J Mater Sci: Mater Med (2015) 26:271
DOI 10.1007/s10856-015-5612-2

BIOCOMPATIBILITY STUDIES

Original Research

In vivo study of the early bone-bonding ability of Ti meshes formed with calcium titanate via chemical treatments

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Received: 17 July 2015 / Accepted: 24 October 2015 / Published online: 29 October 2015

Abstract Alkali and heat (AH) treatment forming sodium titanate has been shown to connect bioinert Ti metal and bone tissue. Artificial joints treated with this method have achieved extensive clinical application. Recently a new chemical treatment of Alkali-Calcium-Heat-Water (ACaHW) treatment forming calcium titanate was proposed. Notably, the apatite-forming ability of this treatment is greater than that of AH treatment, as verified in vitro. However, the early bone-bonding abilities of the two treatments have not been compared in vivo. To simulate clinical application, we treated a commercially pure Ti (Cp-Ti) mesh implant with AH or ACaHW. Then, using mechanical and histological methods, we compared the bone-bonding abilities of the two treatments early during the implantation process (2–4 weeks); untreated Cp-Ti mesh was used as a control. Because the mesh structure might influence bone-bonding ability, we compared these bonding abilities with values obtained at 4 and 8 weeks using a Cp-Ti implant with a plate structure. In the mesh group, histological comparisons at 2 and 3 weeks indicated that ACaHW treatment resulted in a bone-bonding ability similar to that of AH treatment; ACaHW exhibited a greater bonding ability than AH at 4 weeks. However, in tests of the plate group at later time points, such differences were not apparent. The results obtained here indicate that during the early stage of embedment, ACaHW treatment of Cp-Ti mesh implants yields a higher bone-bonding ability than AH treatment, thus providing a positive reference for future clinical applications.

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1 Introduction

In cementless artificial joints, greater early bonding between the implant and bone tissue has always been pursued in clinical treatment. Good early bonding allows earlier weight-bearing movement and effectively shortens the hospitalization period. Therefore, some methods use a mesh or porous structure in artificial joints to enhance the bonding force. Artificial joints using an open, porous mesh structure are effective in practical clinical applications; not only do they realize a closer bonding between the implant and the bone, but they also favor long-term survival and the prevention of osteolysis [1–3]. However, metal implants are not bioactive; thus, for implants with a mesh structure, good osseointegration requires not only physical bonding through bone ingrowth but also chemical bonding between the material surface and the bone tissue [4, 5]. Some researchers have applied a hydroxyapatite (HA) coating to the surface of artificial joints with mesh structures, thus providing a strong bone-bonding force and osseointegration with strong durability [6]. However, some reports have noted that the high-temperature treatment that is used to apply the HA powder to the implant surface via a plasma spray often melted the HA, thus weakening its properties; other reports have shown that the spray method does not uniformly distribute HA onto the material, also weakening the overall bonding effect.

Therefore, many chemical treatment methods have been proposed to improve the bone-bonding ability of such

implants. In 1996, a relatively simple and effective alkali and heat (AH) surface treatment method was proposed by Kokubo [7–11]. After AH treatment, a bone-like apatite layer forms on the surface of metal materials in a simulated body fluid (SBF) and body environment [12–14], thus improving the bioactivity of the implant. In clinical applications, AH-treated artificial joints exhibit good early and long-term bone-bonding results. For example, a joint-bone gap that was observed 2 weeks after an operation was well repaired after 1 year and completely disappeared after 6 years [15, 16]. Unfortunately,When AH treated implant was kept at high humidity, the apatite forming ability of Ti metal decreased. In addition, even though AH treatment could be implemented on traditional Ti alloy such as Ti–6Al–4V, it was hard to form a apatite layer on the surface of new Ti alloy like Ti–Zr–Nb–Ta, because Zr, Nb, Ta were hard to release by AH treatment [20]. In addition, Kizuki et al. reported that the concentration of the NaOH solution used in the AH treatment greatly affects the resulting apatite-forming ability. Among commercially available NaOH solutions, even impurities present at concentrations of less than 0.0005 % result in solutions containing some Ca^{2+} and other ions that can greatly impact the exchange of Na^{+} ions [18]. In order to solve the inconvenient behaviors mentioned above, an alternative method instead of AH treatment was developed, known as ACaHW treatment [17]. In ACaHW treatment's process, Ca^{2+} ions took place of Na^{+} ions in the sodium hydrogen layer and formed a calcium hydrogen titanate layer after soaking in aqueous 5 M NaOH and followed by soaking in 100 mM of $CaCl_2$ solution. After heating at 600 °C under air atmosphere and then soaking in hot water, Ca^{2+} ions in the calcium titanate were partially replaced with H_3O^{+} ions. Thus, ACaHW treated Ti metal in SBF is able to release Ca^{2+} ions from the calcium titanate via exchange with H_3O^{+} ions in the surrounding fluid and Ti–OH groups was formed on the surface of Ti metal. This negatively charged Ti–OH group was combined with positively charged Ca^{2+} ions. Positive charged Ti surface combined with negatively charged phosphate ions in SBF came to form a calcium phosphate layer and finally changed to a bone-like apatite layer. In addition, lower mobility of Ca^{2+} ions than that of Na^{+} in titanate leads to hard decreased Ca content in moisture environment. As a result, the apatite forming ability of ACaHW treated Ti metal and its alloys could be kept even in a humid environment [19, 20]. Release of Ca^{2+} ions from calcium titanate layer increased the ionic activity more effectively than Na^{+} ions in SBF,this is also the reason of better apatite forming ability of ACaHW treatment than AH treatment. Nishio indicated that apatite provides favorable conditions for the differentiation of bone marrow cells [21]. Therefore, we hypothesized that ACaHW-treated metal implants would exhibit greater apatite-forming abilities, improved cell adhesion abilities and, therefore, stronger and more stable bone-bonding abilities versus AH treatment. However, the in vivo bone-bonding abilities of AH and ACaHW treatments at early stages (earlier than or equal to 4 weeks) have not been compared.

