Tissue responses to anti-washout apatite cement using chitosan when implanted in the rat tibia

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The tissue response to anti-washout apatite cement using chitosan (aw-AC(chi)) was evaluated by implanting aw-AC(chi) into bone defects of rat tibiae using conventional apatite cement (c-AC) as a control material. During the experimental period up to 16 weeks, the only difference between aw-AC and c-AC was found at two weeks in the tissue response of soft tissue. At two weeks, c-AC showed a moderate inflammatory response; small particles of c-AC were scattered in the cutaneous tissue and many foreign body giant cells were collected around the scattered c-AC, whereas aw-AC showed only a slight inflammatory response and few foreign body giant cells. We found no difference between aw-AC(chi) and c-AC with respect to bone tissue response. Both AC were almost completely surrounded by mature bone at eight weeks. No promotion or reduction of osteoconductivity was observed by chitosan even though it is considered to promote bone formation.

We concluded, therefore, that enhancement of bone formation cannot be expected by employing chitosan to obtain anti-washout properties, at least in the concentration used in this study, even though aw-AC(chi) is much more useful than c-AC.

1. Introduction

After the development of apatite cement (AC) by Drs Brown and Chow, many studies have been made to evaluate the tissue response to AC [1–6], to analyze the factors which could influence the setting reaction of AC [7–10], and to improve the properties of AC. For example, anti-washout type AC (aw-AC) is an improved AC with respect to setting time and behavior with body fluid. aw-AC is not washed out but sets in 5 min keeping its original shape even when the paste is immersed in fluid such as distilled water or serum immediately after mixing, in contrast to conventional AC (c-AC) which is washed out completely [11–21]. It is reported that aw-AC is formed by controlling two independent processes which occur when the paste is in contact with liquid; one is the formation of AP which is the key step of the cement setting reaction, the other is the penetration of fluid into the cement paste, which induces washout. For the fabrication of aw-AC, the former property was achieved by using fast-setting AC (fs-AC) as the base cement. fs-AC accelerates the formation of AP, i.e., the setting reaction of AC, by adding neutral sodium hydrogen phosphate in the liquid phase. The latter property is achieved by adding a viscous gel into the liquid phase. Various viscous gels such as sodium alginate, chitosan, collagen or other materials are reported to be effective in reducing the liquid penetration process to cement paste. Prepared in this way, aw-AC is thought to be more useful than previous AC, especially in surgical procedures where complete hemostasis is sometimes very difficult.

Among these viscous gels, chitosan has useful properties with respect to bone regeneration. For example, certain modified chitosans possess properties that promote ordered regeneration of soft tissues and osteoconduction [22–27]. It has been demonstrated *in vivo* that endochondral ossification and direct membranous osteoinduction are induced by chitosan [22–27]. Based on the possible pharmacological effects on bone formation, beside fast setting and anti-washout behavior, aw-AC using chitosan (aw-AC(chi)) could be a useful bioactive cement when used for the reconstruction of bone defects.

In our previous study, aw-AC(chi) and c-AC were implanted subcutaneously in rats immediately after mixing to evaluate the response to soft tissue. One week after implantation, we found c-AC was crumbled and caused a severe inflammatory response whereas aw-AC(chi) was surrounded by thin fibrous tissue with a slight inflammatory response. Therefore aw-AC(chi) is superior to c-AC with respect to soft tissue response [18].

In this investigation, to evaluate the feasibility of aw-AC(chi) as a bioactive cement, aw-AC(chi) was

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implanted in rat tibiae and the tissue reaction was examined histopathologically up to 16 weeks using c-AC as a control material.

2. Materials and methods

2.1. Cement powders and liquids

The powder phase of both AC was prepared as described previously [7–9, 11–13, 19, 28, 29]. In brief, an equimolar mixture of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) was mixed using a speed mill (SK-M2, Kyoritsuriko, Tokyo, Japan). The mixture was sterilized by exposure to 20 kGy of gamma radiation and kept in a vacuum desiccator at 60 °C until use. For the liquid phases, c-AC used carbon-free double distilled water. For aw-AC, 0.5% chitosan (PC-100, Ajinomoto, Tokyo, Japan) containing 0.2 mol/l neutral sodium hydrogen phosphate ($Na_{1.8}H_{1.2}PO_4$) solution was used as the liquid phase [13, 18]. The solutions were sterilized by filtration through 0.22 μm Millex-GS filter assemblies (Millipore Corp., Bedford, MA, USA).

