pISSN 1598-298X / eISSN 2384-0749
J Vet Clin 34(6) : 410-413 (2017)
http://dx.doi.org/10.17555/jvc.2017.12.34.6.410

Journal of Veterinary Clinics
www.ksvc.or.kr

Biodegradable Hydroxyapatite/Chitosan Composites on the Bone Defect of Canine Model

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(Received: November 24, 2017 / Accepted: December 13, 2017)

Abstract : Composites of hydroxyapatite (HAp) and chitosan (CS) have been successfully used in bone healing in humans and animals. However, the characteristics of HAp and CS are different. Therefore, the effects of HAp/CS composites on canine bone formation could differ according to their ratio. This study investigated the therapeutic effects of different contents ratios (100, 80:20, 60:40 wt%) on bone defects in a canine model. Thirty intrabony cylindrical defects were created in the humeruses and femurs of 5 beagle dogs, and then the defects were implanted with different composites. The evaluations were performed using radiographs obtained at 10 weeks post-surgery and by histological findings. In radiographic evaluation including the grades of bone filling, periosteal and endosteal reactions, pure hydroxyapatite composite had a significant effect on bone filling, and chitosan containing the composites showed vigorous responses at the periosteum and endosteum. In histological findings, the defect implanted with pure hydroxyapatite had healed completely into mature bony tissue with an obvious osteon structure, and the defect implanted with chitosan containing the composites had the amount of fibrous connective tissue increased significantly within the cortical bone tissue. The results indicate that hydroxyapatite/chitosan composites are therapeutically useful, promoting effective bone healing in defects when the ratio of hydroxyapatite is high and enhanced fibrous connective tissue formation at the periosteum as more chitosan is added.

Key words **:** dog, hydroxyapatite, chitosan, bone defect.

Introduction

Hydroxyapatite (HAp) is an important biomaterial due to its chemical and crystallographic similarity to the apatitic mineral in the inorganic matrix of hard tissues of the body (7,13). Its excellent biocompatibility and overall safety makes it a suitable alternative to bone grafts for repairing hard tissues (5,8). HAp of different sizes and shapes have been used to reconstruct various bone and dental defects, such as cystic defects, surgical bone defects, and periodontal bony defects (3,4,7). However, hardness and porosity of HAp make it brittle and may by susceptible to failure. Moreover, HAp has a tendency to migrate from the implantation site, which can lead to increases in bone loss, trauma to the surrounding tissue, and prolonged surgical time (4,5,13,14).

In order to overcome the disadvantages of HAp, many substances that can play the role of a binder have attracted considerable attention. Recently, chitosan (CS) has attracted considerable attention in space-filling implants and tissue engineering in human medicine. CS can be used in guided tissue regeneration as well as in localizing HAp at the implantation sites. Composites of HAp and CS have been successfully used in bone healing in humans and animals (1,15). However, it has been reported that CS increased the inflammatory reactions in dogs, due to CS-mediated stimulation of the migration of polymorphonuclear neutrophils (PMNs) and macrophages (9-11). In particular, a severe inflammatory reaction can occur in the early stage of implantation, and gradually subside thereafter (6). The characteristics of HAp and CS are different. Therefore, the effects of HAp/CS composites on canine bone formation could differ according to their ratio. This study investigated the therapeutic effects of HAp and CS, and examined the effects of different content-ratios of HAp/CS composites on bone defects in a canine model.

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Material and Methods

Preparation of Gel-formed Composites

The degree of deacetylation was 92% and the viscosity was 600 cps in a 0.5% (w/w) CS solution. Acetic acid, calcium hydroxide ($Ca(OH)_2$), and phosphoric acid (H_3PO_4) were used without further purification. Twenty grams of a H_3PO_4 solution was added drop-wise to 15 g of a $Ca(OH)_2$ suspension with vigorous stirring at 600 rpm and at 25°C. The resulting slurry was aged for 24 hours with constant stirring. The final 100 wt% of HAp was obtained from the slurry by co-precipitation, filtration, washing, and freeze-drying. The 80 wt% of HAp and 20 wt% CS was a suspension containing 9 g H_3PO_4, 4 g CS, and 12 g $Ca(OH)_2$. The 60 wt% of HAp and 40 wt% CS was a suspension containing 7 g H_3PO_4, 8 g CS, and 9 g $Ca(OH)_2$. The three composites were sterilized for 2 hours using ethylene oxide at 55°C. The ethylene oxide

was removed over 12 hours by purified aeration. For implantation, the composites were made as gel-type by mixing with a physiological solution, and were filled into a syringe.