The purpose of this study was to simulate the treatment of artificial joints by applying AH or ACaHW to the surface of a Cp-Ti mesh and to observe the bonding properties at 2, 3 and 4 weeks after implantation in rabbit tibias. Second, a Cp-Ti plate was used to eliminate the influence of surface morphology of implant on bonding properties, and the bone-bonding abilities of AH and ACaHW treatments were also compared at 4 and 8 weeks. We expected that during early implantation, ACaHW treatment would yield similar or even improved bonding performance over that of the current AH treatment, thus providing a reference for future clinical applications.

2 Materials and methods

2.1 Materials

This study examined two groups. Group 1 (the mesh group) was used to simulate actual clinical cases. Mesh-structured implants were used, and the implant surfaces were treated with various chemicals, rendering them bioactive. The early-phase bone-bonding abilities of these treatments were compared in vivo at 2, 3, and 4 weeks. In group 2, Cp-Ti plates were used (the plate group). The plates were chemically treated as with group 1. Differences in the bone-bonding abilities between the mesh and plate groups were evaluated. In the mesh group, commercially pure Ti meshes were used. The diameter of the metal wire used in the mesh was 0.25 mm, and the distance between wires was 1 mm. We pressed 13 layers of Ti net together, and each layer was alternately turned 45° (Fig. 1a). The layers were then cut into a cuboid specimen of 10 × 15 × 2 mm^3 (Teijin-Nakashima Medical Co., Ltd. Japan). For the plate group, commercially pure titanium (Cp-Ti) was used (Ti >99.5 mass%, Teijin-Nakashima Medical Co., Ltd., Japan). The plate was polished using a No. 400 diamond plate; washed consecutively with acetone, 2-propanol and ultrapure water in an ultrasonic cleaner for 30 min; dried at 40 °C; and then pulverized. Finally, a specimen of the same size (10 × 15 × 2 mm^3) as the mesh group was constructed.

The mesh group was further divided into three sub-groups, to which different surface treatments were applied. Samples in sub-group 1 were treated using AH (positive control). Implants were soaked in aqueous 5 M NaOH at 60 °C for 24 h (alkali treatment). After removal from the solution, the implants were gently rinsed with ultrapure

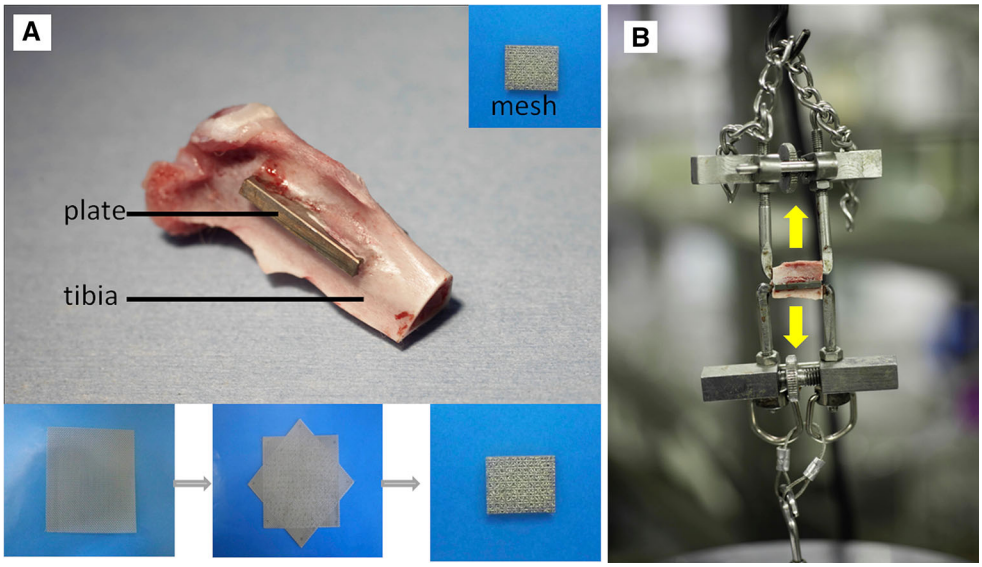

Fig. 1 **a** Plate (or mesh) was implanted parallel to the long axis of the tibia. The *arrow* shows the mesh implant manufacturing process. **b** *Yellow arrows* show the direction of the force acting on the interface between the implant and the tibia in the detaching test (Color figure online)

water for 30 s and then dried at 40 °C for 24 h in air. Next, the implants were heated to 600 °C at a rate of 5 °C min^{-1} in an electrical furnace in air and maintained at that temperature for 1 h, followed by natural cooling. In a previous study, apatite-forming ability was tested after a 3-day SBF soak, and the bioactivity provided by the AH treatment was confirmed [7–9, 14]. Samples in sub-group 2 were treated using ACaHW. Implants were first soaked in 10 ml of aqueous 5 M NaOH at 60 °C for 24 h (alkali treatment). After removal from the solution, the implants were gently rinsed with ultrapure water for 30 s and then dried at 40 °C. The plates were subsequently soaked in 20 ml of 100 mM $CaCl_2$ at 40 °C for 24 h and then washed and dried in a similar manner. Next, the implants were heated to 600 °C at a rate of 5 °C min^{-1} in an electric furnace in air and maintained at that temperature for 1 h, followed by natural cooling. The implants were then soaked in 20 ml of ultrapure water at 80 °C for 24 h and then washed and dried (ACaHW treatment). Apatite-forming ability was tested after a 1-day SBF soak, as described in the previous study; more apatite was observed at the surface of the ACaHW-treated sample than at the surface of the AH-treated sample [17]. Sub-group 3 comprised the control group without any chemical treatment. For the plate group, we processed samples with AH and ACaHW treatments as described for the mesh group. Untreated Cp-Ti was also used as a control.