2.2. Animals and implantation procedure

Twelve week-old male rats of the Wistar strain, obtained commercially (Charles River, Japan) and fed standard pellets and water *ad libitum*, were used for the implantation study.

The rats were anesthetized by i.p. injection of sodium pentobarbital (Nembutal®, Abbott Co., Chicago, IL). The legs were shaved and infiltration anesthesia with 0.6 ml of 2% lidocaine-epinephrine solution (Xylocaine®, Fujisawa Pharmaceutical Co., Osaka, Japan) was applied around the medial end of the tibia to arrest the bleeding from bone marrow and to control early postoperative pain. The medial end of the tibia was exposed, and a 4 × 6 mm bone cavity was formed with a dental round bur (Fig. 1). The powder phases of ACs were mixed with the liquid phases with a powder to liquid (P/L) ratio of 3.5 on a glass using a spatula for 20 s. The cement paste thus prepared was packed with a dental cement condenser in the cavity within 1 min after mixing. The lower half of the rat's body was elevated with a belt for one week after the implantation to prevent fracture of the leg.

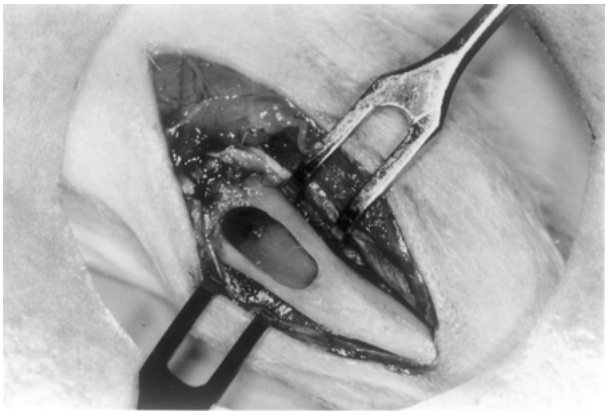


Figure 1 A cavity is made in the rat tibia with a dental round bur, and the bone marrow and cancellous bone are almost totally removed.

2.3. X-ray diffraction analysis

The compositions of cements were analyzed using powder X-ray diffraction (XRD) [7–13, 15–21, 28]. After the specimens were removed from the rat tibiae at 2, 4, 8 and 16 weeks after implantation, they were immediately immersed in liquid N_2. They were then lyophilized and freeze-dried (Automatic Freeze-Dryer 10–010, Virtis Co., Gardiner, NY). In this manner, the conversion of AC into AP was stopped at specific times. The freeze-dried samples were ground to fine powders and characterized by XRD. The XRD patterns of the specimens were recorded with a vertical–mounted diffractometer system (ADG-301, Toshiba, Tokyo, Japan) using Ni-filtered CuKα radiation ($\lambda = 0.1540$ nm) generated at 30 kV and 16 mA. The samples were first scanned from 3° to 60° 2θ (where θ is the Bragg angle) to determine the reaction products in continuous mode (1.0° 2θ/min, time constant 2 s) on a strip-chart recorder and digital recorder (Thermodac E, Eto Denki, Tokyo, Japan).

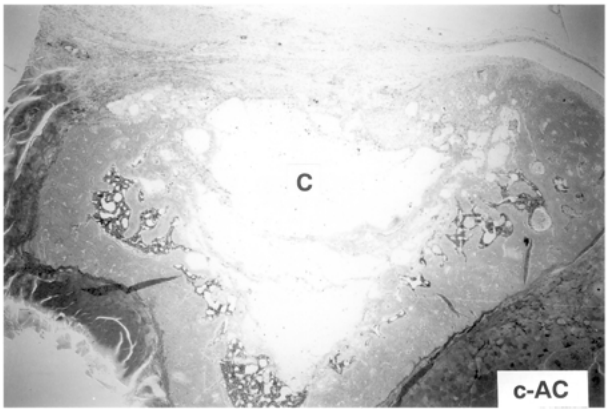
2.4. Histological preparations

Tibiae, containing c-AC or aw-AC(chi), were removed from four rats each at 2, 4, 8 and 16 weeks after surgery. The specimens, each comprising the tibia with surrounding tissue, were fixed in 10% neutral buffered formalin, demineralized in 10% ethylenediamine tetraacetic acid (EDTA) at 4 °C, and embedded in methylmethacrylate (HistoDur® Leica Co., Nussloch, Germany). After polymerization, thin serial sections, 4 μm thick were cut using a rotary microtome (RM 2065, Leica Co., Nussloch, Germany). The sections were stained with hematoxylin-eosin and investigated by light microscopy.

