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A histomorphometric study on collagen-apatite composite as a graft material: the influence of gap size at the titanium–bone interface in animal model

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Abstract The purpose of this study was to evaluate the healing process of collagen-apatite composite (CAC) at the titanium–bone interface in animal model. Small gaps (0.5 or 1.0 mm-sized wells) were prepared in the epoxy-resin block implants coated with pure titanium. The gaps were filled with CAC or demineralized freeze-dried bone (DFDB). The titanium-coated epoxy-resin block implants were inserted in the tibia of rabbit for 4 weeks or 8 weeks. The microscopic features of bony healing process in the grafted gaps were examined and analyzed. In the histomorphometric analysis, CAC group showed higher fraction of newly-formed bone than DFDB group in both 0.5 and 1.0 mm gap subgroup at 4-week specimen ($P < 0.05$). In the transmission electron microscopic examinations, osteoblasts of the newly-formed bone of CAC group showed more cellular activity than that of DFDB group. From the results, it was expected that CAC had more beneficial property on early bony healing process than DFDB at the titanium–bone interface.

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1 Introduction

It is generally accepted that dental implant is a valid treatment option for edentulous patients. With the development of auxiliary surgical procedures including sinus lift procedure, the application of dental implant was expanded to the anatomically vulnerable areas. Also there has been remarkable development of bone graft techniques and bone substitute materials to fill the bony defect adjacent to the dental implants [1, 2]. Numerous bone substitute materials have been used instead of conventional autogenous bone graft. Though the qualities of bone substitute materials have been improved and there are few problems in clinical use, it is suspected that there are differences in effect of bone formation between bone substitute materials [3, 4]. Alloplastic materials are recommended, the framework of which is composed of porous hydroxyapatite (HA). These materials are biologically acceptable, allowing bone ingrowths and bone remodeling while maintaining volume [5]. Since Doi et al. introduced apatite-collagen complexes, many studies have been performed to apply hydroxyapatite-combined collagen as a bone substitute material [6–8]. Kim et al. reported a novel protocol to prepare collagen-apatite composite (CAC) [9]. However, there were few data on the suitability of CAC as a graft material in implant surgery. To evaluate the usefulness of a graft material in the implant surgery, investigation of the bony healing process at the titanium–bone interface is critical.

The purposes of this study were to evaluate the healing process of CAC graft and to evaluate the influence of gap

size at the titanium–bone interface histomorphometrically in animal model.

2 Material and method

2.1 Titanium-coated epoxy-resin block implants preparation

Implants were made of epoxy-resin (Poly/Bed 812, Polyscience Inc. IL, USA) block following Luft method [10]. In the epoxy-resin block implant, well-shaped gaps of different sizes (0.5 mm and 1.0 mm, respectively) were formed (Nanotech Co., Uiwang, Korea) (Fig. 1). Epoxy-resin block implants were coated with pure titanium using ion sputtering coater (BIO-RAD, CA, USA) at the condition of 1200 kVp, 15 mA, 10^{-4} Pa for 180 s. Titanium-coated epoxy-resin block implants were cleansed with 70% of alcohol, dried and autoclaved.

2.2 Graft materials

2.2.1 Collagen-apatite composite (CAC)

Highly supersaturated stable calcium and phosphate ion solution prepared at 4°C. Synthetic calcium phosphate apatite crystals were dissolved in 0.2 N HCl (1.0 mg/ml). Then, 1.0 ml of this acidic ion solution was mixed with 1.35 ml of 0.2 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) solution to form Ca–P precipitate at 4°C. Ca–P precipitate was removed by filtration (pore size: 0.22 μm) to get a metastable calcium and phosphate ion solution (pH 7.3). CollaTape® (IntegraLife-Sciences Co., Plainsboro, NJ, USA) was used as a collagen matrix. CollaTape® was cut into the size of 0.5 × 5.0 mm, and soaked into this Ca–P ion solution at 37°C for 40 min to induce the nucleation of Ca–P on the surfaces. The solution was dropped away and reacted with 2.5 mM solution for 30 min. This CAC was cleaned with HPLC grade distilled water and dried. Temperature was maintained or increased (up to 60°C) to form a thin film coat of low-crystalline apatite crystals (LCAs) by the growth of apatite crystals. Scanning electron microscope (SEM, JEOL Inc., Tokyo, Japan) was used to examine the surface of CAC.