2.2 Animal studies

The experiments were conducted on male rabbits weighing 2.3–3.5 kg (Japan SLC, Inc., Shizuoka, Japan). For acclimation, the rabbits were transported to a feed center and fed individually 2 weeks prior to the experiment. Following a previously described surgical method [9], 1.3 ml of pentobarbital was injected as anesthetic; then, the epidermis covering the bilateral lower limbs was stripped. The rabbit was fixed to the surgical table, and lidocaine was injected on the inner side of the bilateral tibias to provide local subcutaneous anesthesia. Simultaneously, isoflurane-inhalation anesthesia was initiated and sustained throughout the operation. A 3-cm vertical incision parallel to the long axis of the tibia was made on the inner skin of the tibia. The cut was deepened to reveal the tibia, and a 2 × 16-mm incision was made on the tibia surface using a dental burr. The long axis of the implant was placed in the incision (Fig. 1a), and the skin was subsequently sutured. To obtain objective results, different materials were placed in the bilateral tibias of each rabbit. After surgery, the rabbits were individually housed as before. The Kyoto University guidelines for animal experimentation were observed in this study. Eighteen rabbits were used in the mesh group (4 plates of each type per implantation) and 16 rabbits were used in the plate group (8 plates of each type per implantation).

2.3 Mechanical experiments

At 2, 3, 4 and 8 weeks, an intravenous injection of 5 cc of pentobarbital was used to euthanize the rabbits. The tibia segments containing the specimens were removed, and the osseous tissues around the metal specimens were removed using a dental burr. The specimens were maintained in a wet condition throughout the process. A detachment test was conducted as previously described [22]. Load-testing

equipment was used to hook the upper and lower ends of the tibia in a vertical orientation (Fig. 1b) (model 1310VRW; Aikoh Engineering Co. Ltd., Nagoya, Japan). The pull rate was maintained at a crosshead speed of 35 mm per minute. When either end detached from the specimen, the experiment was considered complete. The momentary force (failure load) was recorded as the bonding force between the implant and the bone.

2.4 Histological examination

The tissue specimens were first soaked in 10 % phosphate-buffered formalin for 2 weeks and then fixed consecutively in 70, 80, 90, 99, 100, and 100 vol% ethanol (3 days in each solution). The specimens were then embedded in polyester resin, fixed, and cut into 1000-μm-thick slices using a band saw (BS-3000CP, Exact-Apparatebau, Norderstedt, Germany). Each specimen was polished with #180, #400, #800, #1200, #2000, and #4000 sandpaper using a grinding-sliding machine (Microgrinding MG-4000, Exact-Apparatebau, Norderstedt, Germany) to a final thickness of 50 μm. Finally, the specimens were stained using Stevenel's blue and Van Gieson's picrofuchsin. All stained specimens were evaluated using a digital microscope (DSX 500; Olympus, Tokyo, Japan) [23].

2.5 Bone-bonding calculation

To histologically evaluate bone-bonding further, we determined the bone area (BA), bone-implant contact rate (BIC) and normalized bone-implant contact rate (NBIC) of every specimen:

$$\text{Bone area}\,(\%) = \frac{\text{Bone area in whole implant}}{\text{Whole implant area}} \times 100$$

$$\text{Bone-implant contact}\,(\%) = \frac{\text{Length of bone}-\text{implant contact}}{\text{Perimeter of implant}} \times 100$$

$$\text{Normalized bone-implant contact}\,(\%) = \frac{\text{Length of bone}-\text{implant contact}}{\text{Bone area in implant}} \times 100$$

We calculated the bone area (BA) of the entire implant to evaluate bone ingrowth differences between the applied treatments. However, bonding between implant material and bone tissue is affected both by mechanical bonding due to bone ingrowth and by chemical bonding. We evaluated the bonding performance between the implants and bone tissue. We divided the entire sample by the longitude (5) multiplied by the transverse (20); thus, the material was equally divided into 100 sections. Then, we calculated the bone-implant contact (BIC) of the 3 × 3 area in the lower right corner, i.e., 9 squares (a 9-section area) (Fig. 2). We uniformly observed the lower-right corner because we found considerable bone tissue in this area in all specimens. Moreover, the mesh group was studied to simulate applications involving artificial joints, in which bone ingrowth would occur on the outer side of the implant in the same direction. Therefore, calculations based on this region are appropriate for simulating clinical situations.

Due to the irregular cross-section of the mesh structure, the mesh areas in the 9-section area of each specimen were not identical. The use of a greater number of mesh implants in the 9-section area provided a larger bonding base for the bone tissue. In specimens with less mesh in the 9-section area, little bonding with the bone tissue was observed. Thus, the random distribution of mesh inevitably affected the authenticity of the BIC calculation. Therefore, we added a calculation to normalize for bone-implant contact (NBIC) in the same 9-section area. The equation used to calculate NBIC did not include mesh data, thus eliminating the influence of the random mesh distribution.

The image was binarized using an automated computing procedure. Differences in luminance indicate the location of the material, and the area of the red-colored bone tissue was calculated based on differences in color separation (red, green, and blue). Due to the non-uniform distribution of the material in the area, the normalized bonding rate was added to eliminate the influence of the material on the calculation.

2.6 SEM analysis

The mesh-tissue interface was analyzed using a scanning electron microcope (SEM) with backscattered electrons mode afer coating with platium. The thickness of coating was 25–30 nm.(S-4700, Hitachi Co. Tokyo, Japan). The accelerating voltage was 15 kV. While making the sample, if there was no direct contact between the material and bone tissue, the interface between them would be filled with resin. Therefore we judged whether there was direct contact between the implant and bone tissue by judging whether there was a dark grey region of resin between the white area as "Ti" and light gray area as "bone". If "Ti" and "bone" are connected with no dark gray resin area in between, then we can judge that there was a "direct contact" between "Ti" and "bone", while if there was dark gray resin area between them, we judged that a gap existed. A black crack line often appeared in sample cutting process and SEM observation and could hardly be avoided, but it had no influence on judging whether there was a gap between "Ti" and "bone".