3. Results

Fig. 2 shows transverse sections of c-AC and aw-AC(chi) two weeks after surgery. New bone formation was not observed along the cement at the edge of the pre-existing cortical bone regardless of the type of AC, i.e., aw-AC(chi) or c-AC. Fig. 3 shows a longitudinal section of aw-AC(chi) two weeks after surgery. Along the cement surface in the bone marrow, new bone, like a dentin bridge, was observed. The dentin-bridge-like new bone covered most of the cement surface in the case of c-AC. The area of cement surface covered with dentin-bridge-like new bone was relatively smaller in the case of aw-AC(chi) compared with c-AC. No inflammatory reaction was seen in the bone marrow and no fibrous soft tissue was observed between the cement and new bone. Fig. 4 shows the interface between cement and soft tissue. Small particles of c-AC were scattered in the cutaneous tissue and many foreign body giant cells were collected around the scattered c-AC (Fig. 4a). In contrast, aw-AC(chi) was surrounded by thin fibrous tissue with a slight inflammatory response and few foreign body giant cells were observed (Fig. 4b).

Fig. 5 shows the transverse sections of c-AC and aw-AC(chi) four weeks after surgery. At four weeks, new bone formation was observed along the cement at the edge of the pre-existing cortical bone in both aw-AC(chi)



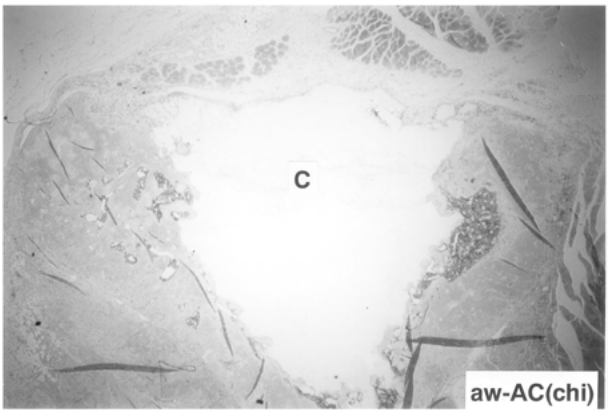
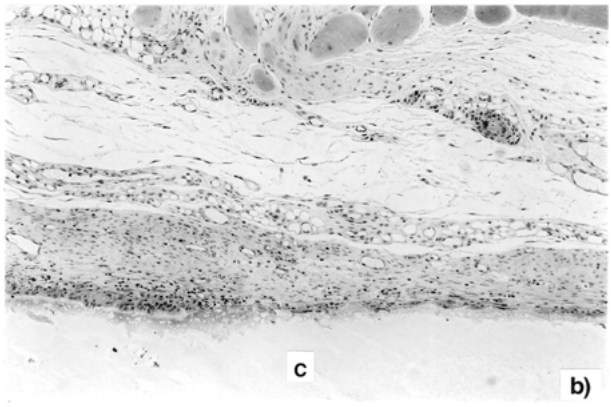


Figure 2 Transverse sections of rat tibia containing c-AC and aw-AC(chi) two weeks after implantation. No new bone formation along the cement is observed extending from the edge of the pre-existing cortical bone. (C: Cement, Original magnification × 18, Hematoxylin-eosin stain.)



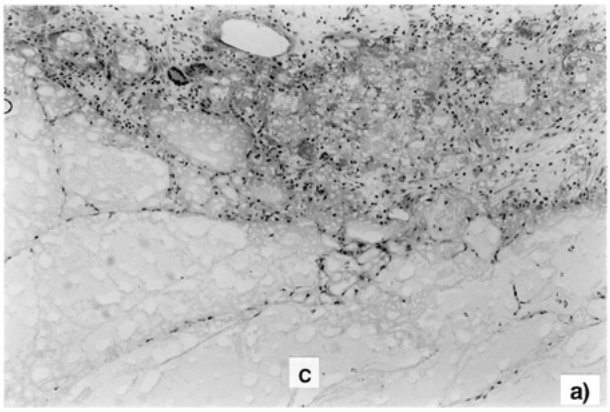


Figure 4 Histologic appearance of the soft tissue apposed to cements two weeks after implantation. (a) c-AC and (b) aw-AC(chi). (C: Cement, Original magnification × 90, Hematoxylin-eosin stain.)

and c-AC. Longitudinal sections of c-AC and aw-AC(chi) obtained four weeks after surgery were similar to those obtained at two weeks (data not shown). Fig. 6 shows the interface between the cements and cutaneous tissue four weeks after surgery. No difference was observed in the soft tissue response to the cements. Both cements were covered with thin fibrous tissue with a slight inflammatory response and few foreign body giant cells were observed.