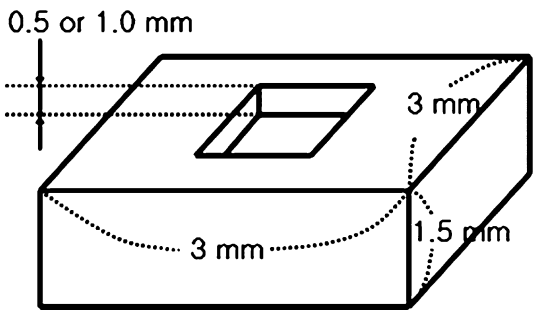

Fig. 1 Titanium-coated epoxy-resin block implant. The surface was coated with pure titanium by use of ion sputtering coater. Each implant had a well of 0.5 or 1.0 mm depth

2.3 Demineralized freeze-dried human bone (DFDB)

In this study, for the comparison of bone healing property with CAC, demineralized freeze-dried bone (DFDB, Dembone®, Demineralized Human Bone Powder, Pacific Coast Tissue Bank, CA, USA) was grafted.

2.4 Surgical procedure

This study was approved by the animal ethics committee at Dental Research Center of Seoul National University. Twelve white New Zealand adult rabbits (weight range: 2.8–3.2 kg) were included in the study. Implantation surgery was performed in an operating theatre using aseptic technique. The animals were anaesthetized with ketamine (Ketalar®, Yuhan Co., Korea) and xylazine-hydrochloride (Rompun®, Bayer Korea Ltd., Korea) at the administration ratio of 50 mg/kg intramuscularly. The skin of left leg was shaved and cleaned with povidone iodine solution (Betadine®, Samil Pharm. Co., Korea). After local anesthetic agent injection, incision and subperiosteal flap elevation was done. Three sites for titanium-coated epoxy-resin block implants were prepared with low speed drill in copious saline irrigation.

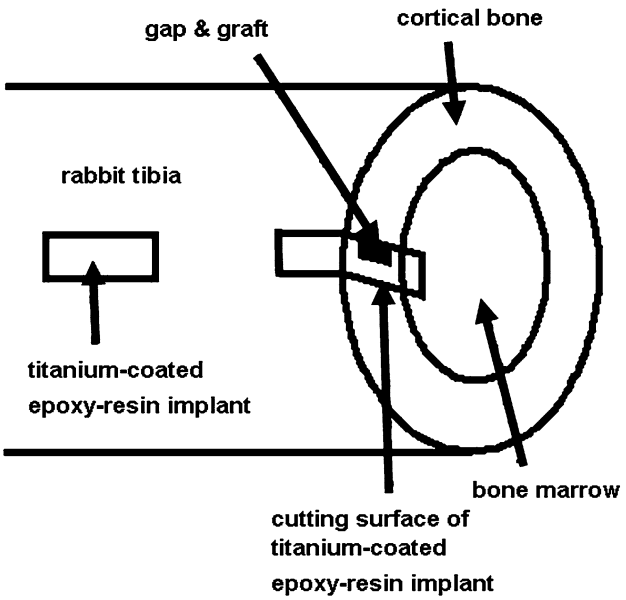

Fig. 2 Schematic diagram of implantation of titanium-coated epoxy-resin block into tibia of rabbit. Three titanium-coated epoxy-resin block implants had different graft. (1) Control (no graft), (2) CAC, (3) DFDB, respectively. The gap size of titanium–bone interface was 0.5 mm in one subgroup and 1.0 mm in the other

