Biocompatibility and osteoconduction of bone defect fillers made from α-tricalcium phosphate and 50%-deacetylated chitosan based binders

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Abstract : New bone defect fillers (BDFs) were made from α-tricalcium phosphate and binders consisting of 50%-deacetylated chitosan with and without sodium alginate and diluted organic acid as solvent. Inflammatory response, bio-resorption and osteoconductivity were examined through studies with subcutaneous implantation of binders and bone implantation of BDFs. Histopathological images of host tissue were observed up to four weeks following implantation.

The concentrations of acetic and citric acid at which chitosan dissolved exceeded 0.1 and 1.0 wt %, respectively. Adopting of chitosan thus made it possible to reduce acidity of the BDFs, which had not been possible in previous studies. Inflammatory response was higher for binder using chitosan alone than with chitosan and alginate, whereas it was scarcely different between kinds and concentrations of acid used. Bio-resorption was also higher for binder using chitosan alone than with chitosan and alginate. And it increased with acid concentration. Therefore, it was thought that inflammatory response and bio-resorption would appear possibly due to the water-soluble chitosan.

In all bone implantations, the gap between the implanted bulk and bone cavity gradually was filled with newly-formed bone, thus diminishing the trabecular bone formation around the bulk. When citric acid was used, significantly higher conductivity was noted compared to acetic acid.

Thus, it was suggested that adequate biocompatibility and osteoconductivity was clearly evident for all BDFs.

Key words : chitosan, bone defect fillers, bone substitute, osteoconductivity, biocompatibility

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Introduction

Granules of calcium phosphate, such as hydroxyapatite, have clinical application in oral and periodontal surgery. But as disadvantages, shape cannot be maintained up until the time decayed bone is remodeled and application is limited to concave cavity restoration.

The authors thus devised new bone defect fillers (BDFs) made from polysaccharides such as chitosan deacetylated at more than 85 percent and sodium alginate and α-tricalcium phosphate with the property of osteoconductivity[1]. These materials can be easily hardened into any shape to meet clinical requirements. Their mechanical strength, setting time, osteoconductivity and biodegradability are also adequate. *In vivo* experiments, however, have demonstrated some inflammatory responses about BDFs in rat, possibly due to organic acids used to dissolve polysaccharides. The alginate gel effectively lessened the binder irritation.

Thus, modified BDFs made with 50%-deacetylated chitosan, which required low concentration acid to achieve solubility and thereby to be expected to minimize inflammatory response, were prepared for use *in vivo* experiments. Also the BDFs which substituted half amount of chitosan by sodium alginate were prepared. In this study, examination was made of the effects of modified BDFs on the biocompatibility through the microscopic observation of tissues and BDFs in rat.

Materials and Methods

Experimental materials

The materials from which BDFs were prepared in this study are listed in Table 1. Water-soluble chitosan with deacetylation of 50% (Marinedue®PC-100, Ajinomoto, Tokyo) and sodium alginate (alginate; Kanto Kagaku, Tokyo) served as the basic components. Acetic (Kanto Kagaku, Tokyo) and citric acids (Kanto Kagaku, Tokyo) were used as solvent of chitosan and distilled water for alginate. Sodium diphosphate decahydrate (SDD; Kanto Kagaku, Tokyo) and sodium polyphosphate (SPPS; Wako pure chemical, Osaka) were used as setting agents of chitosan in acetic acid and citric acid solutions, respectively. Alpha-tricalcium phosphate (α-TCP, mean particle size 300μm; Sankin Kogyo, Tochigi) was used as the BDF filler.

Table 1. Constituents of binders

No.	base	solvent	setting agent
1	chitosan	0.1% Ace	SDD
2	chitosan	1.0% Cit	SPPS
3	chitosan+alginate	0.1% Ace	SDD
4	chitosan+alginate	1.0% Cit	SPPS
5	chitosan	0.5% Ace	SDD
6	chitosan	5.0% Cit	SPPS
7	chitosan+alginate	0.5% Ace	SDD
8	chitosan+alginate	5.0% Cit	SPPS

Ace: acetic acid, Cit: citric acid, SDD: Sodium diphosphate decahydrate, SPPS: sodium polyphosphate.

