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Comparison of ready-made and self-setting alginate membranes used as a barrier membrane for guided bone regeneration

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In order to understand the requirements of guided bone regeneration (GBR) involving alginate base self-setting barrier membranes, GBR was performed in the case of bicortical bone defects formed at the tibiae of experimental animals employing self-setting and ready-made alginate membranes. Connective tissue ingress into the bone defects at the skin side of the tibia was observed when GBR was generated utilizing ready-made alginate membrane. In contrast, bone defects were reconstructed with bone tissue when GBR was generated with self-setting alginate membrane formed from aqueous 3% sodium alginate and 3% $CaCl_2$ solutions. The unreacted aqueous sodium alginate solution inherent to self-setting alginate membrane did not inhibit bone tissue regeneration. Rather, callus bone was formed using sodium alginate as the nucleus. However, when GBR was effected with self-setting alginate membrane formed from aqueous 10% $CaCl_2$ solution, membrane was too thick and thus regeneration of bone tissue in the bone cavity was prevented. Therefore, we concluded that self-setting alginate membrane is very useful as a barrier membrane for GBR upon appropriate adjustment of conditions with respect to preparation of alginate membrane.

Introduction

We previously reported that an alginate membrane is applicable as a self-setting barrier membrane for guided tissue regeneration (GTR) and guided bone regeneration (GBR) [1]. As the name "self-setting" implies, the key advantage of the alginate membrane in comparison to conventional ready-made barrier membranes made from Teflon® or poly-L-lactic acid (PLLA) is the generation of a barrier membrane in the form of the bone defect at the bone defect during the surgical procedure. For example, the bone defect is filled with an aqueous sodium alginate (Na-Alg) solution, followed by dropping aqueous $CaCl_2$ solution onto the surface of the Na-Alg solution. As a result of this simple process, an alginate membrane is formed on the bone defect, which is maintained within the bone defect filled with unreacted aqueous Na-Alg solution. When the bone defect was covered with the alginate membrane, regeneration of bone tissue was observed at the bone defect, whereas the bone defect was filled solely with connective tissue

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when it had been maintained in an open posture [2].

Although our previous results [1, 2] demonstrated that alginate membrane is useful as a barrier membrane for GBR, factors affecting the bone regeneration with alginate based GBR has not been clarified. For example, alginate membrane can be prepared in advance and thus, ready-made alginate membrane may be able to be used at the surgical GBR procedure similar to other barrier membranes. Although the shape of ready-made alginate membrane is different from the shape of bone defect, it will fit the bone defect better than the other barrier membrane such as Teflon® or PLLA since alginate is elastic material.

When GBR was performed utilizing self-setting alginate membrane, the bone defect was filled with unreacted aqueous Na-Alg solution in lieu of blood clot. Na-Alg displays excellent tissue response [1, 3]; additionally, this substance has been applied as a blood expander [4]; however, its role in bone formation at the bone defect has not been thoroughly examined. Effects of unreacted Na-Alg in the bone defect will be understood by comparing GTR using alginate base self-setting and ready made barrier membrane.

In this study, therefore, GBR was conducted on bone defects of experimental animals using alginate base self-setting and ready-made barrier membranes to shed some light for the better understanding of alginate base barrier membrane for GBR.

Materials and methods

Preparation of alginate membrane

Commercially available Na-Alg (Nacalai Tesque, Kyoto, Japan) and calcium chloride (Nacalai Tesque) were used without further purification. Aqueous solution of Na-Alg (1.0, 3.0%) and $CaCl_2$ (1.0, 3.0, 10.0%) were prepared and sterilized by filtration through 0.22-μm Millex-GS filter assemblies (Millipore Corp., Bedford, MA).

A ready-made alginate membrane was generated via exposure of aqueous Na-Alg solution with aqueous $CaCl_2$ solution. In brief, aqueous $CaCl_2$ solution was delivered via a sprayer onto a plastic vessel (size: 30 × 30 × 1 mm) filled with aqueous Na-Alg solution. The aqueous Na-Alg and $CaCl_2$ solutions were allowed to be in contact for 2 min; subsequently, the surface of the alginate membrane was rinsed with distilled water to remove excess aqueous $CaCl_2$ solution. Then, the alginate membrane was removed from the vessel and immediately immersed in water in order to remove unreacted Na-Alg solution.

