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Original Article

Effect of New Bone Substitute Materials Consisting of Collagen and Tricalcium Phosphate

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Abstract

The purpose of this study was to investigate the effect of new bone substitute materials consisting of collagen and tricalcium phosphate (TCP). Prior to the experiment, mandibular dog teeth were extracted. After 3 months, specific cavities were prepared on the alveolar ridge. In one group, cavities were filled with collagen sponge (CS group), in the other, cavities were filled with TCP sponge (TCP group). Cavities with no fillings (Cont group) were created as controls. Mandibular bone was evaluated histopathologically at experimental time periods of 1, 2, 4, and 8 weeks. Due to the non critical inflammatory symptoms that each group showed throughout all the time periods investigated, a low irritation level was observed. Absorption of material was almost complete at after 4 weeks in the CS group, and at after 8 weeks in the TCP group. At the top of the cavity, the TCP group exceeded the Cont group in amount of neogenesis at after 8 weeks. The materials examined in this study showed good osteoconduction and biodegradable character. The TCP Group, in particular, showed highly acceptable results, demonstrating that the materials used were excellent candidates as bone substitute materials.

Key words: Bone substitute—Collagen—α-TCP

Introduction

The regeneration or augmentation of alveolar bone defects and gingiva are routine approaches in the treatment of exposed roots, and are often followed by esthetic regeneration of the tissue around the cervix of the tooth, if necessary.

Recently, bone substitutes have been used to regenerate lost bone tissue in clinical practice. These include bioactive ceramic materials, represented by hydroxyapatite (HAp), and biodegradable ceramic materials, represented by tricalcium phosphate (TCP). Klinge *et al.*[12] assessed the effects of ceramic materials and concluded that an ideal bone substitute should be completely absorbed and replaced by the newly formed bone. Many reports have also suggested that absorbent materials are biologically appropriate as bone substitutes.

This study is part of the thesis submitted by Ken Takahashi for a doctoral degree originally published in the Shikwa Gakuho (97:509–536, 1997) in Japanese.

Table 1 Preparation of filling materials

Collagen sponge	TCP sponge
[A] 0.3% (w/v) pepsine-treated Type I collagen solution	[A] 0.3% (w/v) pepsine-treated Type I collagen solution
[B] 2×McIlvaine's buffer solution	[B] 2×McIlvaine's buffer solution containing 10% (w/v) α-TCP particles
• Mixed ice cold [A] and [B] at ratio of 1:1	• Mixed ice cold [A] and [B] at ratio of 8:2
↓	↓
• Incubated at 37°C incubator for 3 hr	• Incubated at 37°C incubator for 3 days
↓	↓
• Lyophilized	• Lyophilized
↓	↓
• UV-irradiated for 2 hr	• UV-irradiated for 2 hr

Conventional granular materials tend to leak from pores and are difficult to apply to bone defects with complicated surfaces. However, such shortcomings are now being resolved by the development of materials such as a collagen coating[3] and collagen sponge[9,22].

This study assessed the effects of bone substitute consisting of collagen and TCP on the regeneration or augmentation of bone defects. Collagen has both biocompatible and bio-absorbent properties and is a highly effective biomaterial.

Materials and Methods

1. Filling materials

1) Components of sponge

The following two types of material were used in the experiment:

(1) Collagen sponge: freeze-dried material created from pepsin-treated type I collagen gelated at 37°C (Experimental production, Nitta Gelatin Inc., Osaka, Japan).

(2) TCP sponge: a composite material consisting of α-TCP particles incorporated with the collagen sponge mentioned above in 1) (Nitta Gelatin Inc.).

Preparation methods are shown in Table 1.

2) Scanning electron-microscopical analysis

Surface microstructure observed with a scanning electron microscope (SEM S-800, Hitachi High-Technologies Corporation, Tokyo, Japan).

2. Experimental design

1) Animals

This study used 10 healthy male or female dogs aged one year or older and weighing approximately 20 kg each. It was performed according to the guidelines for the use of experimental animals stipulated by Tokyo Dental College.

2) Preparation of bone cavity

Prior to the study, the dogs had their mandibular teeth, including the second, third and fourth premolars and first postmolar, extracted on both sides under general anesthesia (pentobarbital sodium 0.5 mg/kg) and infiltration anesthesia (1.8 ml 2% lidocaine hydrochloride and 1:80,000 epinephrine).