Twenty-four male Sprague-Dawley rats aged 8 weeks (weight about 300 g), raised for one week or more at Iwate Medical University Animal House, were used for the *in vivo* study. None had any abnormality.

Specimen preparation for implantation

BDFs hardened without α-TCP were used for subcutaneous implantation. The preparation of each BDF was as follows:

The chitosan based specimens were made

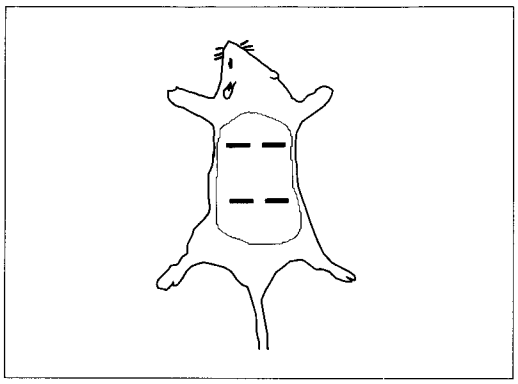

Fig. 1. Schematic view of four sites of dorsal skin incisions

by mixing chitosan (1.0 g) dissolved in acid (50ml) with the setting agent (0.2 g) placed in a cylindrical acrylic mold 6.0 ㎜ in diameter and 1.0㎜ in depth. 5ml mixture were used for each specimen. 0.1 and 0.5% acetic acid and 1.0 and 5.0% citric acid were used. The chitosan-alginate based specimens were prepared essentially in the same manner, whereas chitosan and alginate were premixed to produce the base component. BDF specimens for implantation in bone were prepared by kneading binders No. 1, 2, 3 and 4, respectively with α-TCP (6.0 g) and then putting them in the acrylic mold (diameter 2.0×2.0㎜). There were four different compositions of BDF. Prior to implantation, the specimens were stored at constant temperature and humidity for 5 hours under ultraviolet rays for disinfection.

Measurement of binder pH

This parameter was measured immediately after the mixing of constituents, using a pH electrode (Model 85-35, Orion, Boston, USA) and pH/ion meter (Model 720A, Orion, Boston, USA) at 25℃.

Subcutaneous implantation

Eighteen rats were divided into low and high acid concentration groups, 9 animals each. Sodium pentobarbital (Nembutal®, Abbott laboratory, North Chicago, USA; 0.8 ㎎/kg) was administered via the celiac of rats, then the dorsal skin was shaved and scrubbed with 80% ethanol. Skin incisions of 10㎜ at 4 sites were made as shown in Fig. 1 for binder implantation. Intramuscular injection of antibiotics (Procaine penicillin G®, Tamura medicine manufacture, Tokyo) was then carried out to prevent infection (30,000 units/on each of three days). Three animals in each group were killed by an overdose of sodium pentobarbital at 1, 2 and 4 week(s). The binders were removed along with surrounding tissue and immersed in 10% phosphoric acid buffered formalin solution (pH 7.2). Following fixation, they were dehydrated in a series of increasingly stronger alcohols and embedded in paraffin for sectioning into thin specimens. This was followed by staining with hematoxylin-eosin for light microscopic observation.

Bone defect filler implantation

Six rats were used for BDF implantation. Subsequent to anesthetization, shaving and disinfecting in the same manner as for subcutaneous implantation, the femur surface was exposed by removing the skin 40mm along the femur and by separating the femoral segment from the bone. BDFs were implanted into two cavities prepared in bilateral femoral diaphysis using dental fissure burrs under cooling with saline.

Three animals in each group were killed 2 and 4 weeks after implantation. The extracted BDFs with the femur were fixed and demineralized with Plank-Rychlo solution. Sectioned specimens were prepared for observation by light microscope as above.