A self-setting alginate membrane was prepared at the bone defect. First, the bone defect was filled with Na-Alg aqueous solution. Also, the Na-Alg aqueous solution was applied around the bone defect. Then calcium chloride aqueous solution was dropped on the Na-Alg solution to form an alginate membrane. After 60 s, physiological saline solution was poured to wash out excess calcium chloride solution. (Fig. 1)

Thickness and tensile strength measurement of alginate membrane

The thickness of the alginate membrane was determined with a micrometer. Ten specimens were prepared independently under identical conditions; thickness was measured at least three times for each specimen. In terms of tensile strength measurement, alginate membranes were shaped in the form of dumbbells; membranes were placed under tension on a universal testing machine (DSC-2000, Shimadzu, Japan). Ten specimens were measured under each condition.

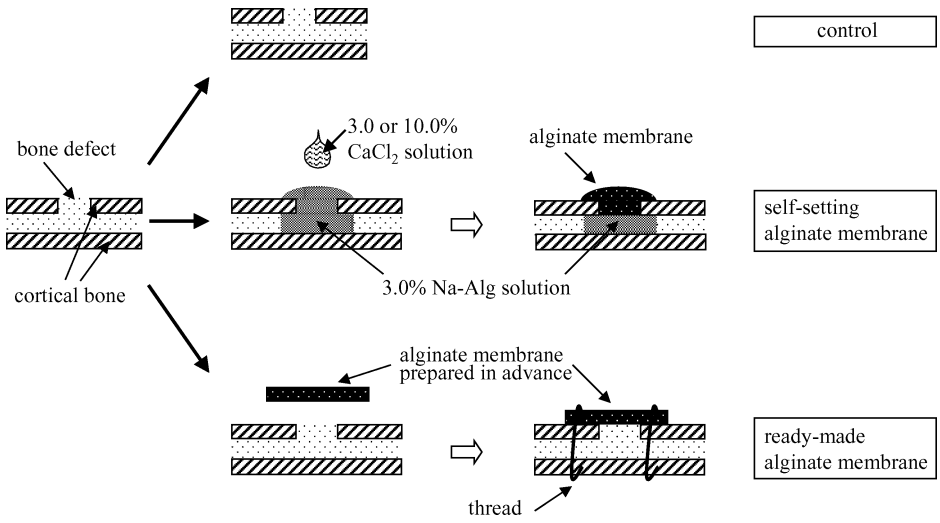

Figure 1 Schematic illustration of ready-made and self-setting alginate membrane employed in the present study. Note that monocorotical defect was used for simplicity in this figure even though bicorotical bone defect was used in the actual study.

Linear shrinkage ratio

Two sides of a ready-made membrane were measured and calculated the mean after it was made by the methods mentioned above. Linear shrinkage ratio was calculated in the following formula. The experiment was repeated under each condition five times.

$$\text{Linear shrinkage ratio (\%)} = \{30 - \text{mean (mm)}/30\text{(mm)}\} \times 100$$

Animals and operative procedure

Fifteen-week-old male Wistar rats, obtained commercially (Shimizu Experimental Animals, Kyoto, Japan) and fed standard pellets and water ad libitum, were used in the animal study. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal® ; Abbott Co., Chicago, IL). The legs were shaved and infiltration anesthesia with 0.4 mL of 2% lidocain-epinephrine solution (Xylocaine® ; Fujisawa Pharmaceutical Co., Osaka, Japan) was applied around the medial end of the tibia to arrest bleeding from the bone marrow and to control early postoperative pain. The medial end of the tibia was exposed, and a 3 × 10 mm bicortical bone defect was formed with a fissure bar. In the experiments using animals, concentration of the Na-Alg was fixed to 3% and concentration of $CaCl^2$ was 3 or 10%. In the case of "self-setting" alginate membrane group, the bone defect was filled with 3% Na-Alg aqueous solution. Also, the 3% Na-Alg aqueous solution was applied around the bone defect. Then 3 or 10% calcium chloride aqueous solution was dropped on the Na-Alg solution to form an alginate membrane. After 60 s, physiological saline solution was poured to wash out excess calcium chloride solution. This simple procedure forms alginate membrane on the surface of the bone defect. In the case of "ready-made" alginate group, alginate membrane was prepared in advance from 3% Na-Alg and 3 or 10% $CaCl_2$, and the membrane was placed on the bone defect similar to the other commercially available ready-made barrier membrane. Since ready-made alginate membrane would not stick on the surface of bone defect by itself, the membrane was fixed at the bone defect with threads. For the control group, neither Na-Alg nor calcium chloride aqueous solutions were applied. After the GTR procedure, the flap was closed and sutured.