2.7 Statistical analyses

All data were recorded as means ± standard deviations (SDs). The results of the experiment were analyzed using

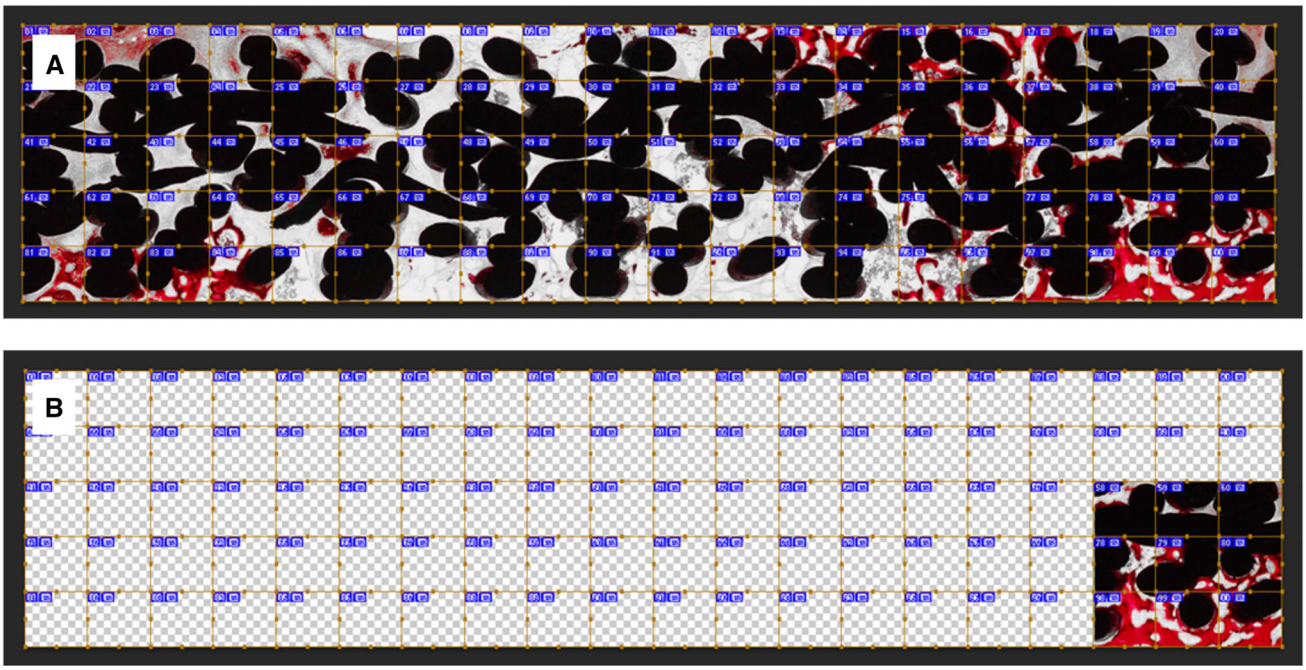

Fig. 2 The material was divided equally into 100 squares (**a**); the *right*, *bottom* 3 × 3 corner 9-section area was used to calculate bone area, bone-implant contact and normalized bone-implant contact (**b**)

one-way ANOVA analyses followed by Tukey–Kramer multiple comparison post hoc tests (SPSS v.19) to determine the statistical significance of differences. Differences were considered significant when $P < 0.05$.

3 Results

3.1 Detaching test (failure load)

Figure 3a shows the failure load of the mesh group 2 weeks after implantation. No significant difference was observed between the ACaHW-treated group (48.5 ± 6.8 N), the AH group (47.4 ± 17.8 N) and the control group (56.8 ± 4.4 N) (*P* values are presented in the figure). After 3 and 4 weeks, almost all ruptures occurred in bone tissues rather than at the interface between the implant material and the bone tissues. In other words, even at failure loads sufficient to cause bone-tissue rupture, the implant material and the bone tissues remained tightly connected. Thus, the bonding strength could not be measured.

For the plate group, as shown in Fig. 3b, the failure load of the AH-treated group increased from 4 to 8 weeks (7.82 ± 4.85–16.96 ± 5.37 N, respectively). The same trend was also observed in the ACaHW group (6.65 ± 3.34–19.80 ± 10.49 N at 4 to 8 weeks, respectively). As the data shows, the effects of the surface treatments did not differ, but the failure loads of the samples subjected to chemical treatment were significantly higher than that of the control group (the failure load of the control was 0.17 ± 0.05 N at 4 weeks and 0.63 ± 0.32 N at 8 weeks). The *P* value for AH versus the control was 0.01 at 4 weeks and 0.004 at 8 weeks. The *P* value for ACaHW versus the control was <0.01 at 4 weeks and 0.012 at 8 weeks (for additional *P* values, please see Fig. 3b).

3.2 Histological results

3.2.1 Mesh group

Based on general observations, all bone tissues grew into the meshes after 2 weeks, regardless of the treatment used. However, the bone tissues on either side of the mesh did not continuously connect (Fig. 4a–c). As the specimens observed at 3 weeks (Fig. 4d, f) and 4 weeks (Fig. 4g–i) show, bone tissue continued to grow into the mesh over time. Bone tissues on top of and underneath the implant were bonded together in most samples, and a large amount of bone tissue appeared in the center of the specimens. After extended observation at 4 weeks, the AH and ACaHW groups presented numerous direct contacts between the bone tissue and the implants. In contrast, for the control group, fibrous tissue commonly intervened at the interface between the bone tissues and the implant, and little BIC was observed, indicating that in the control group, the bone tissue and the implants failed to connect (Fig. 5a–c).