Results

Binder pH

Values for this parameter are indicated in Table 2. The lowest pH, 3.68, was for binder 6, a chitosan-based material prepared using 5.0% citric acid as solvent. For binder 8, a chitosan-alginate based material prepared using the same acid, the pH was a slightly higher 4.51. The highest pH, 8.96, was for binder 3, a chitosan-alginate based material obtained with 0.1% acetic acid. Slightly lower pH of 8.71 and 8.35 were for binders 1 and 7, chitosan based using 0.1% acetic acid and chitosan-alginate based using 0.5% acetic acid, respectively. Binders 2, 4 and 5 had intermediate pH of 6.62, 7.72 and 7.58, respectively.

Table 2. Binder pH data

No.	1	2	3	4	5	6	7	8
pH	8.71	6.62	8.96	7.72	7.58	3.68	8.35	4.51

Subcutaneous implantation

Microscopic images of binders implanted and surrounding tissue are shown in Fig. 2. Histopathological findings for the 1-week implantation were as follows: Extended acute inflammatory infiltration mainly by neutrophils was evident in specimen 1. The original size of the binder was maintained in the tissue, but resorption could not be confirmed. In specimen 2 the same inflammatory response as in specimen 1 was noted but binder size had slightly decreased, thus enhancing bio-resorption. In specimen 3 resorption was not seen and acute inflammatory infiltration by neutrophils was restricted to about the binders. Specimen 4 findings were similar to those of 3. In specimen 5, acute inflammatory infiltration as in specimens 1 and 2 and slight bio-resorption were evident. In specimen 6, the binder was obscure in the tissue and there was no acute inflammatory response. In specimens 7 and 8, limited acute inflammatory infiltration by neutrophils as in specimen 3 was seen, along with decreased size due to resorption, compared to specimens 3 and 4.

Two-week implantation findings were as follows: In specimen 1, inflammatory infiltration the same as at 1-week and bulk fragments with partial resorption was noted. In specimen 2, the same inflammatory response and resorption were observed as at 1-week. In specimen 3, acute inflammatory infiltration by neutrophils was limited to about the binders similarly as at 1-week and resorption was evident. In specimen 4, no resorption was observed, but inflammatory response was the same as in 3. In specimen 5, the bulk was obscure by resorption, and inflammatory response was less than at 1-week. In specimen 6, inflammatory response and resorption were the same as at 1-week. In specimens 7 and 8, limited acute inflammatory infiltration by neutrophils could be seen along with decreased size compared to 1-week findings.

Four-week implantation findings were as follows: In specimens 1 and 2, inflammation was less intense compared to that at 2 weeks and binders were obscure situated in tissue, with consequently more advanced resorption. In specimen 3, acute inflammation was less intense and chronic inflammatory infiltration of phagocytosis more advanced. Resorption was more than at two weeks and encapsulation of the binder by fibrous membrane could be seen. In specimen 4, the same inflammatory