All the animal experiments were performed according to NIH guidelines.

Histological preparations

Two, four and eight weeks after surgery, each rat was anesthetized with sodium pentobarbital to a level at which respiration was markedly suppressed; subsequently, rats were perfused with 10% neutral buffered formalin and fixed. Following the aforementioned procedure, each operative field in the tibia with surrounding tissue was removed and prepared with the aforementioned fixative for an additional three hours. Subsequently, specimens were decalcified with 10% formic acid for two weeks. After dehydration in an ascending graded series of ethanol solutions, specimens were embedded in paraffin. Coronal sections 4 μm in thickness were prepared utilizing a microtone (Leica SM 2000R). Finally, sections were stained with hematoxylin-eosin for microscopic observation.

Statistical analysis

For the statistical analysis, one way factorial ANOVA and Fisher's PLSD method, used as a post-hoc test, were performed using the program "Stat View 4.02" (Abacus Concepts Inc, Berkeley, CA). p values < 0.05 were considered to indicate statistically significant differences.

Results

Fig. 2 displays effects of $CaCl_2$ concentration on the thickness of alginate membrane when Na-Alg aqueous solution was exposed to $CalCl_2$ aqueous solutions for 2 min. Basically, alginate membrane was thicker when we employed concentrated Na-Alg and $CaCl_2$ aqueous solution. Thickness of the alginate membrane made with 3.0% aqueous Na-Alg was significantly ($p < 0.05$) thicker than that of the alginate membrane made with 1.0% aqueous Na-Alg regardless of the concentration of $CaCl_2$. Similarly, thickness of the alginate membrane made with 3 and 10% $CaCl_2$ aqueous solution was significantly ($p < 0.05$) thicker than those made with 1 and 3% $CaCl_2$ aqueous solution when regardless of the concentration of Na-Alg.

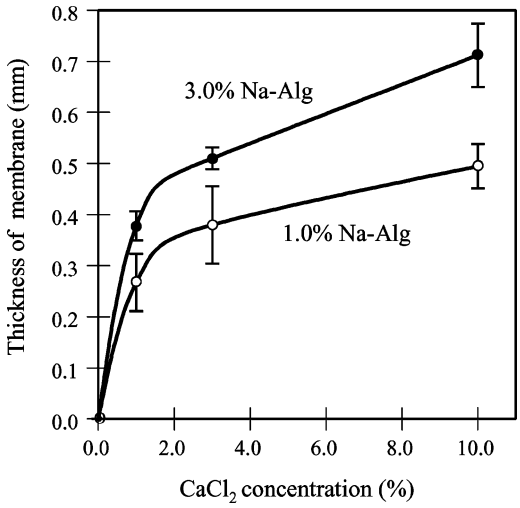

Figure 2 Thickness of alginate membrane as a function of the concentrations of aqueous sodium alginate and calcium chloride solutions.

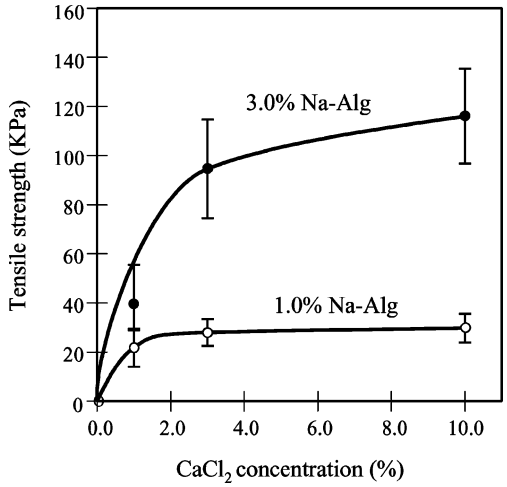

Figure 3 Tensile strength of alginate membrane as a function of the concentrations of aqueous sodium alginate and calcium chloride solutions.