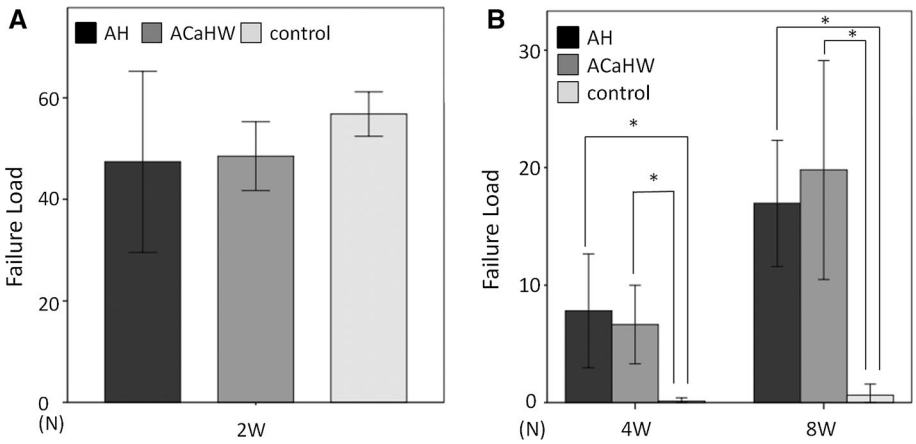

Fig. 3 **a** Failure load in the detaching tests of the mesh group at 2 weeks after implantation (*error bars* indicate standard deviations); the *P* values of differences between the AH and ACaHW, AH and control, and ACaHW and control groups are 0.995, 0.712, 0.763, respectively, indicating that there were no significant differences between the 3 groups. **b** Failure load in the detaching tests of the plate group at 4 and 8 weeks after implantation (*error bars* indicate standard deviations). The *P* values of differences between the AH and ACaHW groups are 0.187 at 4 weeks and 0.435 at 8 weeks. The *P* values of differences between the AH and control groups are 0.01 at 4 weeks and 0.004 at 8 weeks. The *P* values of differences between the ACaHW and control groups are <0.01 at 4 weeks and =0.012 at 8 weeks. There were no significant differences between the AH and ACaHW groups at 4 and 8 weeks

3.2.2 Bone area calculation

Bone area calculations showed no significant difference between the AH treatment and the ACaHW treatment at all times (2 weeks, AH 4.937 % ± 2.302 vs. ACaHW 4.120 % ± 1.205; 3 weeks, AH 5.933 % ± 1.642 vs. ACaHW 6.703 % ± 1.976; and 4 weeks, AH 5.345 % ± 1.580 vs. ACaHW 7.079 % ± 2.325). At 2 weeks, the AH group presented a higher BA than the control group (AH 4.937 % ± 2.302 vs. control 2.507 % ± 1.062; $P = 0.018$), but at 3 weeks, the control group surpassed the AH group (AH 5.933 % vs. control 8.102 % ± 2.372; $P = 0.044$) (Fig. 6a).

3.2.3 Bone-implant contact calculation

With respect to the BIC, both the AH and ACaHW treatment groups exhibited a higher bonding rate than the control group after 2 weeks (AH 17.517 % ± 9.861; ACaHW 13.344 % ± 4.70; control 5.001 % ± 2.985) and 3 weeks (AH 23.266 % ± 7.784; ACaHW 22.618 % ± 7.46, and control 8.112 % ± 3.373); however, according to the *P* values (Fig. 6b), the AH and ACaHW treatment groups did not significantly differ. At 4 weeks, the ACaHW group presented an obviously higher BIC than the AH group (ACaHW: 45.998 % ± 10.172; AH: 26.239 % ± 2.67), and this difference was significant ($P = 0.001$). Unlike the AH group, the ACaHW group exhibited remarkable BIC growth from 2 to 4 weeks (Fig. 6b).

3.2.3.1 Normalized bone-implant contact calculation To exclude the effect of structure on the calculation, we calculated the NBIC for each 9-section area. The resulting NBIC values presented similar trends to the BIC values. At 2 weeks, the NBICs of samples in the AH, ACaHW and control groups were 3.147 % ± 2.393, 1.564 % ± 0.482, and 1.417 % ± 1.061, respectively; at 3 weeks, the NBICs were 3.650 % ± 2.609, 2.613 % ± 1.178 and 1.340 % ± 0.637, respectively; at 4 weeks, the values were 2.661 % ± 0.27, 3.877 % ± 0.978 and 1.293 % ± 0.52, respectively. The ACaHW group showed significant NBIC growth from 2 to 4 weeks, but no significant growth was observed for the AH treatment over the same period. Although AH treatment resulted in a higher NBIC than ACaHW before 4 weeks, this difference was not statistically significant (for *P* values, please see Fig. 6c.) At 4 weeks, the NBIC of the ACaHW group exceeded that of the AH group, and this difference was statistically significant ($P = 0.032$) (Fig. 6c).

3.2.4 SEM analysis

The interface between the implant and the bone tissue was observed on the same plane using SEM with backscattered mode. Immature bone tissue was observed in all three groups at 2 weeks (Fig. 7a, b, c). Small amounts of contact were observed between the implant and the bone tissue in the AH and ACaHW treatment samples (Fig. 7a, b). However, at 2 weeks, a large resin area showing gap was observed at the border between the implant and bone tissue in the untreated sample, indicating a lack of direct contact between the implant and the bone tissue (Fig. 7c). At 4 weeks, bone tissue appeared to be more mature than at 2 weeks, and more direct contact between the implant and