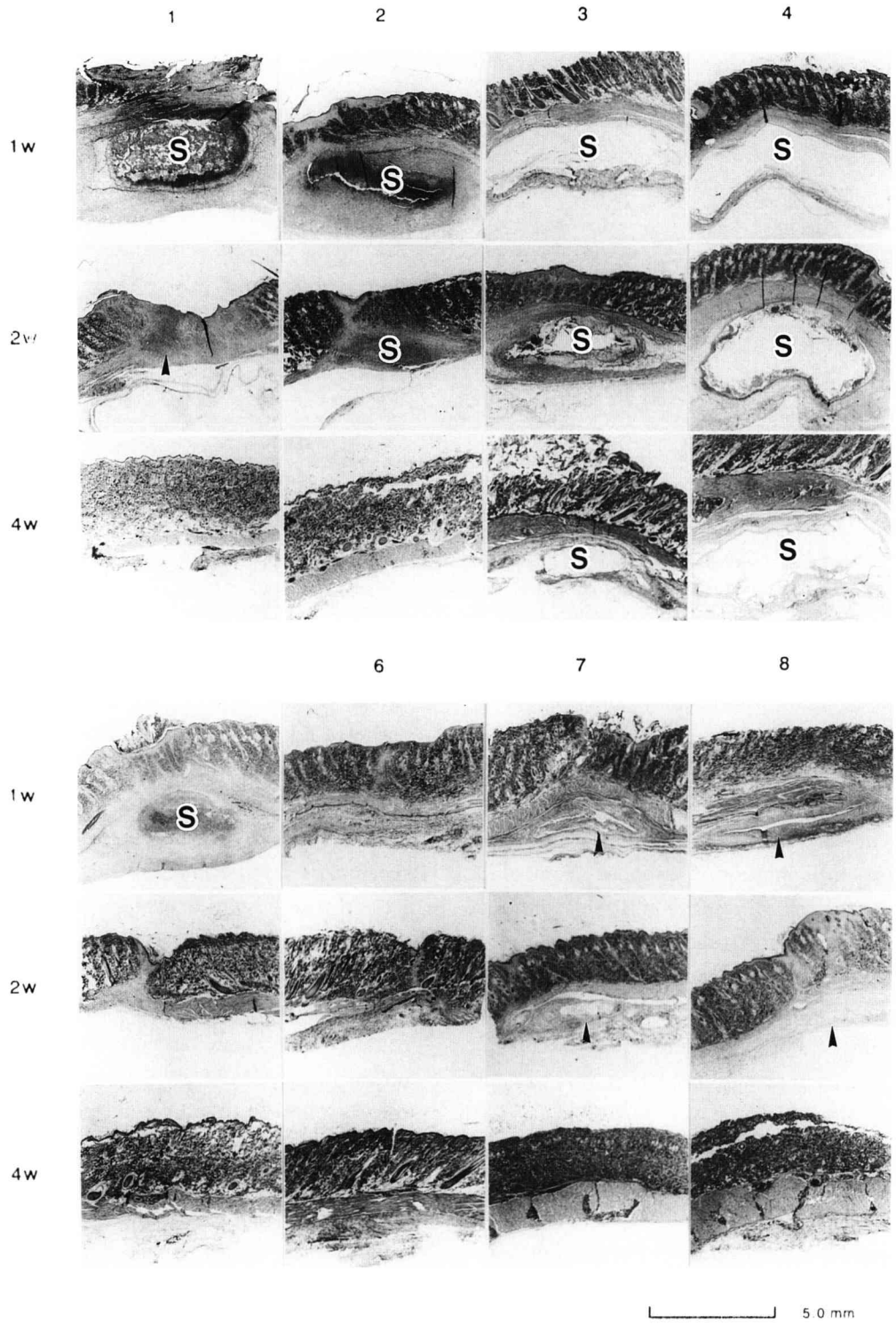

Fig. 2. Histopathological images of binders (No. 1–8) in the subcutaneous tissue at 1, 2 and 4 week(s) following implantation. S and arrowhead in images indicate specimen. Strong inflammatory response was evident soon following implantation of binders prepared with chitosan alone. Bio-resorption would appear due to reaction with acid and chitosan. Stain: hematoxylin and eosin stain.