Fig. 7 shows a transverse section of aw-AC(chi) eight weeks after surgery. At eight weeks, the cement was almost completely surrounded by mature bone in some

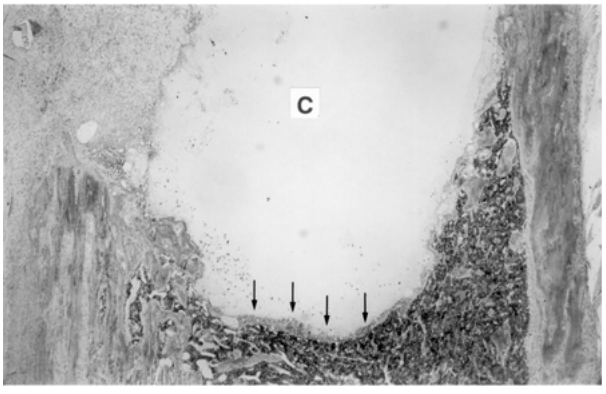
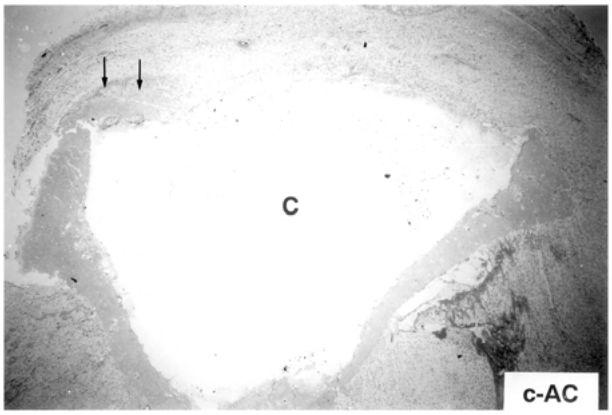


Figure 3 Longitudinal section of rat tibia containing aw-AC(chi) two weeks after implantation. New bone (arrows) like a dentin bridge is formed along the cement surface in the bone marrow. No inflammatory reaction is seen in the bone marrow. (C: Cement, Original magnification × 18, Hematoxylin-eosin stain.)



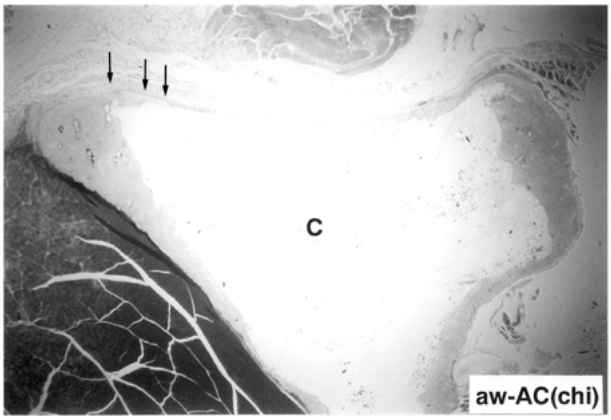


Figure 5 Transverse sections of rat tibia containing c-AC and aw-AC(chi) four weeks after implantation. Elongated new bone (arrows) has formed along the cement from the edge of the cortical bone. (C: Cement, Original magnification × 15, Hematoxylin-eosin stain.)

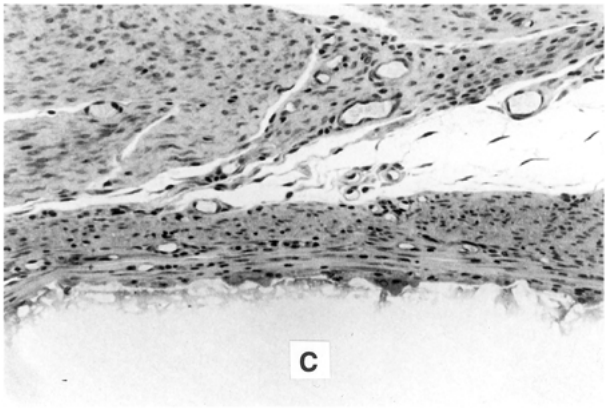


Figure 6 Histologic appearance of the soft tissue apposed to aw-AC(chi) four weeks after implantation. (C: Cement, Original magnification × 90, Hematoxylin-eosin stain.)