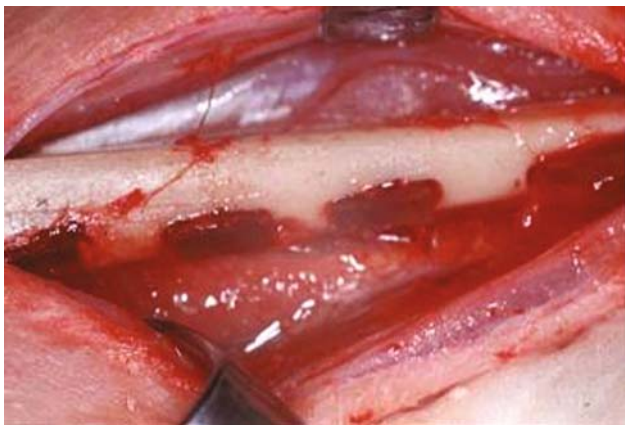

Fig. 3 Titanium-coated epoxy-resin block implants were inserted into the tibia of rabbit

Gaps (0.5 mm or 1.0 mm) on the titanium-coated epoxy-resin block implants were filled with graft materials in experimental groups (Figs. 2, 3). Periosteum and skin were closed with Vicryl® 4-0 (Ethicon, Somerville, NJ), respectively. For preventing infection, gentamycin (Gentamycin®, Kukje Pharm. Co., Korea) was administrated intramuscularly into all experimental animals at the ratio of 3.0 mg/kg/day for three days. The experimental animals were fed commercial solid food (Samyoung Oil & Food, Korea) manufactured for experimental animals.

2.5 Histology and histomorphometry

At the time of 4 and 8 weeks after surgical procedure, the animals were anaesthetized with the same method of above. The animals were euthanized through direct injection of 2.5% glutaraldehyde solution (0.1 M phosphate buffer, pH 7.2) into the left ventricle of heart for vascular perfusion. The titanium-coated epoxy-resin block implants were retrieved en bloc with surrounding bone tissue followed by immersion in 2.5% glutaraldehyde for prefixation. The samples were decalcified with 0.2 M EDTA for 16 weeks and post-fixed with 1% OsO_4 (0.1 M cacodylate buffer, pH 7.2) at 4°C. For the microscopic examination, the samples were dehydrated in a graded series of ethanol and treated with propylene oxide and finally embedded in epoxy-resin (Poly/Bed 812, Polyscience Inc. IL, USA). The samples were cut horizontally from cortical bone of tibia to marrow. At the depth of 300 μm from the outer cortex of tibia, 5 serial sections were gained and toluidine blue staining was performed. The cortical bone healing pattern at 4 and 8 weeks with different gap sizes and different graft materials was observed through digital microscope (Qimaging Co., BC, Canada). Histomorphometric evaluation was performed by an investigator using a computer-assisted image analyzing software (Global Lab Image V.2.10®, Data Translation Inc., MA, USA) at the Dental Research Center of Seoul National University. The mean percentages of newly-formed bone in the gaps were calculated. The differences between CAC and DFDB group were evaluated by independent *t*-test. The differences between 0.5 and 1.0 mm gap subgroups were evaluated by paired *t*-test. A P value of <0.05 was considered to be statistically significant. The statistical evaluation was performed with SPSS 15.0.

2.6 Transmission electron microscopy (TEM)

Following semi-thin sections for light microscopy (LM), ultra-thin sections of 60–80 nm were made with the use of ultra-thin microtome (Microtome 2050 Supercut®, Teichert-Jung, Germany) and double-stained in uranyl acetate and lead citrate for transmission electron microscopy (TEM). And these samples were observed through TEM (TEM-1200 EX II®, JEOL, Tokyo, Japan) at a accelerating voltage of 80 kV.

3 Results

3.1 SEM image of CAC

The CAC in this study demonstrated collagen lattice structure on which thin film of apatite crystal was coated (Fig. 4). Apatite-aggregated granules were also observed.

3.2 Light microscopy (LM)

In low magnification, newly-formed bone was found in the grafted gap in all groups. For the convenience, titanium surface of the gap was located at the bottom in all photographs.