Three months after extraction, X-ray was performed to make sure that the resulting cavities on were completely healed. A horizontal incision was made at the mucogingival junction, extending from the distal of the mandibular first premolar to the mesial of the second postmolar, on both sides. A full thickness flap was then elevated to expose bone surface. Five specific bone defects (referred to hereafter as cavities) approximately 3.7 mm in diameter and 5.0 mm in depth were created on the alveolar crest on both sides with twist and tap drills used for implantation under

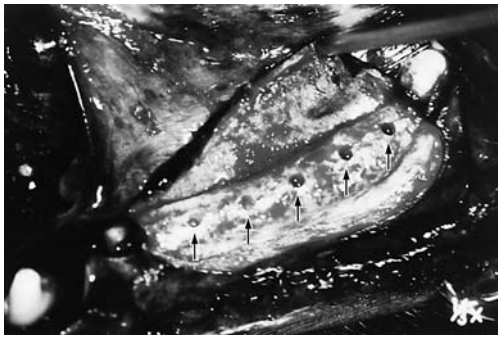

Fig. 1 Intraoral picture after preparation of specific bone defects (arrows)

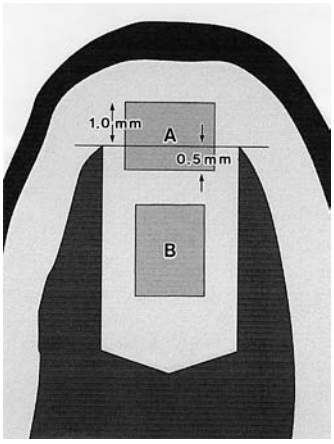

Fig. 2 Schema showing areas measured the bone mass
A indicates the measured portion, 1.5×2.0 mm, at the entrance of the bone defect. B shows the measured portion, 2.0×1.5 mm, at the center of the bone defect.

injection of isotonic sodium chloride solution (Fig. 1). Of the five cavities, some were used for filling with one of the two types of material, while others were given no fillings as controls.

After the sponge was cut into pieces using scissors, the cavity was filled with the pieces of sponge up to the level where the bone wall remained under low pressure. After filling, a full thickness flap was repositioned and sutured to complete the procedure.

Cavities were divided into the following three groups: CS group, with collagen sponge fillings; TCP group, with TCP sponge fillings; and Cont group, with no fillings. Cont group served as the controls.

In order to minimize the effect of differences in wound-healing arising from area of filling, the two types of filling were assigned to different locations among the dogs.

3) Tissue preparation

Six cavities were studied at each follow-up time in each group. Tissues were histopathologically assessed at one, two, four and eight weeks after surgery. At each time period, the dogs were submitted to perfusion fixation with 10% neutral buffered formalin (Lillie) under general anesthesia. Mandibular bone was extracted, and exposed to decalcification solution (nitrate dissolved in solutions of ethanol) and embedded in celloidin according to the conventional method. Consecutive slices were cut in the buccolingual direction at approximately 7 μm intervals and processed for histopathological examination (microscopic evaluation) with hematoxylin-eosin stain.

4) Histopathological analysis

Bone mass per unit area was measured in order to assess bone neogenesis at the center and top of the cavities. Prior to measurement, the outlines of newly formed bone in each sample were traced and subsequently analyzed with a multi-purpose image processor (SPICCA-II, Nippon Avionics Co., Ltd., Tokyo, Japan). Newly formed bone was measured at two weeks after surgery in the center of the cavity, in the created 2.0×1.5 mm space, as shown in Fig. 2. Newly formed bone per 1 mm^2 was calculated based on this volume of newly formed bone in each cavity. The calculated area was defined as bone mass per unit area. In the top of the cavity, bone mass per unit area was measured by the same method at four and eight weeks after surgery.

5) Statistical analysis

Statistical analyses were performed with the Mann-Whitney test, and p values of less than 0.05 were regarded as significant: highest and lowest bone mass were excluded from this study.

Fig. 3 Surface microstructure by SEM observation of collagen sponge

SEM image reveals irregular arranged collagen fibers with pores, 100 to 200 μm in diameter. Bar indicates 100 μm.