Fig. 3 shows the tensile strength of alginate membranes as a function of identical Na-Alg and $CaCl_2$ concentrations per Fig 1. Tensile strength also increased with increasing concentrations of aqueous Na-Alg and $CaCl_2$ solutions. However, we found no significant difference on tensile strength between $CaCl_2$ 3 and 10% when Na-Alg concentration was 3%. We also found no significant difference among 1.0% Na-Alg group based on the concentration of $CaCl_2$.

Fig. 4 presents the linear shrinkage ratio of alginate membrane as a function of the identical Na-Alg and $CaCl_2$ concentrations per Fig 1. Shrinkage ratio of the alginate membrane increased in linear fashion with increasing $CaCl_2$ concentration. In the case of 1.0% Na-Alg, concentration change of $CaCl_2$ did not bring a large change to shrinkage ratio. A larger shrinkage percentage for alginate membranes prepared with lower concentrations of aqueous Na-Alg solution when the concentration of $CaCl_2$ was held constant was observed.

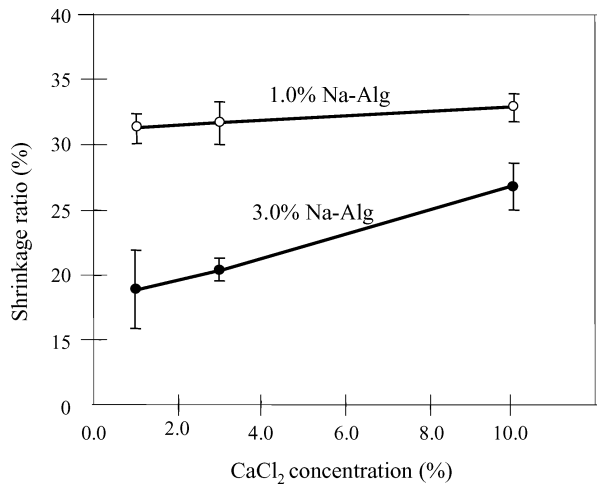

Figure 4 Linear shrinkage of alginate membrane as a function of the concentrations of aqueous sodium alginate and calcium chloride solutions.

Fig. 5 exhibits transverse sections two weeks after surgery. In the control group, ingress of connective tissue into bone defects was apparent. Most of the blood clot had disappeared; moreover, new bone was formed toward the cancerous bone at the edge of pre-existing cortical bone. In the self-setting alginate membrane (3%) group, the alginate membrane prevented ingress of connective tissue from bone cavity. Callus formation was evident not only from the edge of pre-existing cortical bone but also at the center of the bone defect as if callus were covering the alginate membrane. No inflammatory response was detected around the alginate membrane. In contrast, the bone defect was filled with alginate membrane; thus, no bone formation occurred in the self-setting alginate membrane (10%) group. In the ready-made alginate membrane group, new bone formation was observed from the edge of pre-existing cortical bone at the muscle side. However, the alginate membrane was broken and ingress of connective tissue was present for the bone defect at the skin side.

Fig. 6 displays a transverse section four weeks after surgery. In the control group, the bone defect was filled with connective tissue and the tibia was divided into two portions. In the self-setting alginate membrane (3%) group, ingress of connective tissue into the bone cavity was nearly completely prevented by the membrane. As a result, new bone formation from the edge of pre-existing cortical bone proceeded further when compared with the results at 2 weeks. In addition, regeneration of hematogenous bone marrow was evident. In the self-setting alginate membrane (10%) group, new bone was formed along the alginate membrane inside the bone defect. However, thick alginate membranes prevented bone formation between the cortical bones. In the ready-made alginate membrane group, the alginate membrane prevented ingress of connective tissue into the bone cavity at the muscle side; consequently, bone formation was observed. However, continuity of the formed bone to the pre-existing cortical bone was poor in comparison with the results obtained with self-setting alginate membrane (3%) group. In addition, connective tissue invaded the bone cavity from the bone defect at the skin side. As a result, new bone formation was not detected. At the bone marrow, granulation tissue was apparent; however, regeneration of hematogenous bone marrow was absent.