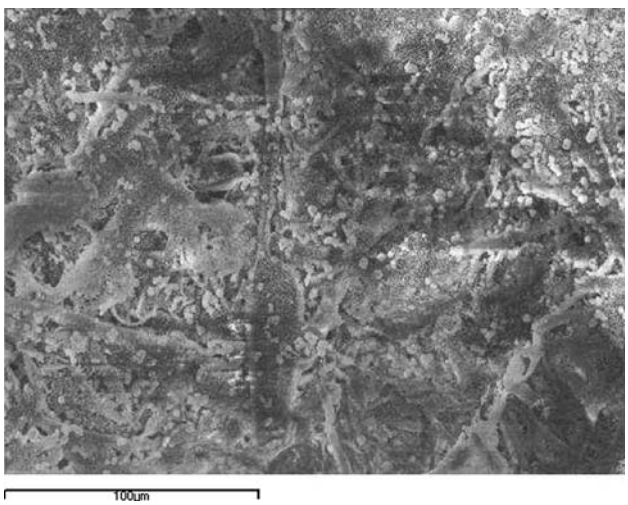

Fig. 4 SEM image of the CAC: It was composed of collagen lattice structure on which thin film of apatite crystal was coated (original magnification: ×500)

3.2.1 4-Week specimen

3.2.1.1 Control group Newly-formed bone in close contact with the titanium surface was seen in some area, but there were ingrowths of fibroblast-like cells between titanium and newly-formed bone in almost all area (Fig. 5). The newly-formed bone generally showed immature features, i.e., parallel-fibered bone and lamellar bone dominated with remnants of woven bone only. Old bone was discriminated from the newly-formed bone by cement line. Osteoblasts of newly-formed bone trabeculae were rectangular form in some area or flat in another.

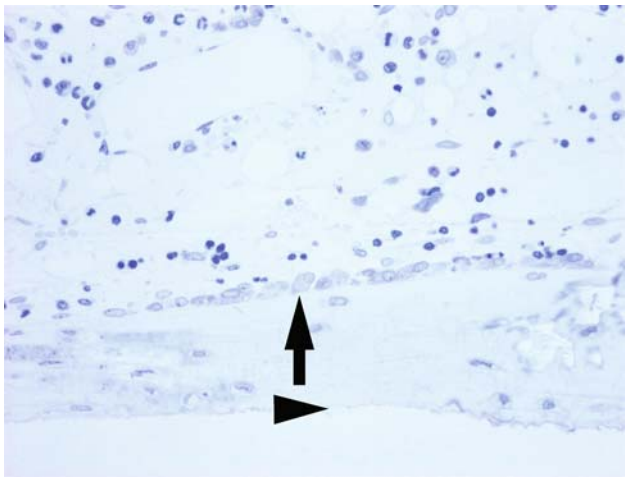

Fig. 5 (LM) Osteoblasts (*arrow*) were arranged with the newly-formed bone. Newly-formed woven bone was changing into lamellar bone. Newly-formed bone was in direct contact with titanium surface (*arrowhead*) in some area (0.5 mm gap, 4 weeks, Control group, original magnification: ×400)

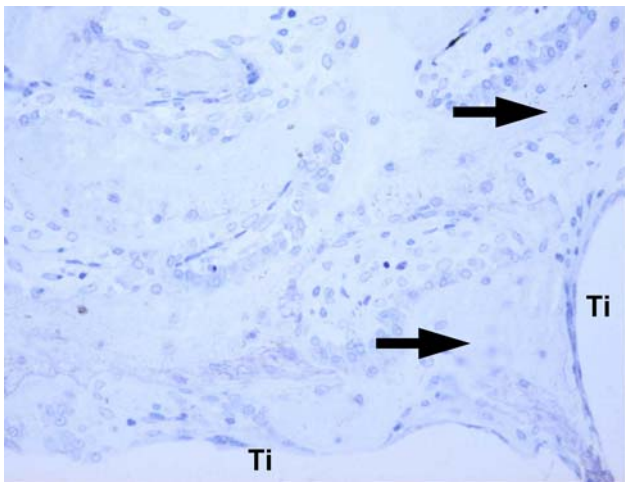

Fig. 6 (LM) Osteoblasts (*arrow*) lining newly-formed bone. The active osteoblasts had large nucleus and plentiful cytoplasm. The gap between titanium implant surface (Ti) and old bone was more crowded with newly-formed bone than DFDB group (0.5 mm gap, 4 weeks, CAC group, original magnification: ×400)