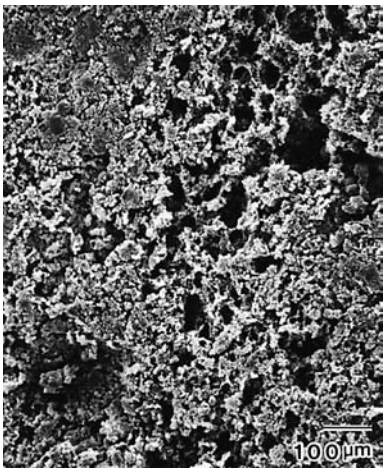

Fig. 4 Surface microstructures of TCP sponge

SEM image shows aggregations of fine collagen fibers with pores, approximately 100 μm in diameter, and TCP micro particles in these pores. Bar indicates 100 μm.

Results

1. Microstructure of filling materials

Surface microstructure observed with a scanning electron microscope were shown in Figs. 3 and 4. SEM images revealed that collagen fiber looked like a sponge with pores (100 to 200 μm in diameter) in the case of the collagen sponge. Similarly, SEM images revealed that collagen fiber looked like a sponge with pores, many of which were approximately 100 μm in diameter, in case of TCP sponge. Tricalcium phosphate micro particles (5 to 10 μm in diameter) were incorporated into the collagen fibers.

2. Experimental and histopathological results

1) Cont group

Blood clot was extensively noted in the cavities at one week after surgery (Fig. 5), but was almost absorbed after two weeks.

A mild infiltration of neutrophils into the oral mucosa was detected in most cavities at one post-surgery week, but was observed in 33% of cavities after two weeks, finally disappeared in virtually all cavities at four post-operative weeks. Similarly, infiltration of lymphocytes and plasma cells into the cavity gradually reduced and completely disappeared in all cavities after eight weeks.

New small blood vessels grew in all cavities at

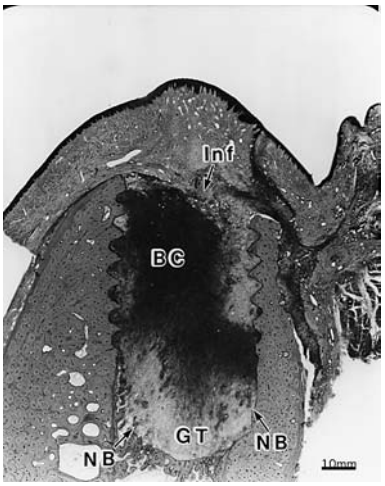

Fig. 5 Histopathological picture of Cont group 1 week after surgery

Blood clot (BC) and granulation tissue (GT) are detected in the bone defect. Inflammatory cells infiltration (Inf) can be observed between the bone defect and oral mucosa. NB: New bone. Bar indicated 10 mm.

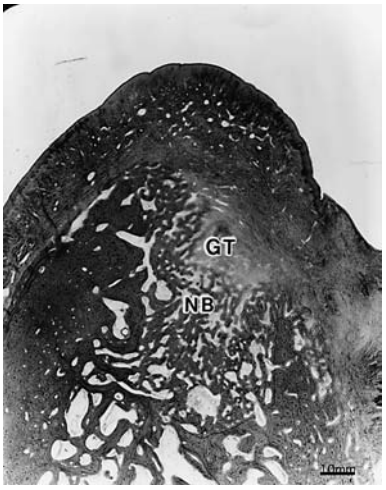

Fig. 6 Histopathological picture of Cont group at 4 post-surgery week

The bone defect is filled with new bone (NB). Granulation tissue (GT) can be observed at the entrance portion of the defect. Bar indicated 10 mm.

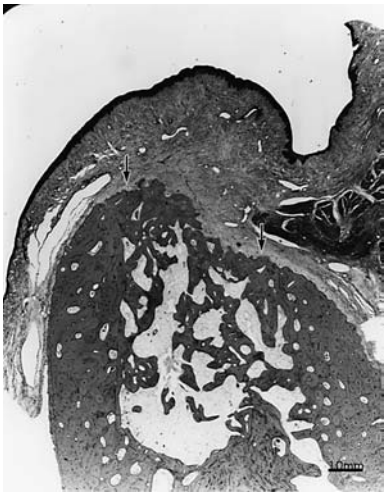

Fig. 7 Histopathological picture of Cont group at 8 post-surgery week

The bone defect is completely filled with new bone. Arrows indicate contours of pre-existing bone defect. Bar indicated 10 mm.

post-operative one week. However, they were extensively discernible in 50% of cavities at one post-operative week, and in most cavities at two weeks after surgery. An abundant supply of blood vessels was found in the cavities after four and eight weeks.