Fig. 7 displays the transverse section eight weeks after surgery. In the control group, the tibia was perfectly divided into two portions in order to effect repair; furthermore, the thickness of cortical bone in eight weeks had increased in comparison with that of four weeks. In the self-setting alginate membrane (3%) group, the alginate membrane prevented ingress of connective tissue into the bone cavity; moreover, remodeling of the newly formed bone was observed. Regeneration of the

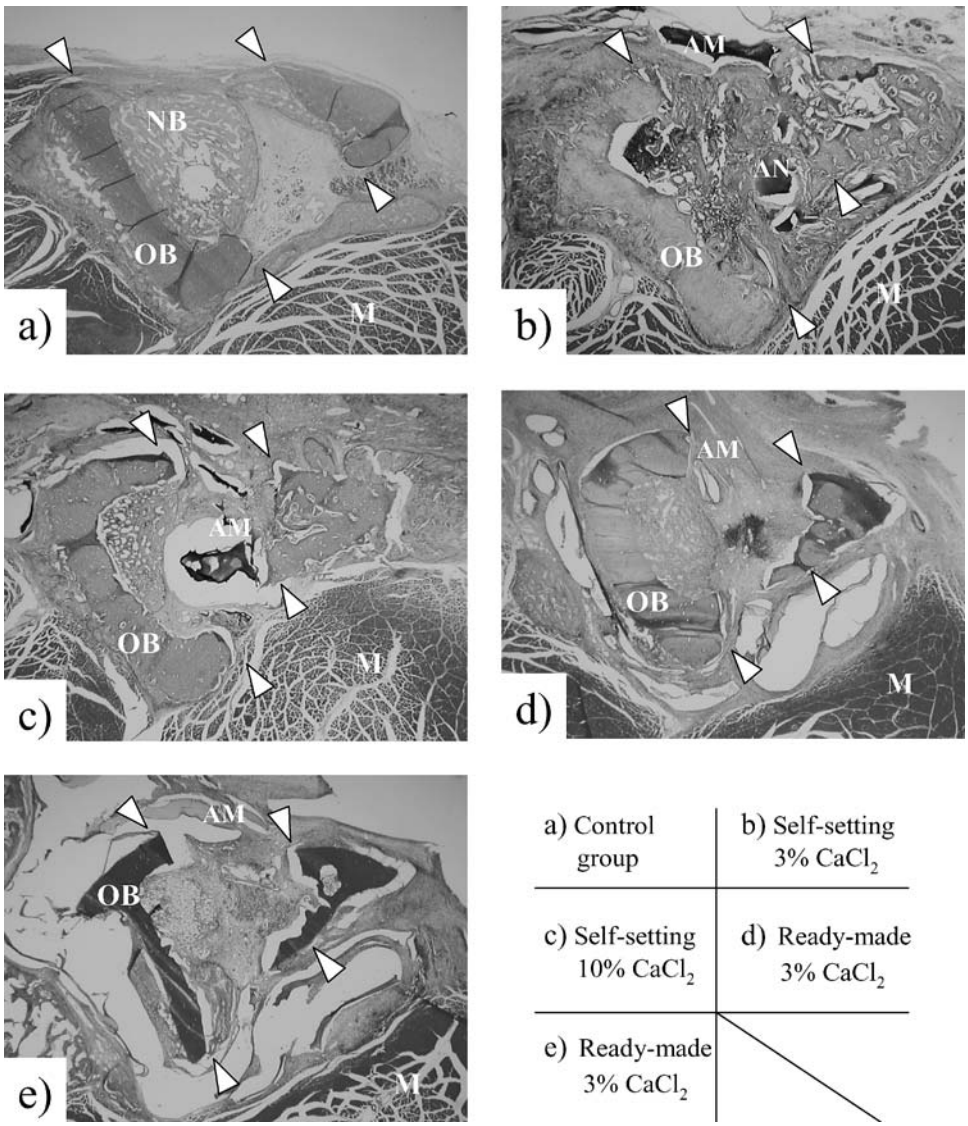

Figure 5 Transverse section of a rat tibia two weeks after operation. Hematoxylin-eosin stain. (a) Control group, (b) Self-setting alginate membrane (3%) group, (c) Self-setting alginate membrane (10%) group, (d) Ready-made alginate membrane (3%) group, (e) Ready-made alginate membrane (10%) group.
Arrow: edge of pre-existing cortical bone, OB: Original Bone, NB: new bone, AM: Alginate Membrane, AN: Sodium Alginate, M: Muscle.

hematogenous bone marrow remained apparent at this stage. Although resorption of the alginate membrane proceeded to some degree, it was not completely absorbed. In the self-setting alginate membrane (10%) group, new bone was formed along the surface of the alginate membrane. Due to the thick alginate membrane, the tibia was divided into the bone. In the ready-made alginate membrane group, regeneration was observed at the muscle side. However connective tissue invaded the bone cavity and prevented bone formation at the skin side.