3.2.1.2 CAC group There was more new bone formation in the grafted gap in CAC group than the control or DFDB group (Fig. 6). Newly-formed bone was cross-linked each other to form many trabeculae showing mature form. Each osteoblast had large nucleus and plentiful cytoplasm, which showed highly active form. These osteoblasts were arranged along the newly-formed bone trabeculae. Homogenous matrix-like area showing deficiency of cellular component was regarded as CAC matrix. Fibroblast-like cells or fibrous tissue was interposed between titanium and newly-formed bone.

3.2.1.3 DFDB group The remaining DFDB graft particles were all highly integrated with surrounding newly-formed bone (Fig. 7). Osteoblasts lining newly-formed bone had more flat nucleus and less cytoplasm than CAC group, which was considered as inactive form. Less osteocytes were observed in the newly-formed bone matrix than CAC group. There were also some multinucleated giant cells around DFDB particle.

3.2.2 8-Week specimen

3.2.2.1 Control group The newly-formed bone was more matured than 4-week group and completely integrated with old bone. Precise distinction between old bone and newly-formed bone was difficult due to the loss of cement line. In 0.5 mm gap, some titanium surface was in direct contact with newly-formed bone. But almost all area had soft tissue contact in 1.0 mm gap. Some osteoblasts were still generating new bone (Fig. 8).

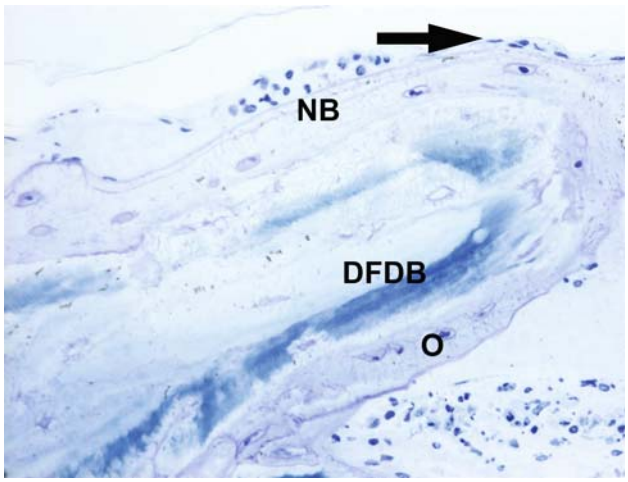

Fig. 7 (LM) DFDB particle remained without being absorbed. Osteoblasts (*arrow*) had more flat nucleus and less cytoplasm than CAC group, which indicated inactive state. DFDB: demineralized freeze-dried bone, NB: Newly-formed bone, O: Osteocyte (0.5 mm gap, 4 weeks, DFDB group, original magnification: ×400)

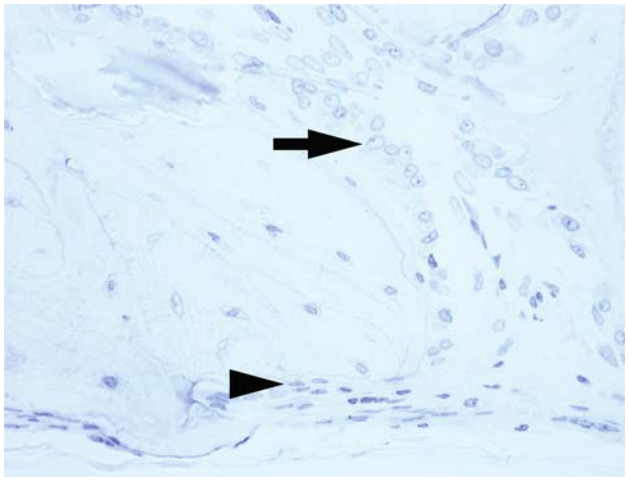

Fig. 8 (LM) New trabecular bone showing lamellar pattern. Some fibroblast-like cells (*arrowhead*) were located between new bone and titanium implant. Some osteoblasts (*arrow*) still made new bone (1.0 mm gap, 8 weeks, Control group, original magnification: ×400)

3.2.2.2 CAC group Newly-formed bone filled almost all the gap space. Plentiful newly-formed bone trabeculae showed matured lamellar pattern. Some newly-formed bone area still showed active bone formation by osteoblasts and immature form was observed in the deep portion of 1.0 mm gap (Fig. 9). In some area, multinucleated giant cells were detected.