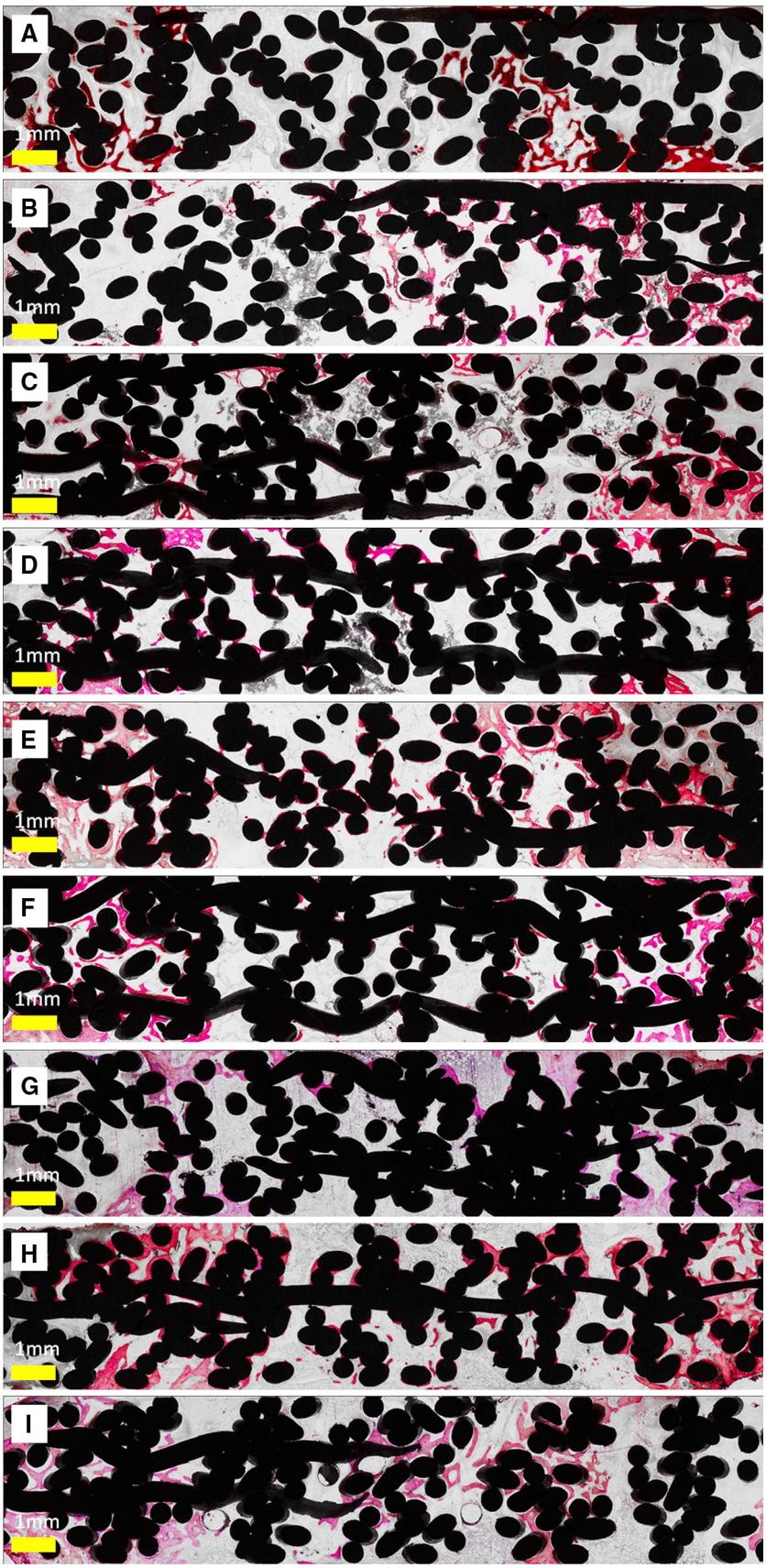

Fig. 4 Bone tissue was observed in each group at 2 weeks (**a** AH, **b** ACaHW and **c** control). At 3 weeks (**d** AH, **e** ACaHW and **f** control) and 4 weeks (**g** AH, **h** ACaHW and **i** control), more bone tissue was found in the specimens, and bone tissue was observed growing throughout the entire implant. A large quantity of bone tissue was also found in the middle of the specimens at 3 and 4 weeks, which was rarely observed at 2 weeks

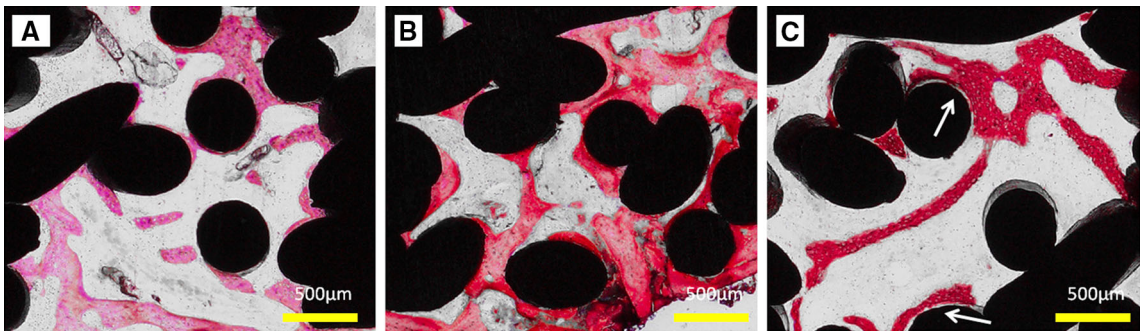

Fig. 5 At 4 weeks, the AH- (**a**) and ACaHW-treated (**b**) implants (*black*) exhibited direct contacts with the bone (*red*). The *white arrow* indicates fibrous tissue at the interface between the implant and bone tissue in the control group (**c**) (Color figure online)