A small amount of newly formed bone was distinct at the base of most cavities at one week after surgery. Virtually all cavities were half-filled or more with newly produced bone after two weeks. 33% of cavities were completely supplied with new bone, while four remaining cavities were covered with granulation tissue at four weeks after surgery (Fig. 6). All cavities were completely filled with new bone after eight weeks with half of cavities showing a concave top and 17% of cavities showed a convex top (Fig. 7).

2) CS group

Fewer blood clots were observed in the cavities at one week after surgery than in the Cont group at the same time period. However, these were extensive in most the cavities at one post-operative week, still remaining slightly in most cavities, even after two weeks.

Lymphocytes and neutrophils infiltrated into the oral mucosa of all the cavities and into all the cavities at one and two weeks after surgery. This was evident in approximately half of all the cavities at four weeks after surgery, and disappeared in all cavities after eight weeks.

Pattern of blood vessel growth was nearly analogous to that seen in the Cont group.

Extensive and abundant collagen sponges were obvious in most cavities at one week after surgery, but these were gradually absorbed at two post-operative weeks, becoming unidentifiable at four weeks after surgery. As well as neutrophils and lymphocytes with regressive change, macrophages infiltration into the collagen sponge could be found at one week after surgery (Fig. 8). Macrophages infiltration was still recognizable in those areas where collagen sponge slightly remained after two and four weeks.

A small amount of bone was newly produced on the base of most cavities at one week after surgery. Most cavities were half filled with newly formed bone after two weeks. Two cavities were completely filled with newly formed bone after four weeks. All cavities were

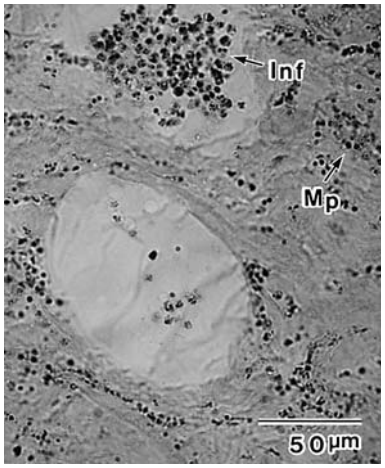

Fig. 8 Histopathological picture of CS group 1 week after surgery

Inflammatory cells infiltration (Inf) is discernible into collagen sponge as well as macrophages (Mp). Bar indicated 50μm.

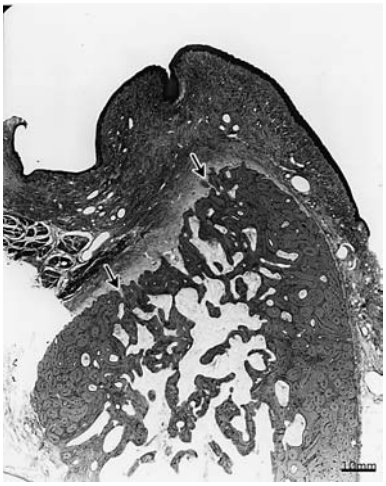

Fig. 9 Histopathological picture of CS group 8 weeks after surgery

The bone defect is completely filled with new bone. Arrows indicate contours of pre-existing bone defect. Bar indicated 10 mm.

completely filled with newly formed bone after eight weeks, with two cavities showing a concave top and three cavities a convex top (Fig. 9).

3) TCP group

Blood clot was detected in most cavities at one week after operation, but was mostly less than that in the Cont group. It could not be found in all cavities at two weeks after treatment.

Lymphocytes and neutrophils into the oral mucosa were discerned in all the cavities after one and two weeks, completely disappeared in all cavities after four weeks. Infiltration of lymphocytes into the cavity also stopped to observe in all cavities after four weeks.

Pattern of blood vessel growth was almost similar to that in the Cont group.