3.2.2.3 DFDB group More newly-formed bone was found than 4-week specimen (Fig. 10). The amount of newly-formed bone was somewhat less than CAC group in 8 weeks. Around the DFDB particle, many multinucleated giant cells were observed. There were active and inactive osteoblasts on the newly-formed bone trabecula.

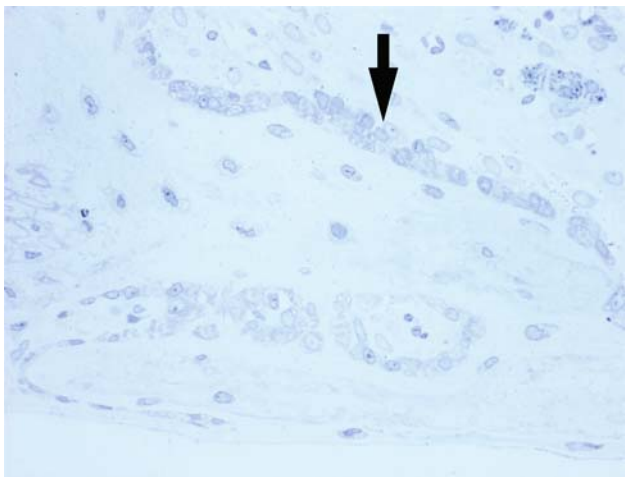

Fig. 9 (LM) Newly-formed bone was matured more than at 4-week specimen. But, in the deep portion of the gap, there was still active bone formation by osteoblasts (*arrow*) (1.0 mm gap, 8 weeks, CAC group, original magnification: ×400)

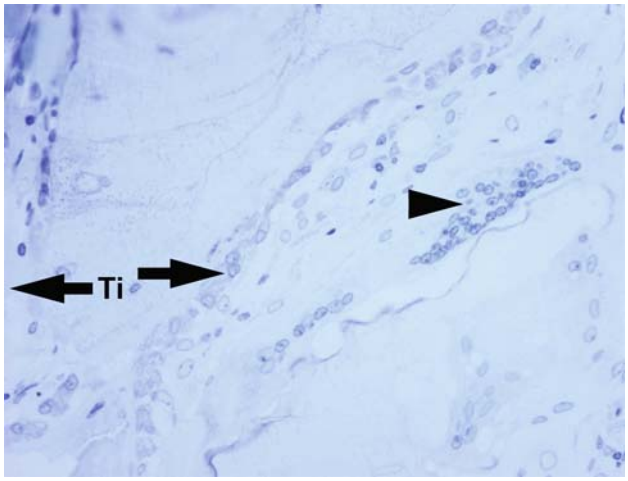

Fig. 10 (LM) New bone was formed still less than CAC group at 8-week specimen. Around the DFDB particle, many multinucleated giant cells (*arrowheads*) were observed. There were active and inactive osteoblasts (*arrow*) on the newly-formed bone (Ti: Titanium surface. 1.0 mm gap, 8 weeks, DFDB group, original magnification: ×400)

3.3 Histomorphometric analysis

CAC group showed higher fraction of newly-formed bone than DFDB group in both of 0.5 ($P = 0.030$) and 1.0 mm gap ($P = 0.035$) subgroups at 4-week specimen. But the difference was not significant at 8-week specimen (Table 1).