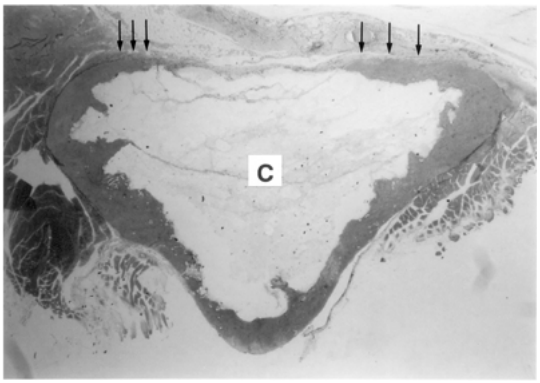


Figure 7 Transverse section of rat tibia containing aw-AC(chi) eight weeks after implantation. The cement is almost totally surrounded by mature bone (arrows) extending from both edges of the pre-existing cortical bone. (C: Cement, Original magnification × 25, Hematoxylin-eosin stain.)

places. No difference was observed in the amount mature bone due to the type of AC. No inflammatory response was observed in the bone marrow (data not shown).

Fig. 8 shows a transverse section 16 weeks after surgery. New bone covered most of the cement surface. No difference was observed due to the type of c-AC. Fig. 9 shows a longitudinal section section 16 weeks after surgery. At 16 weeks, abundant new bone, like a bridge,

Figure 8 Transverse section of rat tibia containing aw-AC(chi) 16 weeks after implantation. New bone (arrows) covers most of the cement surface. (C: Cement, Original magnification × 22, Hematoxylin-eosin stain.)

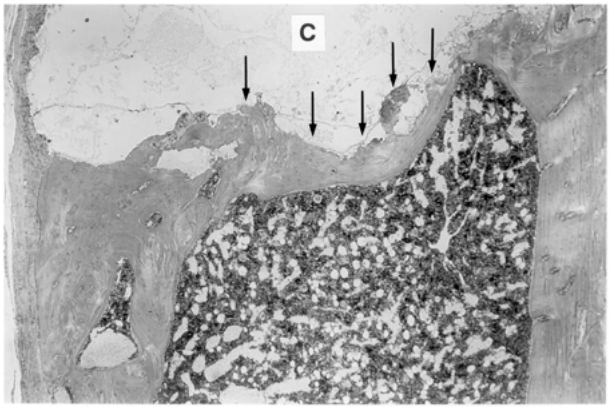


Figure 9 Longitudinal section of rat tibia containing aw-AC(chi) 16 weeks after implantation. Abundant new bone (arrows) like a bridge is formed along the cement in the bone marrow. (C: Cement, Original magnification × 19, Hematoxylin-eosin stain.)

was formed along the cement in the bone marrow when compared with that obseved at eight weeks. Fig. 10 shows the XRD patterns of aw-AC(chi) and c-AC,2,4,8 and 16 weeks after implantation in rat tibiae. In this figure the powder phase of AC, an equimolar mixture of TTCP and DCPA, and poorly crystallized AP is presented for compasison. At two weeks, although the dominant component of the set mass was AP, some TTCP still remained unreacted in both cements, i.e. c-AC and aw-AC(chi). At four weeks, the XRD patterns were similar to those at two weeks even though the amount of unreacted