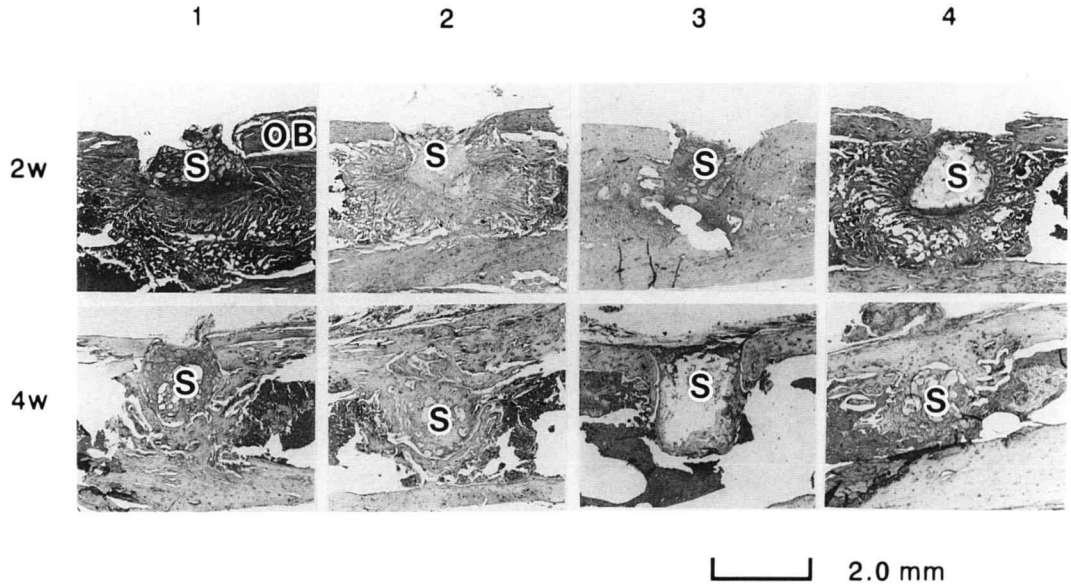

Fig. 3. Histopathological images of a bone cavity implanted with each BDF in the femur at 2 and 4 weeks following implantation. S and OB indicate specimen and original bone. The images for specimens 2 and 4 at 4 weeks showed the bulk to be covered with newly-formed bone due to remodeling. Stain: hematoxylin and eosin stain.

findings as for 3 were noted and the size of binder was the same as at two weeks. In specimens 5 and 6, no inflammatory response such as at 2 weeks was evident. In specimens 7 and 8, there was virtually no acute inflammatory response compared with 2-week findings and the chronic inflammatory response noted for chitosan-alginate based materials (No. 3 and 4) was absent. In specimens 5, 6, 7 and 8, implants were obscure in tissue. In specimens 7 and 8, resorption was greater than at two weeks.

Bone implantation

Microscopic images of BDF implanted in femurs are presented in Fig. 3. Two-week histopathological findings were as follows:

In specimen 1, the implanted bulk had collapsed at the site where it faced the original bone. Trabecular bone-like cancellous was apparent radially about the bulk. No intervening fibrous connective tissue could be seen between the bulk and trabecular bone. In specimen 2, trabecular bone was noted bound to the bulk and fibrous connective tissue was absent as in specimen 1. In specimen 3, trabecular bone seen in 1 and 2 was absent. A gap between the bulk and original bone was apparent. Specimen 4, findings were the same as for 2.

At 4 weeks specimen 1 trabecular bone could be seen about the bulk. There was no intervening of fibrous connective tissue such as noted at 1 week. The bulk was connected to the original bone. The specimen 2 image showed the bulk to be covered with newly-formed bone due to remodeling. In specimen 3, only little trabecular bone could be seen about the bulk in contrast to specimens 1 and 2 and the bulk was in contact with original bone. Specimen 4 bulk was covered with

newly-formed bone.

Discussion

Binder pH

The solubility of chitosan varies with the degree of deacetylation[2-5]. In this study, chitosan with 85% required higher citric acid concentration more than 5.0% to achieve solubility. More than 1.0% citric acid as solvent was used in the present work, with consequent reduction in binder pH. There was concern that this might lead to inflammatory reaction in tissue. Chitosan with 50%, solubility was found to dissolve at lower acid concentration than previously used. With acetic acid, chitosan could be dissolved to ever less than 0.1% while with citric acid, dissolution was always more than 1.0%. The pH of binders prepared with acetic acid ranged from 7.58 to 8.96. This acid should thus lessen binder irritation. Chitosan required higher citric acid concentration for dissolution compared to acetic acid. Nevertheless, moderate binder acidity was achieved through the use of 1.0% citric acid at pH from 6.62 to 7.72. Thus, citric acid should also prove useful as a solvent of chitosan in binder preparation.

Subcutaneous implantation

In BDF implantation in a living body, assessment should initially be made of inflammatory response and bio-resorption, both vital factors in this process as well as osteoconduction. BDFs and chitosan-based binders were previously found to give rise to inflammatory response, possibly due to the action of the constituents of binders[1], chitosan, acid and the setting agent. A water-soluble chitosan was used in this study to lessen acid concentration and thereby to minimize inflammatory response. Inflammatory response and bio-resorption following subcutaneous implantation were then examined.