3.4 Transmission electron microscopy (TEM)

3.4.1 CAC group

A layer of osteoblasts were secreting newly-formed bone matrix. These osteoblasts were secreting bone matrix and growing in the direction of collagen matrix of CAC. These osteoblasts had large nucleus which had plentiful euchromatin and also had much rER and Golgi complexes (Fig. 11). In some area around CAC, there were multinucleated giant cells which had many nuclei scattered throughout the cytoplasm and also has numerous vacuoles and vesicles. Collagen mesh of CAC was found around multinucleated giant cells. Therein, denatured protein-like round materials were observed. Under the high magnification, the lattice frameworks of collagen fibers were detected. Some collagen mesh was degraded by the multinucleated giant cells. Multinucleated giant cells showed lots of mitochondria and free ribosomes.

3.4.2 DFDB group

Osteoblasts were arranged in a row on the newly-formed bone matrix. The osteoblasts were secreting bone matrix. However, the osteoblasts of newly-formed bone were caught between DFDB particle and newly-formed bone

Table 1 The fraction of newly-formed bone in 0.5 and 1.0 mm gap between titanium-coated epoxy-resin block implant and host bone after CAC and DFDB application

	Control		CAC		DFDB	
Gap size	0.5 mm	1.0 mm	0.5 mm	1.0 mm	0.5 mm	1.0 mm
4 weeks	33.9 ± 6.2	27.8 ± 3.8	49.3 ± 7.4	58.0 ± 13.1	30.6 ± 6.3	35.6 ± 9.0
8 weeks	35.3 ± 25.3	27.2 ± 18.0	63.1 ± 4.7	41.9 ± 15.5	44.7 ± 8.6	30.1 ± 15.3

Values are percentage of newly-formed bone detected in the gap (means ± SD). CAC group showed higher bone regeneration than DFDB group in both 0.5 and 1.0 mm gap at 4-week specimen ($P < 0.05$). However, the difference of means was not significant at 8-week specimen

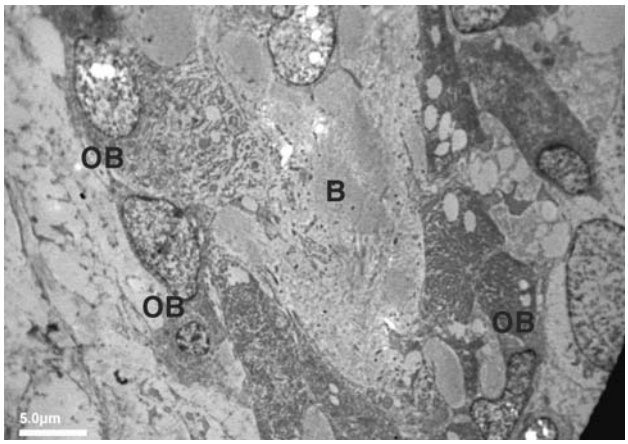

Fig. 11 (TEM) Osteoblasts (OB) were lined in a row. These osteoblasts were secreting bone matrix (B). They had large nucleus which had plentiful euchromatin and also had much rER and Golgi complexes (TEM, 0.5 mm gap, 4 weeks, CAC group, original magnification: ×3000)

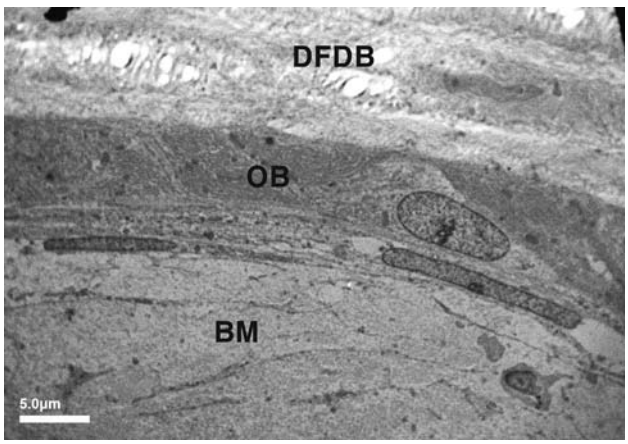

Fig. 12 (TEM) The osteoblasts (OB) of newly-formed bone were caught between DFDB particle and newly-formed bone matrix (BM). They had large nucleus which was full of heterochromatin and also had much rER and Golgi complexes. But, the osteoblasts in DFDB group had more flat nucleus and less cytoplasm than CAC group (TEM, 1.0 mm gap, 8 weeks, DFDB group, original magnification: ×3000)