Fewer sponges remained at one post-operative week, becoming unidentifiable at two weeks after treatment. Particles tended to clump together at one week post-surgery. A small amount of particle agglutination surrounded by spindle-shaped cells or multi-nucleated giant cells was observed after two weeks. Concurrently, part of this particle agglutination was absorbed by newly formed bone (Fig. 10), with complete taking place at four weeks post-surgery. Only a small amount of agglutination was identified in the newly formed bone after eight weeks (Fig. 11).

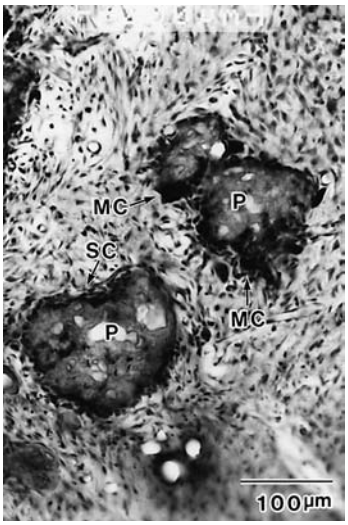

Fig. 10 Histopathological picture of TCP group at 2 post-surgery week

A part of TCP particles (P) is absorbed by multinuclear giant cells (MC) and replaced by newly formed bone. SC: spindle cells. Bar indicates 100μm.

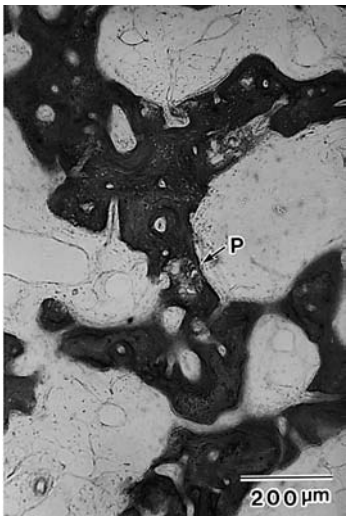

Fig. 11 Histopathological picture of TCP group at 8 post-surgery week

A small amount of the particles (P) can be identified in the new bone. Bar indicates 200 μm.

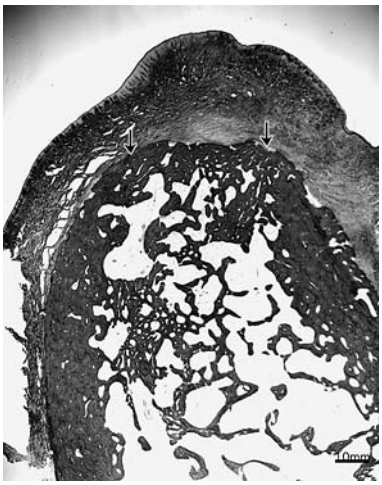

Fig. 12 Histopathological picture of TCP group at 8 post-surgery week

The bone defect is completely filled with new bone. Arrows indicate contours of pre-existed bone defect. Bar indicated 10 mm.

A small amount of bone was newly produced on the base of most cavities at one week after surgery. Most cavities were half or more filled with newly formed bone after two weeks. All cavities were completely filled with newly formed bone after four weeks. All cavities showed a convex top after eight weeks (Fig. 12).

3. Statistical analysis

1) Bone mass per unit area in center of cavity

Table 2 and Fig. 13 showed average bone mass per unit area in each group.

The cavity was less than half filled with newly formed bone in all three groups at one week after surgery, and so bone mass was zero.

Bone mass per unit area in the Cont and TCP groups was greater than that in the CS group at two post-operative weeks.

No significant differences in bone mass per unit area were discernible between the Cont and CS groups or the Cont and TCP groups at four post-surgery weeks.

No significant differences in bone mass per unit area were observed between the Cont and CS groups or the Cont and TCP groups after eight weeks.

2) Bone mass per unit area in top of cavity

Bone mass per unit area in the TCP group was greater than that in the CS group at four weeks after surgery. Bone mass per unit area in the TCP group was greater than that in the Cont group at eight weeks after surgery.