Discussions

The results obtained in this study revealed clearly that self-setting alginate membrane is superior to a ready-made alginate membrane when both are employed as a barrier membrane for GBR. In the case of self-setting alginate membrane (3%), the alginate membrane prevented ingress of connective tissue from bone cavity completely and thus, the bone regeneration was confirmed. In contrast, alginate membrane was broken at two weeks after surgery and thus, ingress of the connective tissue was present at skin site bone defect in the case of ready-made alginate membrane (3%). Although the detailed mechanism of this distinction was not clarified in the present study, one possible explanation may be attributable to greater mechanical strength of self-setting alginate membrane compared with the ready-made alginate membrane. As depicted in Fig. 4, alginate membrane shrinks during its formation, i.e., upon exposure of Na-Alg solution to $CaCl_2$ solution.

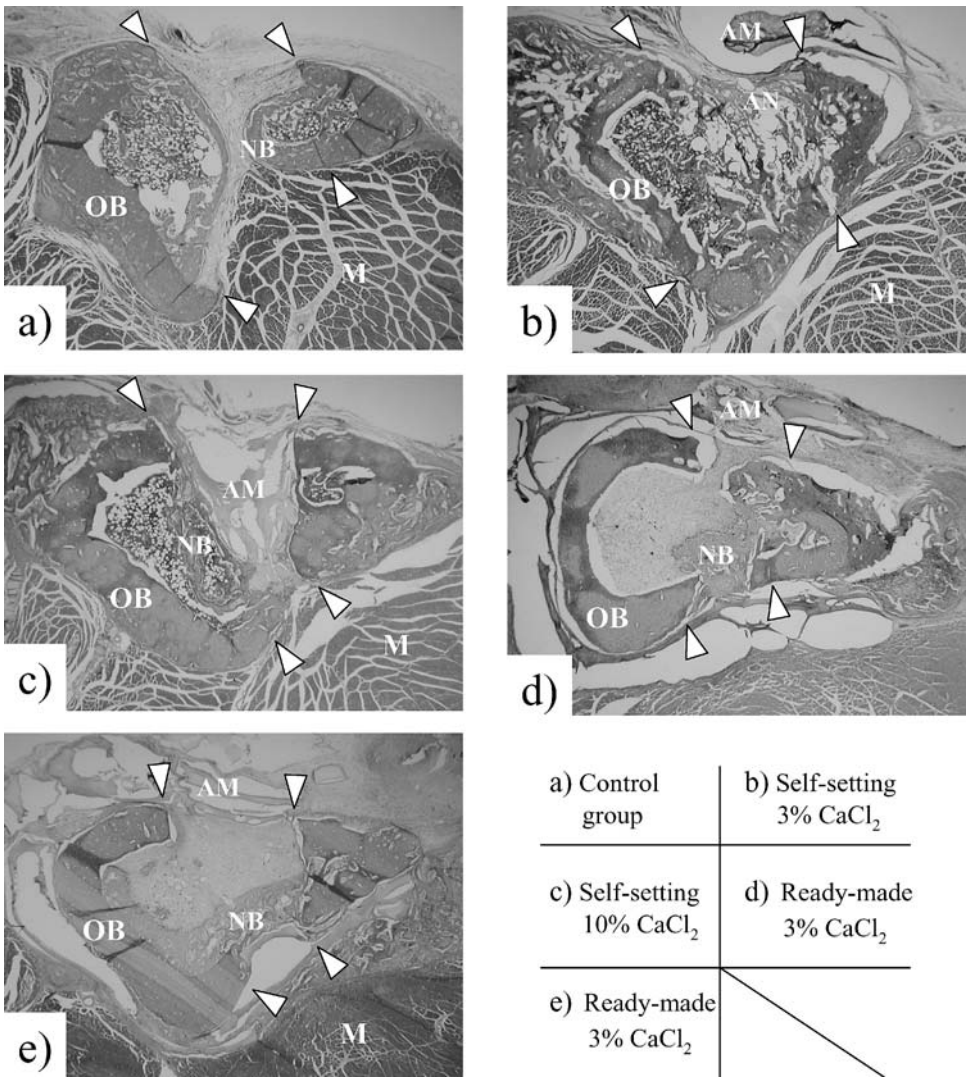

Figure 6 Transverse section of a rat tibia four weeks after operation. Hematoxylin-eosin stain. (a) Control group, (b) Self-setting alginate membrane (3%) group, (c) Self-setting alginate membrane (10%) group, (d) Ready-made alginate membrane (3%) group, (e) Ready-made alginate membrane (10%) group.
Arrow: edge of pre-existing cortical bone, OB: Original Bone, NB: New Bone, AM: Alginate membrane, AN: Sodium alginate, M: Muscle.

The self-setting alginate membrane adhered to the surface of bone; therefore, the membrane was formed with some tension. On the other hand, the ready-made alginate membrane was placed on the bone defect under no tension. It is well known that an extended membrane exhibits greater tensile strength, as is the case for expanded polytetrafluoroethylene (e-PTFE)[8]. In other words, an extended membrane would not experience further extension, whereas a membrane lacking extension treatment would undergo extension upon the application of a tensile load. An additional difference between the self-setting and the ready-made alginate membranes involves the presence of unreacted aqueous Na-Alg solution in the bone defect. In the case of GBR utilizing the self-setting alginate membrane, unreacted aqueous Na-Alg solution remains in the bone defect, which may contribute to retention of the shape of the alginate membrane at least in the initial stage. When GBR was performed with a ready-made alginate membrane, the bone defect would be filled with blood clot. However, the amount of blood clot may be insufficient with respect to retention of the shape of the alginate membrane.

The results obtained in this study demonstrated that unreacted Na-Alg solution in the bone defect contributed to retention of the shape of the alginate membrane. Originally, we anticipated that aqueous Na-Alg solution could delay bone formation, as we believed that the blood clot is superior to Na-Alg solution, at least with respect to factors that are required for bone formation. However, we found bone regeneration of GBR involving the self-setting alginate membrane was not delayed in comparison with that using ready-made alginate membrane. Moreover, more rapid callus formation

