure 6: Histological observation of collagen sponge (a), carbonate apatite-collagen sponge (b), carbonate apatite-collagen sponge + recombinant human bone morphogenetic protein-2 (c), carbonate apatite-collagen sponge + basic fibroblast growth factor (d), and open defect (e) after 3 weeks implantation at femur of rabbits with H and E staining

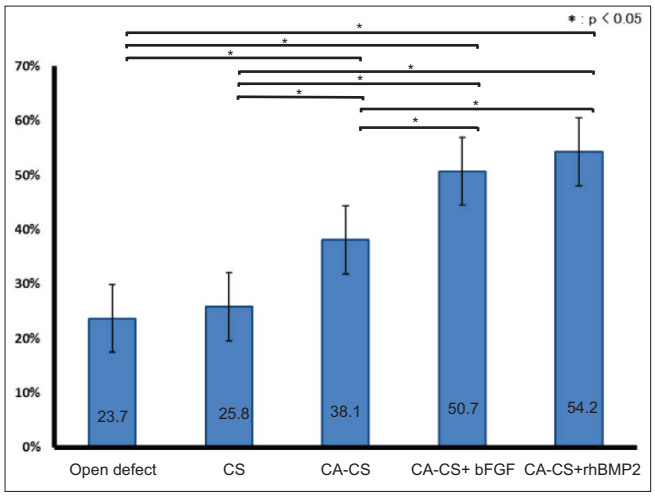

Figure 7: Bone formation area ratio at femur of rabbit (3 weeks)

and bFGF may induce cell proliferation and the results of the *in vivo* study showed that CA-CS + rh-BMP2 and CA-CS + bFGF had the ability to enhance bone formation. It may be suggested that combined application of CA-CS with rh-BMP2 and bFGF can provide as a novel bone graft material for bone formation in clinical use.

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Nil.

Conflict of interest

There are no conflict of interest.

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