Experimental and control groups

Thirty intrabony cylindrical defects (4 mm) were created in the humeruses and femurs of 5 beagle dogs from an inbred colony. The dogs were 2 years old and weighed 8 ± 1.3 kg. In the experimental groups, 6 defects were implanted with the 100 wt% HAp composite (HAp100 group), 6 defects were implanted with the 80:20 wt% HAp/CS composite (HAp-80CS20 group) and 6 defects were implanted with the 60:40 wt% HAp/CS composite (HAp60CS40 group). In the control group, 12 defects were not implanted but underwent a sham operation (Control group).

Surgical Procedure

Surgery was performed under general anesthesia. The animals were premedicated with 0.04 mg/kg atropine sulfate (Atropine sulfate, Daihan, Korea) and 0.5 mg/kg diazepam (Melode, Donghwa, Korea). In order to induce and maintain anesthesia, 6 mg/kg propofol (Anefol, Hana, Korea) was injected intravenously, and followed by 1~3% enflurane inhalation (Geloran, Choongwae, Korea).

The surgical procedure consisted of the creation of one or two defects, 4 mm in diameter, in the humerus or femur and the implantation the HAp/CS composites. The defects were produced using round stainless steel bur on a low speed drill. During penetrating the bone, sterile saline was used for irrigation and to minimize thermal damage to the tissues. The defects were implanted with different composites for each group.

Ten weeks after implantation, the animals were euthanized. Immediately after sacrifice, humerus and femur were undertaken radiography and harvested for histological examination.

Radiographic Evaluation

Lateral radiographs of humerus and femur were taken immediately after euthanasia under identical exposure setting (42 kVp, 2.5 mAs and 40 inch FFD). The radiographic evaluation was performed by using grading system established in this study. The grading system consisted of changes in bone filling, periosteum, and endosteum (Table 1).

Histological findings

The harvested humerus and femur specimens from each group were fixed with the 10% formaldehyde, and then stained with a Villanueva stain for 4 days. The specimens were dehydrated with a graded series of ethanol (50%, 70%, 80%, and 90%). After dehydration, the specimens were substituted twice with propylene oxide for 2 h. Infiltration was then performed three times as follows: 1:1 of propylene oxide and resin for 3 h, 1:2 of propylene oxide and resin for 16 h, and pure resin for over 3 h. For the next process, polymerization was performed with a pure resin in a 37°C incubator over the next 24 h, then in a 60°C incubator for 3 days. The unnecessary part was removed for cutting and grinding. The specimens were cut to a thickness of 1 mm using a precision cut-off machine, and mounted onto a slide. Undecalcified sections 40 μm in thickness were prepared using a grinding and polishing machine. The microscopic examinations were performed using an optical microscope.

Statistical analyses

The data on the grades of bone filling, periosteal and endosteal reactions were analyzed using a one-way analysis of variance and Scheffe's test to determine the level of significance between the groups.

Table 1. Grading system for radiographic evaluations

Grade	Bone filling	Periosteal Reaction	Endosteal Reaction
1	0-10%	None	None
2	10-30%	Mild	Mild
3	30-60%	< 3 mm	Distinguished with cortical bone
4	60-90%	< 6 mm	Intermediate distinguished with cortical bone
5	90-100%	> 6 mm	Undistinguised with cortical bone

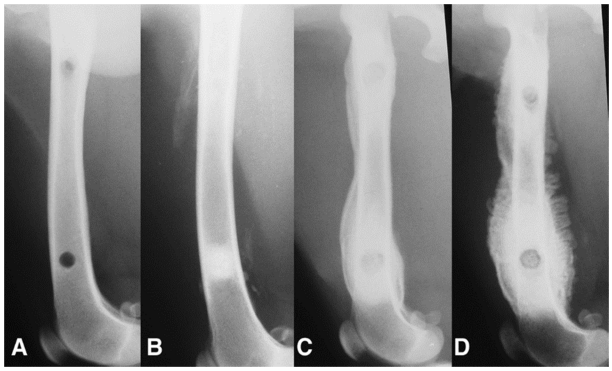

Fig 1. Radiographs of left femurs obtained at 10 weeks postoperatively. A Control group (B) HAp100 group (C) HAp80-CS20 group (D) HAp60CS40 group.