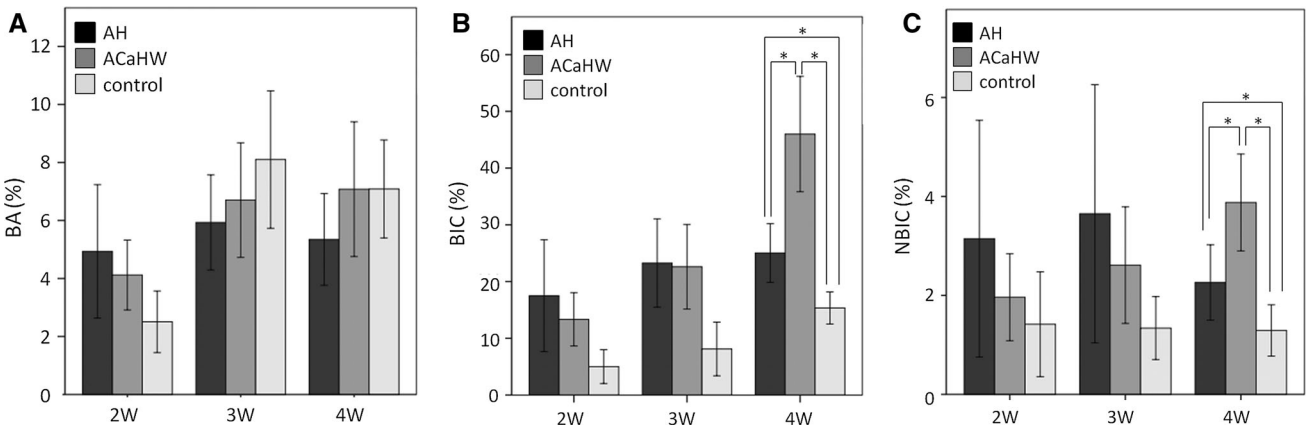

Fig. 6 **a** Bone area (BA) for the mesh group at 2, 3 and 4 weeks after implantation (*error bars* indicate standard deviations). The *P* values of differences between the AH and ACaHW groups are 0.58 at 2 weeks, 0.647 at 3 weeks, and 0.349 at 4 weeks. There was no significant difference between the 2 treatments. **b** Bone-implant contact (BIC) for the mesh group at 2, 3 and 4 weeks after implantation (*error bars* indicate standard deviations). The *P* values of differences between the AH and ACaHW groups are 0.423 at 2 weeks, 0.973 at 3 weeks, and 0.001 at 4 weeks. The *P* value of the difference between the AH and control groups is 0.044 at 4 weeks. The *P* value of the difference between the ACaHW and control groups is 0.05 at 4 weeks. There was no significant difference between the 2 treatments before 4 weeks. $*P < 0.05$. **c** Normalized bone-implant contact (NBIC) for the mesh group at 2, 3 and 4 weeks after implantation (*error bars* indicate standard deviations). The *P* values of differences between the AH and ACaHW groups are 0.318 at 2 weeks, 0.335 at 3 weeks, and 0.016 at 4 weeks. The *P* value of the difference between the AH and control groups is 0.017 at 4 weeks. The *P* value of the difference between the ACaHW and control groups is 0.01 at 4 weeks. There was no significant difference between the two treatments before 4 weeks. $*P < 0.05$

bone tissue was observed in the AH- and ACaHW-treated samples (Fig. 7d, e); in contrast, a large resin area showing gap remained in the control samples, confirming that the implant and the bone tissue were less strongly connected in the control samples (Fig. 7f).

3.2.5 Plate group

Histological images at 4 and 8 weeks are shown in Fig. 8. No significant differences were observed between the AH- and ACaHW-treated groups at all times. At 4 weeks, new bone (NB) formation between the implant and the original bone (OB), together with a small amount of soft tissue, was observed for the ACaHW- and AH-treated plates. Few connections existed between the bone and the implant, whereas a notable amount of soft tissue formed around the NB (Fig. 8a, b). For the control group, direct contacts between the NB and the implant were scarce (Fig. 8c). At 8 weeks, mature bone tissue was observed in both treated samples. For the samples treated with AH or ACaHW, more NB and more direct BIC was observed at 8 weeks than at 4 weeks (Fig. 8d, e). For the control group, a fibrous tissue layer was observed at the interface between the bone and the implant, bone-implant direct contact was not exist (Fig. 8f).

4 Discussion

In this study, Cp-Ti mesh implants were untreated or treated with AH or ACaHW, and the mechanical and histological properties of the implants were compared at 2, 3 and 4 weeks after implantation. Moreover, to consider the

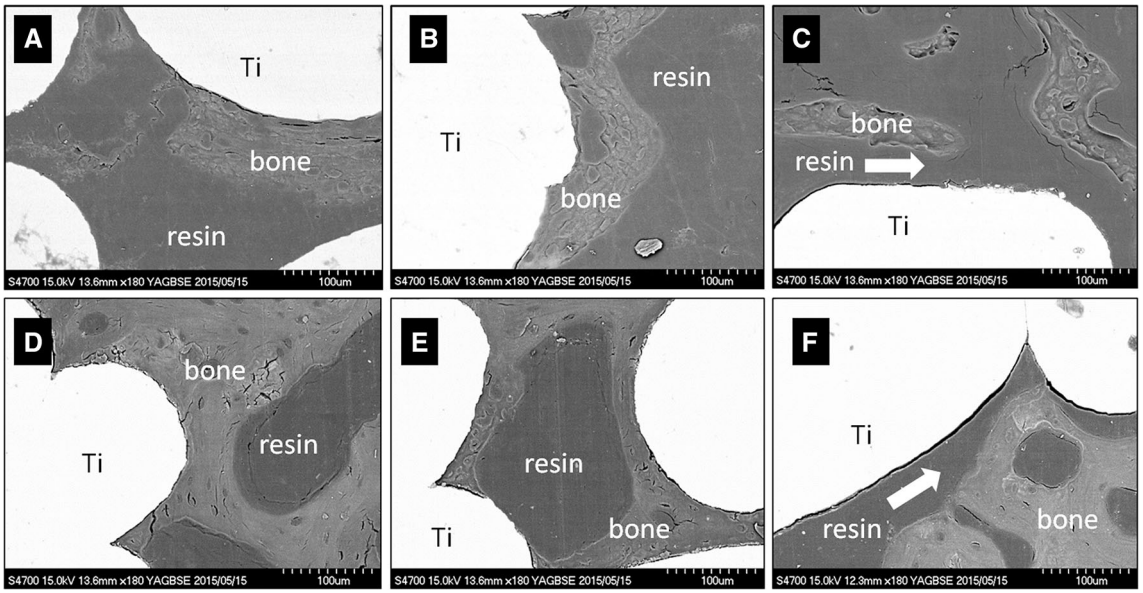

Fig. 7 SEM for AH treatment (**a**), ACaHW treatment (**b**) and the control (**c**) at 2 weeks; bone tissue exhibited an immature form. Bone-implant direct contacts were observed in the AH and ACaHW groups, whereas in the control group, resin filling gaps (*white arrows*) was visible between bone tissue and the implant, and few bone-implant direct contacts were observed. At 4 weeks, bone tissue was more mature in all groups. AH (**d**) and ACaHW (**e**) treatments produced a larger number of direct bone-implant contacts, but gaps remained present in the control group (**f**)