Discussion

1. Properties and structures of collagen sponge and collagen plus TCP sponge

Since telopeptide, which is the end of the collagen molecule, was removed with pepsin treatment prior to the study, the antigenicity of the collagen was not a matter of concern. Different properties of collagen that may affect bone formation have been reported. The structure of collagen fibers serves as the basis for the development of osteocytes[2] or provides a microenvironment that supports osteocyte growth, transfer, differentiation and calcification[17]. When implantation materials are coated with collagen fiber, they have a good affinity to osteoblast-like cells, and

Table 2 Average bone mass per unit area in each group and statistical analyses with Mann-Whitney test (n=4)

	Center of cavity									Top of cavity					
	2W			4W			8W			4W			8W		
	Cont	CS	TCP	Cont	CS	TCP	Cont	CS	TCP	Cont	CS	TCP	Cont	CS	TCP
Average	0.26	0.03	0.41	0.61	0.48	0.61	0.57	0.51	0.55	0.21	0.17	0.37	0.39	0.54	0.68
STD	±0.05	±0.04	±0.16	±0.10	±0.12	±0.06	±0.09	±0.07	±0.07	±0.08	±0.08	±0.13	±0.11	±0.17	±0.15
Cont		*	—		—	—		—	—		—	—		—	*
CS			*			—			—			*			—

*: $p<0.05$, —: not significant

Cont: control group, CS: collagen sponge group, TCP: collagen with TCP group.

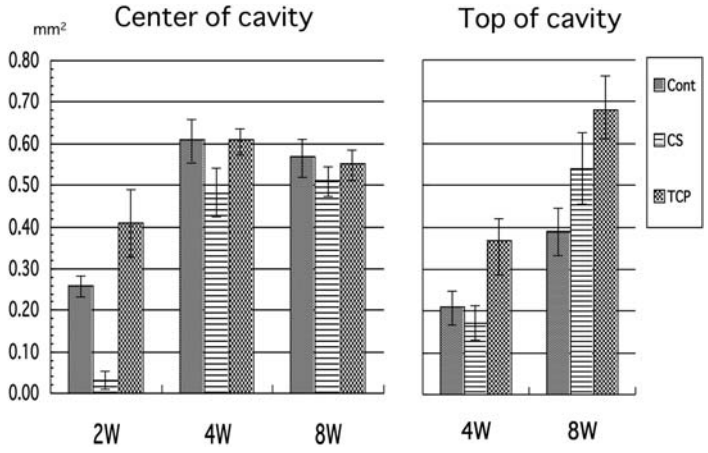

Fig. 13 Average bone mass per unit area in each group

this promotes early calcification in osteoblast-like cells[3].

Joos *et al.*[10] reported that trabecular bone was formed at the early stage in a group with collagen sponge filled bone defects compared to that in a group with no filling materials. Sugaya *et al.*[18,19] reported increased amounts of newly formed bone in a group with collagen-coated HAp and β-TCP particles were greater than that in a group without collagen-coated HAp and β-TCP particles. The effect of collagen-based bone substitutes has been demonstrated by many studies.

This study aimed to assess the effect of a bone substitute consisting of collagen and TCP on regeneration or augmentation of bone defects. Among calcium phosphates with biodegradable properties[7,12], we chose to use α-TCP, which is converted to octacalcium phosphate (OCP) after hydration[1], in this study: OCP is a precursor to HAp during the calcification process *in vivo*[1,4,5].

Conventional non-absorbent HAp particles should be approximately 500 μm in diameter to gain pores big enough to penetrate newly formed bone or blood vessels[9]. When a bone defect was densely filled with non-absorbent HAp particles (10 μm in diameter), some problems occurred[16]. It is difficult for newly formed bone to penetrate pores, as each pore between particles is too small. Furthermore, conventional non-absorbent HAp particles require a high filling technique. After filling is completed, non-absorbent HAp particles tend to leak from the pores between a gingival flap and the surface of the root.

The sponges in this study showed scattered pores (approximately 100 to 200 μm in diameter). Particles (5 to 10 μm in diameter) were incorporated into the spongy structure of the TCP sponge. Therefore, even when a bone defect was densely filled with particles, the pores between those particles still maintained. Klawitter *et al.*[11] studied the effect of different sizes of porous calcium aluminate specimens implanted in the thigh bone of dogs, and found that pores should be 100 μm and 50 μm in diameter to allow penetration of calcified bone and capillary blood vessels, respectively. In this study, there were no marked differences regarding the area where blood vessels were newly formed and the volume of blood flow in the newly formed blood vessels between the CS and Cont groups or the TCP and Cont groups at all follow-up times. This suggests that the three groups had similar characteristics in terms of new blood vessels. These results agree with those found by Toda *et al.*[22] in a study in which α-TCP was applied to a monkey's jaw bone. Materials with spongy structures were, therefore, appropriate as bone substitutes due to their effect on blood vessel formation. One factor in the absorption of TCP particles by newly formed bone was the early incorporation of particles into the newly formed bone developed in the pores of the sponge.