matrix in some area (Fig. 12). The osteoblasts had large nucleus which was full of heterochromatin and also had much rER and Golgi complexes. But, the osteoblasts in DFDB group had more flat nucleus and less cytoplasm than CAC group. Collagen frameworks of the newly-formed bone matrix were apparent. There was osteocyte which had small amount of cytoplasm and apparent canaliculi. Unabsorbed DFDB particle showed orderly aligned collagen framework and still remained around newly-formed bone at 8-week specimen. Along the unabsorbed DFDB particle, osteoclast-like multinucleated giant cells were found. In the cytoplasm of the multinucleated giant cell, Golgi complexes, vacuoles and vesicles were found, which were considered as the specific features of foreign body giant cell.

4 Discussion

The CAC used in this study was made by soaking collagen membrane into the Ca–P ion solution to mimic the calcification of Calcium and Phosphate in normal bone tissue [11]. This structural characteristic is thought to enhance the easy vascularization and bone induction in the gap between titanium implant and bone.

At 4-week specimen, CAC group presented higher fraction of newly-formed bone than DFDB group in both 0.5 and 1.0 mm gap significantly. Also, at 8-week specimen, CAC group showed higher fraction of newly-formed bone than DFDB group, but no significant differences were found between two groups. It could be speculated that CAC had the advantage of initial bony healing process than xenograft material. However, there was no significant difference of the fraction of newly-formed bone in the histomorphometric study between 0.5 and 1.0 mm gap subgroups, which would indicate that the gap size difference under the 1.0 mm was not significant in bony healing process on the grafted gap.

Although HA is osteoconductive and acts as a scaffold for bone formation, CAC showed osteoinductive properties in this study [12–14]. It induced active bone formation by osteoblasts and showed slow phagocytosis of collagen matrix by multinucleated giant cells. DFDB group demonstrated inferior properties to CAC group in the point of the fraction of newly-formed bone and the activity of osteoblasts. The histomorphometric findings of this study

were not corroborated with the results of other study [15]. It was partly due the fact that the DFDB of human bone acted as a xenograft material in this study. At 8-week specimen, a considerable number of DFDB particles near the titanium surface of the gap still remained unabsorbed form.

Usually there may be gap between implant and surrounding bones. In most of cases, if the gaps are small, the influence of gaps would be negligible. The effect of the gap size between titanium implant and bone on bony healing process was also studied by many researchers. Carlsson et al. studied on the critical gap for direct bone apposition on the implant [16]. Cameron et al. investigated the rate of bone deposition around a porous metal implant with a pore size of 100 μm [17]. Sandborn et al. used implants with a uniform spacing of 0.0–2.0 mm in the intramedullary canal for histologic and microradiographic evaluations [18]. However, it is still unclear whether different gap size at the titanium–bone interface play an important role in the bone healing process of graft materials.

The bony healing process detected in the grafted gap was limited in the cortical bone level in this study. So, it was suspected that the remodeling rate of newly-formed bone was slow in all groups. If the bone substitute materials were grafted in the bone marrow or cancellous bone, bone formation and bone remodeling cycle would be improved. Du et al. implanted nano-HA/collagen composite in a marrow cavity of rabbit femur and found composite resorption and new bone formation in the early period [19]. In this study, Haversian systems are involved and the remodeling process leads to the formation of a 'cutting and filling cone', so the bone formation and remodeling was thought to be relatively slow [20].

For transmission electron microscopic study of titanium implant, Johansson et al. used titanium-coated plastic plug implant and Linder studied bone-titanium interface with cylindrical polycarbonate implants covered with 120–250 nm thick layer of pure titanium [21, 22]. The titanium-coated epoxy-resin block implant used in this study was also useful model for the transmission electron microscopic study.

5 Conclusions

From the results, it was expected that CAC had more beneficial property on early bony healing process than DFDB at the titanium–bone interface. CAC would be considered as an option of bone substitute material in the implant surgery. For the clinical application of CAC, further studies should be followed on the physical and biological properties of CAC.

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