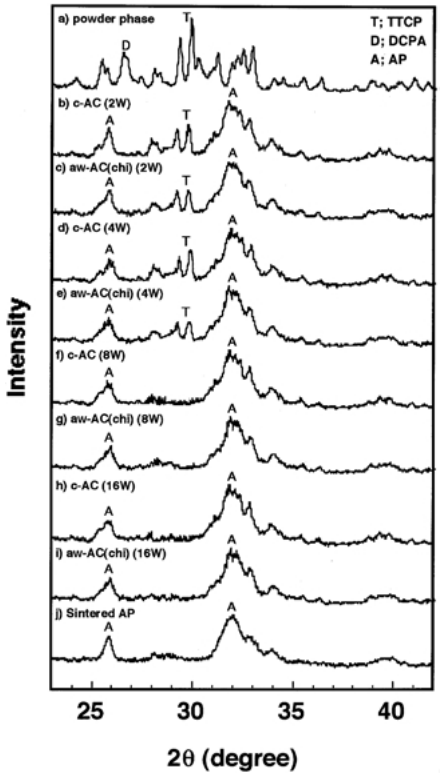


Figure 10 Powder XRD patterns of the c-AC and aw-AC(chi), 2, 4, 8 and 16 weeks after implantation, the powder phase of the cement, and of sintered AP for comparison. (a) Powder phase of cement, i.e., an equimolar mixture of TTCP and DCPA; (b) c-AC two weeks after implantation in rat tibia; (c) aw-AC(chi) two weeks after implantation; (d) c-AC four weeks after implantation; (e) aw-AC(chi) four weeks after implantation; (f) c-AC eight weeks after implantation; (g) aw-AC(chi) eight weeks after implantation; (h) c-AC 16 weeks after implantation; (i) aw-AC(chi) 16 weeks after implantation; (j) Sintered AP.

TTCP was reduced when compared to those obtained after two weeks. At eight weeks, the amount of unreacted TTCP was significantly smaller than that at four weeks. In other words, the transformation to AP was almost complete within eight weeks. At 16 weeks, the XRD patterns were the same as those obtained at eight weeks.

4. Discussion

Miyamoto *et al*. reported severe inflammatory response one week after implantation when c-AC was implanted subcutaneously in rats immediately after mixing, whereas fs-AC or aw-AC using sodium alginate, aw-AC(Alg), caused no inflammation [19]. One of the key differences between c-AC and fs-AC or aw-AC is the mechanical strength at the initial stage. c-AC takes a long time to show mechanical strength whereas fs-AC and aw-AC show some mechanical strength from the initial stage. When a cement paste is implanted subcutaneously, the paste is subject to pressure from the covering skin. In the case of c-AC, the paste crumbled since in the initial stage it has no mechanical strength to resist this pressure. In contrast, fs-AC or aw-AC have much higher mechanical strength in the initial stage and can maintain their original shape at implantation while setting. Crumbling of the c-AC paste could present a serious problem even where the cement is used to fill a bone defect in which the cement is considered to be subject to low mechanical stress. Ueyama *et al*. compared c-AC and aw-AC(Alg) in the reconstruction of premaxilla of rats where complete haemostasis is very difficult, and found that particles of c-AC flowed out and many giant foreign cells appeared around the particles [21]. As a result, an inflammatory response was found surrounding the c-AC leading to poor bone regeneration. In contrast, in the case of aw-AC(Alg) no inflammatory response was observed in the surrounding areas of bone and the bone defect was covered with new bone. In our experimental conditions, complete haemostasis was not so difficult and thus the cement paste did not flow out even in the case of c-AC. However we found cement particles had been scattered in the cutaneous tissue and many foreign body giant cells were collected around the scattered particles in the case of c-AC two weeks after surgery. Since we filled a bone defect with AC, the stress to AC should have been much smaller than the case where cement paste is implanted subcutaneously. The scattered particles may have been formed due to rubbing of the cement paste by the covering skin. It should be noted the bone defects we made in the rats were not so large. There is a good chance that c-AC will cause a severe inflammatory response even when used to fill bone defect if the defect is large since a larger number of particles will flow out due to rubbing by the skin.

Although aw-AC(chi) showed a better soft tissue response than c-AC at two weeks after implantation, we found no promotion of bone formation when compared with c-AC, at least within our experimental periods. As stated previously one of the reasons we chose a chitosan as an additive to form aw-AC is its pharmacological effect for bone regeneration [22–27]. Chitosan may not have sufficient pharmacological effect to regenerate bone in the amount used to prepare aw-AC. The concentration of chitosan in the liquid phase is 0.5%, and thus was calculated to be as low as 0.14% in the set AC since the powder to liquid mixing ratio was 3.5. Such small amounts of chitosan may not have sufficient pharmacological effect to be observed in histological specimens. If bone regeneration can not be promoted by chitosan, there may be a better additive for the preparation of aw-AC. Many additives have been proposed to yield effective anti-washout properties [11, 13, 17, 30–32]. The mechanical strength of the set mass, setting time, and injectability of the cement paste are all governed by the type of additive. Further study is awaited to determine the best additive for the fabrication of aw-AC.

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