2. Treatments for the filling materials, tissue response, and bone formation

In this study, we assessed the process of collagen absorption. Infiltration of neutrophils and macrophages into the collagen sponge was seen at one week after surgery. Absorption of collagen was therefore attributed to enzyme dissolution by neutrophil and macrophage phagocytosis.

Compared to the TCP group, the CS group presented a delayed absorption of collagen sponge, the cause of which remains to be clarified.

Levin *et al.*[15] filled dog alveolar bone with TCP particles (100 to 300 μm in diameter) and found that they were absorbed at 22 weeks after surgery. Kurihara[13] filled rabbit mandibular bone with α-TCP particles (500 μm in diameter) and found that a small number of particles were still present at 48 weeks after surgery. The particles used in this study were absorbed earlier than those in past studies.

The mechanism underlying the absorption of TCP particles probably involved phagocytosis of multinucleated giant cells, as suggested by many studies which found such particles were surrounded by multinucleated giant cells[13,15,20]. It is also possible that some particles were not phagocytized, but embedded in newly formed bone and consequently replaced with bone tissue.

Differences in amount of newly formed bone between the center and top of the cavities were assessed based on measurement of bone mass per unit area. We focused on the center of each cavity, as the amount of newly formed bone in these areas was an index of healing and was likely to differ under varying conditions. The amount of newly formed bone in the top of cavity was compared between at four and at eight weeks after surgery, at which time the cavity was filled with newly formed bone.

Bone mass per unit area in the center of the cavity in the CS group was less than that in the Cont and TCP groups at all follow-up times. This finding was not consistent with the findings of Joos *et al.*[10] suggesting that the collagen sponge was effective in bone formation at the early stage. The TCP group showed an increased bone mass per unit area at the early stage. Differences in bone mass per unit area between the two types of sponge may be a result of the presence or absence of TCP. Tatsumi *et al.*[21] reported that calcification occurred in an area where decayed β-TCP particles were identified and its surroundings *in vitro*. In this study, bone conduction may have been better due to increased Ca and P ion concentrations inside the cavity following dissolution of particles.

Bone mass per unit area in the top of cavity in the TCP group was greater than that in the Cont group at eight weeks after surgery. This may be attributed to improvement in bone conduction due to a combination of three

factors: 1) prevention of early infiltration of lamina propria mucosa into the cavity due to the presence of bone substitutes or their effect, 2) increased Ca and P ion concentrations inside the cavity following dissolution of particles, and 3) a collagen-based microenvironment. This suggests that collagen coupled with TCP is more effective than collagen alone.

Some problems have been reported with the use of non-absorbent HAp, which is a commonly-used bone substitute. In addition to leaked particles, 1) bone or cement is absorbed when root movement occurs due to differences between HAp properties and bone tissue[8], 2) the establishment of a blood vessel network is prevented when the cavity is filled with excessively dense particles[7], and 3) normal bone marrow tissue is not formed during bone remodeling due to the presence of particles[7].

This study assessed the possibility that new composite materials consisting of absorbent collagen and TCP might be clinically applied. They have already been reported to provide a scaffold for development of osteocytes[2].

The sponge materials used in this study achieved high operability and were easily applied to bone defects with complicated surfaces. Use of such materials may prevent particles from leaking, which occurs when particles are independently used.

In particular, α-TCP sponges showed soft tissue irritation, and presented a similar tendency as that seen in the Cont group in the area where blood vessels were newly formed and in terms of the volume of blood flow in newly formed blood vessels. Blood vessels were newly formed at the early stage. Moreover, α-TCP particles were gradually absorbed or replaced by bone, which resulted in morphological regeneration or augmentation of bone defects. These findings suggest that α-TCP sponges are effective bone substitutes.

The results suggest that collagen contributed to the creation of a scaffold and that TCP plays a role in osteoconduction in bone defects. Recently, several studies have demonstrated that TCP with platelet-derived growth factor is effective for high regeneration of bone. These results imply the significance of cells, signals and scaffolds in bone regeneration[6,14].

Acknowledgements

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