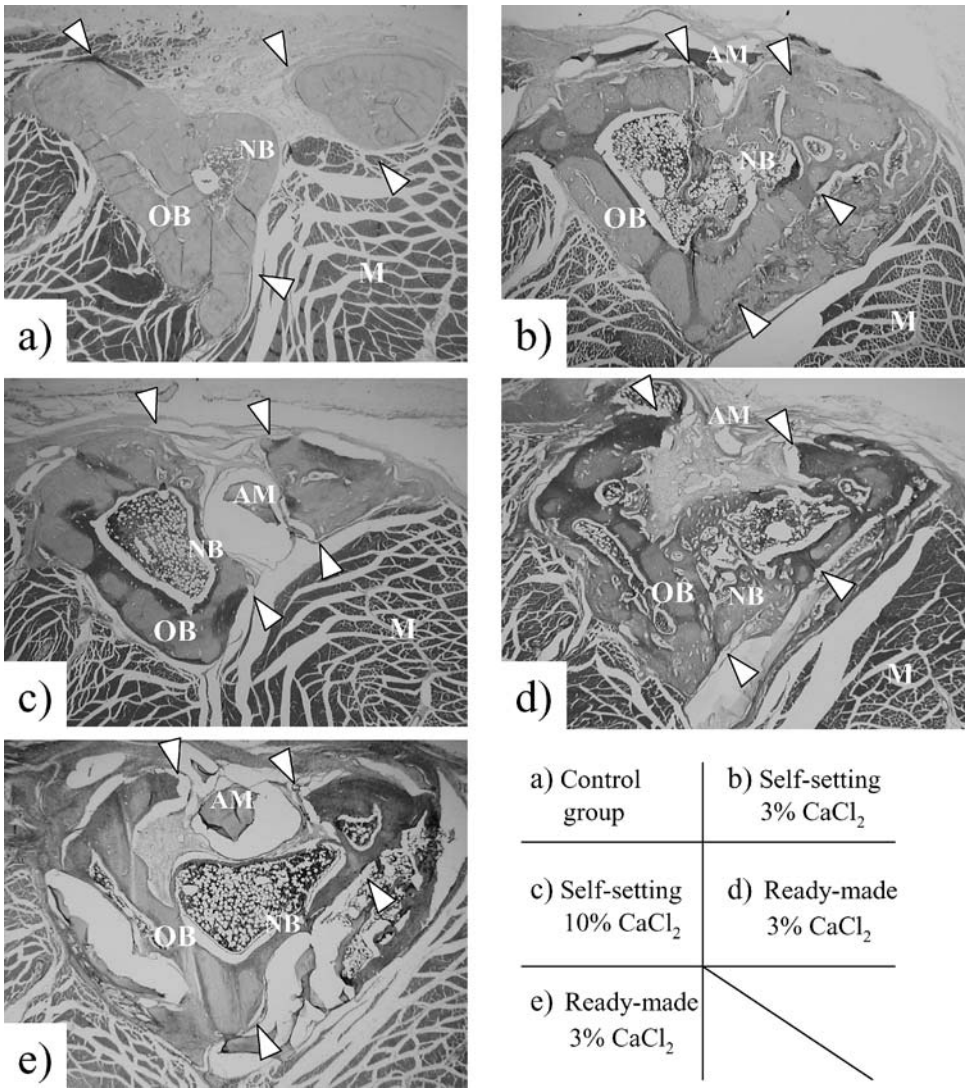

Figure 7 Transverse section of a rat tibia eight weeks after operation. Hematoxylin-eosin stain. (a) Control group, (b) Self-setting alginate membrane (3%) group, (c) Self-setting alginate membrane (10%) group, (d) Ready-made alginate membrane (3%) group, (e) Ready-made alginate membrane (10%) group.
Arrow: edge of pre-existing cortical bone, OB: original bone, NB: new bone, AM: Alginate Membrane, AN: Sodium alginate, M: Muscle.

was observed with Na-Alg nuclei when unreacted Na-Alg remained in the bone defect. Na-Alg is employed in the incubation of liver cells; moreover, it has been applied as a blood expander in the past. Although the detailed mechanism of Na-Alg in terms of bone formation remains unknown, the absence of inhibitory effects on bone formation is a boon for GBR with self-setting barrier membrane.

Although self-setting alginate membrane (3%) is superior to ready-made alginate membrane, it should be noted that preparation conditions for the alginate membrane including the concentration of $CaCl_2$ were important. When aqueous 10% $CaCl_2$ solution was utilized in the preparation of the alginate membrane, no bone regeneration was observed. These findings are explained by the mechanism entailing the interference by the alginate membrane with ingress of connective tissue into the bone defect as well as regeneration of bone in the bone defect. Therefore, regulation of alginate membrane thickness is extremely important. As shown in Fig. 2, the thickness of the alginate membrane increased with increasing $CaCl_2$ concentration. The alginate membrane prepared with 10% $CaCl_2$ solution was too thick; consequently, this situation interfered with regeneration of bone tissue in the bone defect.

We also found that location of the bone defect was one of the factors to predict prognosis of bone regeneration. In the ready-made alginate membrane (10%) group, bone regeneration was observed at the muscle side (Figs. 6(e) and 7(e)). In contrast, connective tissue invaded the bone cavity and prevented bone formation at the skin site. Note that thick alginate membrane obtained by using 10% $CaCl_2$ solution should not be a problem at least with respect to bone formation when the

ready-made alginate membrane is used for GBR since the membrane is placed basically on the outer surface of the bone defect. The difference with respect to these circumstances regarding the bone defect at the muscle and skin sides is due to the fact that the former side faced the muscle whereas the latter side faced the skin. As a result, the membrane can retain its shape with support from muscle at the muscle side. At any rate, retention of the shape of the barrier membrane is an important factor for GBR [6, 7]. The difference of bone regeneration based on the location of bone defect was not observed in the case of self-setting alginate membrane group. This does not mean self-setting alginate membrane is free from such factor, but we could not find the difference using the condition used in the present study.

In conclusion, we found that the self-setting barrier membrane is superior to the ready-made alginate membrane when Na-Alg and $CaCl_2$ concentrations were adjusted correctly. In addition, we found that unreacted Na-Alg in the bone defect did not inhibit bone formation.

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