Table 2. Radiographic evaluation of effects of hydroxyapatite/chitosan composites on the bone defect in dogs (mean ± SD)

Groups	Control *(n = 12)*	HAp100 *(n = 6)*	HAp80CS20 *(n = 6)*	HAp60CS40 *(n = 6)*
Bone filling	2.3 ± 0.61^{a}	4.3 ± 0.67^{b}	2.8 ± 0.57^{a}	2.3 ± 0.27^{a}
Periosteal reaction	1.0 ± 0.00^{a}	1.8 ± 0.17^{b}	2.7 ± 1.87^{b}	4.5 ± 0.30^{c}
Endosteal reaction	1.0 ± 0.00^{a}	2.8 ± 0.97^{b}	4.8 ± 0.17^{c}	5.0 ± 0.00^{c}

Between groups different letters denote significant difference ($P < 0.05$).

Results

Radiographic Evaluations

Lateral radiographs of all groups at 10 week postoperatively were obtained (Fig 1). Based on the established grading system, data from the lateral radiographs were used as the outcome measure. The post-hoc tests found a significant effect on the grade of bone filling of HAp100 group ($p < 0.05$). HAp80CS20 and HAp60CS40 groups did not have any further significant effect ($p > 0.05$) (Table 2). On the grade of periosteal reaction, HAp100 and HAp80CS20 groups had a significant effect ($p < 0.05$), and HAp60CS40 group had a further significant effect ($p < 0.05$) (Table 2). On the grade of endosteal reaction, HAP 100 had a significant effect ($p < 0.05$), and HAp80CS20 and HAp60CS40 groups had a further significant effect ($p < 0.05$) (Table 2).

Histological Findings

Control group (Fig 2)

The surgery site of the control group was filled with adipic bone marrow, and consisted of a number of adipocytes, sporadic reactive bone formation in addition to a small amount of trabecular bone formation along the margin of the bone defect. While one end of the cylinder bone defect showed some recovery of cortical bone tissue, of which an osteon structure was obvious, the other end had not recovered and was filled with adipic bone marrow tissue. However, there was no significant inflammation.

HAp100 group (Fig 3)

The implanted coccoid HAp granules were located in the hemopoietic bone marrow without certain inflammation or a foreign body reaction. While new bone had deposited around the granules, the implants appeared fused to each other. The bone defect had healed completely into mature bony tissue with an obvious osteon structure.

HAp80CS20 group (Fig 4)

Most of the implanted HAp80CS60 composite was eliminated by polynucleated giant cells, and infiltrated macrophages and chronic inflammatory cells substituted for the fibrous connective tissue. Mild new bone formation was observed at the fibrous tissue. The bone defect become narrower through new bone but had not healed completely. The region that had not recovered was filled with dense collagenous connective tissue with weak inflammatory responses. New bone formation at the periosteum was vigorous, and the structure was reticular with loose connective tissue including a large number of vessels. The new bone formation caused narrowing of the bone marrow cavity and an expansion of the bone diameter.

HAp60CS40 group (Fig 5)

The implanted HAp60CS40 composite was located in the bone tissue with no changes and no new bone formation. The implant was partly absorbed by a number of inflammatory cells along its surface. There were no obvious changes between the original bone and the implant. However, while the absorption of original bone was definite, the amount of fibrous

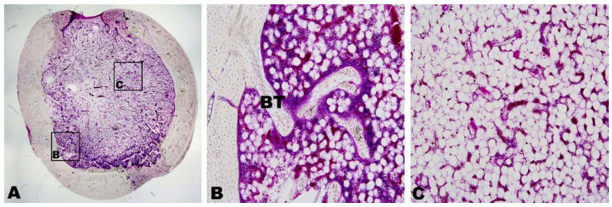

Fig 2. Microphotographs of the control group. (A) Transverse section (B) Bone formation and small bony trabecular formation (TB) were observed along the margin of the bone defect. (C) Adipic bone marrow consists of a number of adipocytes.