No absolute standard is presently available for the quantitative assessment of inflammatory response based on histopathological findings from previous studies though fibrous membrane thickness was used by Wolfaardt, et al.[6] and Cutright, et al.[7] for this purpose. In this study, the continual presence of inflammatory cell infiltration due to macrophages, thin fibrous membrane encapsulation of implanted materials, indicated by histopathological findings served as the standard. As shown in Table 3, inflammation in tissue images was evaluated from these two factors. Bio-resorption was evaluated based on change in the size of implanted binder. Five of eight binders were concluded to cause none or moderate irritation based on these criteria while severe inflammatory response was indicated for the other three, all chitosan-based with pH from 6.62 to 8.71, and thus by weak acid or alkali. The severe response would thus not be due to either acid, but to chitosan itself. Specimens 1 to 5 and 2 to 6 showed less intense response at lower acidity. Inflammatory response for specimen 6 was low even soon following implantation though pH was lowest. Binders prepared from chitosan and alginate were attended with less inflammation than when chitosan alone was used. Thus, the use of chitosan with the same amount of another constituent would appear to lessen inflammation. Sodium alginate would not likely lead to subcutaneous inflammation. But more significant is the finding that the severe inflammatory response in histopathological images of specimens 1, 2

Table 3. Inflammatory response and bio-resorption of binders *in vivo*

No.	inflammatory response			bio-resorption		
	1 week	2 weeks	4 weeks	1 week	2 weeks	4 weeks
1	severe	severe	none	−	++	+++
2	severe	severe	none	+	+	+++
3	moderate	moderate	mild	−	+	++
4	moderate	moderate	mild	−	−	−
5	severe	none	none	+	+++	+++
6	none	none	none	+++	+++	+++
7	moderate	moderate	none	+	++	+++
8	moderate	moderate	none	+	++	+++

Inflammatory response evaluated as "none": bulk obscured, "mild": thin fibrous membrane about bulk confirmed, " moderate": acute inflammatory infiltration confirmed in limited area about bulk, and "severe": extensive acute inflammatory infiltration confirmed.
Bio-resorption graded as "−": bulk unchanged in size or resorption not confirmable, "+": resorption confirmed from decrease in size, "++": considerable resorption confirmed or fragments of bulk apparent, and "+++": no bulk confirmable.

and 5 was due to phagocytosis of chitosan with simultaneous resorption of the implant.

Table 3 shows that resorption occurred most rapidly and soonest for the chitosan-based binder with lowest pH for specimen 6 using 5.0% citric acid. Bio-resorption was least for chitosan-alginate specimen 4 using 1.0% citric acid, for which there was no resorption even after 4 weeks. Resorption was more rapid for binders made from chitosan alone, in the case of specimens 1, 2, 5 and 6, compared to the use of chitosan and alginate together. Bio-resorption would thus appear to be determined primarily by chitosan than acid. For binders with lower pH, resorption appeared greater than at high pH based on a comparison of specimens 1, 2, 3 and 4 with 5, 6, 7 and 8. Water-soluble chitosan more effectively enhances bio-resorption and is more suitable for preparing less acidic solution. The chitosan-based binders in this study showed resorption at about one month, though chitosan with higher deacetylation has been shown to require six months for resorption [8-9]. More soluble chitosan may possibly promote phagocytosis with increase in solubility in tissue fluids.

Inflammatory response and bio-resorption of binders may thus be due to reactions between tissue and chitosan. When chitin is present in damaged tissue, phagocytosis promotes wound healing which in turn induces leukocyte formation through fibroblast activation, as a defense reaction in a living body[10]. Bio-resorption proceeds through successive enzymatic degradation of chitin to oligo chitin by lysozyme, and then to *N*-acetylglucosamine by *β*-*N*-acetylglucosamidase and finally glycoproteins and carbon dioxide in saccharide decomposition[9]. The inflammatory response and bio-resorption of binders should thus result from reactions between tissue and chitosan.

Chitosan is thus shown useful for preparing binders that lead to higher bio-resorption of bone defect fillers, though some inflammatory reaction *in vivo* may be

Table 4. Osteoconduction of bone defect fillers implanted in femur

No.	2 weeks	4 weeks
1	low	moderate
2	moderate	high
3	low	moderate
4	moderate	high

low: bulk not in contact with original bone. moderate: bulk covered with trabecular bone and/or in contact with original bone. high: defect filled with newly-formed bone.

expected.

Bone implantation

Histopathological images indicated that at 4 weeks following implantation, four BDFs differing in composition have the capacity for osteoconductivity, the extent differing in each case. A universal standard to assess this parameter based on histopathological data has become available only recently though quantitative determination of newly-formed bone area on X-ray contact microradiographs has been shown possible by Zellin, et al.[11].

Osteoconduction was determined in this study from contact between bulk and original bone, trabecular bone generation about bulk, and substitution by newly-formed bone, as indicated from the histopathological findings in Table 4.

Osteoconduction was graded low to moderate for bone defect fillers using 0.1% acetic acid as solvent and chitosan alone or with alginate. It was graded moderate to high for fillers using 1.0% citric acid and chitosan alone or with alginate. Citric acid would thus appear to have greater effect on osteoconduction than acetic acid and the effect on bony regenesis by chitosan alone or with alginate might essentially the same. The reason as to why citric acid has greater effect than acetic acid remains unclear. Differences in acidity may possibly be explanation in that inflammatory response and bio-resorption were basically the same in subcutaneous implantations of binders, as evident from Table 3. Acetic and citric acids differ considerably in their action in living tissue. The effects of acid on the osteoconduction should thus be clarified in greater detail.

All BDFs in this study had sufficient osteoconductivity, since the gap between the implanted bulk and original bone was gradually filled by newly-formed bone with consequent reduction in trabecular bone.

Conclusion

1. Strong inflammatory response was evident soon following implantation of binders prepared with chitosan alone. Response was weaker when other materials were used with chitosan.
2. Bio-resorption would appear due to reactions with acid and chitosan. Chitosan should thus be useful for producing binders that lead to greater bio-resorption of bone defect fillers, though some inflammatory reaction *in vivo* may be expected.
3. All bone defect fillers showed sufficient osteoconductivity based on the histopathological observation of newly-formed bone around the implantation.

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50% 脱アセチル化キチンを基剤とする賦形材とα型リン酸三カルシウムを複合した骨補塡材の生体適合性および骨伝導能

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抄録：低濃度の酸で溶解可能な脱アセチル化度50%の水溶性キトサンおよびキトサンにアルギン酸塩を加えた混合物を基材とする賦形材にα型リン酸三カルシウムを加えた骨補塡材を作製した。賦形材のみを用いたラット皮下埋入試験および骨補塡材の骨内埋入試験により炎症反応，生体吸収性および骨伝導能を調べた。埋入後，4週までの病理組織像を光顕的に検索した。

脱アセチル化度を50%にすることにより，キトサン溶液を作製するための酢酸およびクエン酸の濃度はそれぞれ0.1，1.0%と従来の賦形材よりも低くすることができた。炎症反応の程度はキトサン単独系がキトサンとアルギン酸塩の混合系より高く，酸の種類および濃度による差は少なかった。生体吸収性は，単独系が混合系より高く，高濃度の酸を用いたものが低濃度より高くなる傾向を示した。したがって本系材料による炎症反応および生体吸収性は主として水溶性キトサンによるものと考えられた。

骨伝導能に関しては，すべての試料において埋入期間の経過とともに，徐々に新生骨によって間隙が満たされ，周囲に生じた梁状骨は消失してゆく所見が得られた。各試料間の比較では，クエン酸を用いた試料が酢酸を用いた場合と比較して有意に骨伝導能が高いことが認められた。

以上のことより，今回作製した材料は，いずれも骨補塡材として十分な生体適合性および骨伝導能を有する材料であることが示唆された。