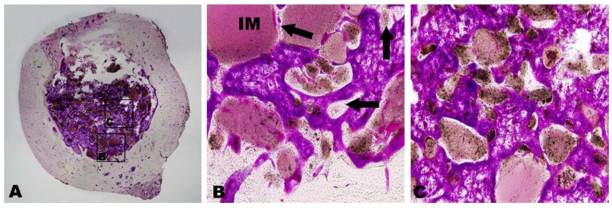

Fig 3. Microphotographs of the HAp100 group. (A) Bone defect was healed completely with obvious osteon structures. (B) New bone (Arrow) was deposited surround the coccoid HAp100 granules. (C) Granules were located in hemopoietic bone marrow.

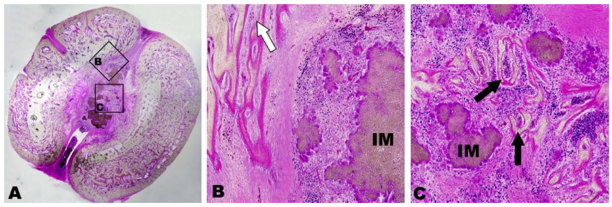

Fig 4. Microphotographs of the HAp80CS20 group. (A) Bone defect was become narrow through new bone, but be not completely. (B, C): While implant(IM) was absorbed, dense connective tissue filled (open arrow) and which new bone (closed arrow) formed. Mild inflammatory response observed.

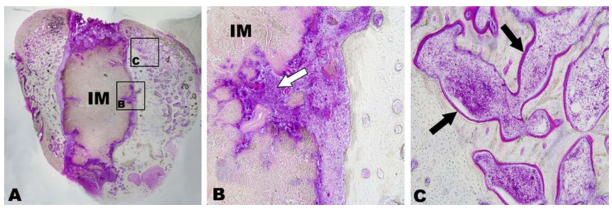

Fig 5. Microphotographs of the HAp60CS40 group. (A) The implant (IM) was located in bone tissue with no change and no new bone formation. (B) Implant was mild absorption by a number of inflammatory cells (open arrow) along the surface of that. (C) Fibrous connective tissue (closed arrows) was increased within cortical bone tissue.

connective tissue had increased significantly within the cortical bone tissue. The infiltration of chronic inflammatory cells was observed in the fibrous connective tissue, which enveloped the implant and formed a thin layer. The bone defect was filled with granulation tissue in which a number of chronic inflammatory cells had infiltrated. Moreover, bone

healing did not progress.

Discussion

The HAp/CS composites have two therapeutic effects in dogs: effective cortical bone healing in defects when the ratio of HAp is high and a more aggressive reaction at the periosteum as more CS is added.

The bone defects in the HAp100 group were almost cured with less response at the periosteum and endosteum in the radiographs. The histological finding showed new bone had deposited around the implants, and cortex area had closed completely with obvious osteon structures. Because of HAp is similar to the inorganic component of bone tissue of the body (7), it is considered to be an effective compound for treating bone defects.

The implanted sites of CS containing the composites showed vigorous responses at the periosteum and endosteum. Furthermore, some sites showed HAp/CS composites with the appearance of hypertrophic osteopathy (palisading). These reactions were considered to be a callus formed by CS, which accelerates the infiltration of PMNs through the complement C5a (9). An inflammatory response occurs at the early stage but new bone formation occurs at a later stage. As CS has a hydrophilic surface, it can promote angiogenesis as well as cell adhesion, proliferation, and differentiation (2,11, 12). Angiogenesis induced by CS leads to enhanced bone density around the implanted site, and a hypertrophic periosteal reaction. In this study, it is believed the CS can induce classical bone healing. However, the bone defects implanted with the CS-containing composites did not heal completely. The histological findings revealed the new bone but very weak formation in the bone defects. In addition, a quantity of composites remained in the bone marrow.

In this study, although the pure HAp composite was most effective in healing bone defects, it was difficult to manipulate when applied clinically. Although bone healing was delayed as more CS was added, it is believed that the 80:20 wt% HAp/CS composite can improve healing and overcome the disadvantage of the pure HAp composite. For better effects, further studies will be needed to reveal the detailed ratio. Because the therapeutic effects of HAp and CS are different, it is believed that there are various indications for application to several bone diseases such as bone defects or comminuted fractures.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2013R1A1A-2009546).

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