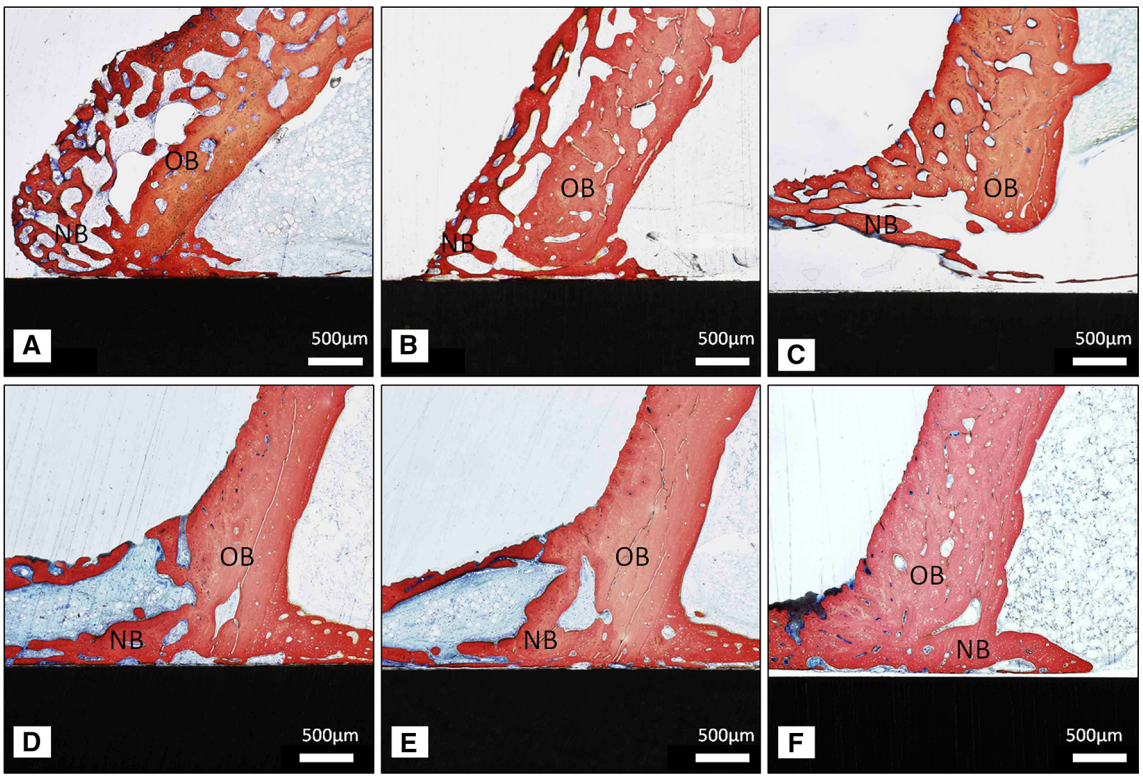

Fig. 8 Histological specimens were stained with Stevenel's blue and Van Gieson's picrofuchsin. At 4 weeks, in both the AH- (**a**) and ACaHW-treated (**b**) groups, a mount of new bone (NB) was generated around the original bone (OB). Fibrous tissue was observed between the NB and the implant materials. Small quantity of direct contacts between the bone and the materials were observed in the AH and ACaHW groups. In contrast, no bone-implant contact were observed in control group (**c**). At 8 weeks, The boundary between the NB and the OB was not clear. Similar osseointegration properties were observed in both chemically treated groups (**d** for AH and **e** for ACaHW). For the control group, a fibrous tissue layer was observed at the bone tissue/implant interface (**f**)

influence of mesh structure on bone bonding, we also used Cp-Ti plate implants and compared their bone-bonding abilities after various chemical treatments at 4 and 8 weeks. In the mesh group, no significant differences were found between the bone-bonding properties of the AH- and ACaHW-treated groups until 4 weeks, and the ACaHW group exhibited greater BIC. However, this difference was not reflected in the behavior of the implants in the subsequent plate group experiment.

The aim of the detaching test is to determine the bonding strength (failure load) between the implant and bone tissue by measuring the transient force required to detach them [22]. For the mesh group, no significant difference was observed between the various treatments at 2 weeks. Bone ingrowth was observed at 2 weeks in the mesh group samples, even without chemical treatment, and little bone tissue was observed in the central area of the implants. Bone ingrowth did not completely traverse the implants at 2 weeks (Fig. 4a–c). After 3 weeks, we observed bone tissue completely growing across the implants, and bone ingrowth was greater in most samples. In most samples, the bone tissues on either side of the implants were connected, and large amounts of bone tissue were observed in the central area of the mesh implants (Fig. 4d–i); for this reason, use of the detaching test was inappropriate after 3 weeks. In contrast, at 2 weeks, the mesh group failure load far surpassed that of the plate group at 4 or 8 weeks (Fig. 3a, b), further demonstrating that the mesh structure exerted a positive effect on bone ingrowth and that the mesh structure provided better bone-bonding force than the plate structure during the early stages of implantation.

BA calculations showed no significant differences among the AH, ACaHW and control groups from 2 to 4 weeks, demonstrating that the effect of the porous structure was greater than that of chemical treatment on bone ingrowth before 4 weeks. Previously, researchers chemically treated Cp-Ti implants with a mesh structure and showed that, compared with a control group, the BIC of the mesh group was greatly improved after 4 weeks [24, 25], a finding that is consistent with our results: at 4 weeks, the BICs and NBICs of the AH- and ACaHW-treated meshes were greater than those of the control group. Additionally, histological observations at 4 weeks showed that after AH or ACaHW treatment, the meshes resulted in more direct BICs than those observed in the control group (Fig. 5). Because tissue sample thickness might have affected these observations, the same surface was examined using SEM; the results of the SEM were similar to those obtained using a digital microscope, indicating that more direct contacts were formed between the implants and the bone tissues in the AH and ACaHW groups than in the control group after 2 and 4 weeks (Fig. 7). From 2 to 3 weeks, although the average BIC and NBIC values of the meshes after AH or ACaHW treatment were higher than those of the control group, these differences were not significant; these results indicate that the porous structure of the mesh might play a more important role than chemical treatment during the early stages of implantation.

The logic underlying the replacement of AH treatment by ACaHW treatment has been elaborated in the study of Kokubo [26]; ACaHW treatment can be applied to a wide range of Ti alloys, such as Ti-Zr–Nb-Ta, and is not limited to use with Ti–6Al–4V, Ti–6Al–2Nb–Ta or Ti–15Mo–5Zr–3Al. Moreover, ACaHW forms apatite more reliably than AH treatment. In this study, at 2 and 3 weeks, implants treated using AH and ACaHW exhibited similar BIC properties; however, at 4 weeks, the BIC and NBIC values yielded by AH treatment were inferior to those yielded by ACaHW treatment (Fig. 6b, c); this result can be attributed to the lower apatite-forming ability provided by AH treatment. Previous researchers have noted that whereas the apatite-forming ability of AH treatment was reduced in vitro when used at high temperatures in a moist environment for a week, the apatite-forming ability of ACaHW treatment remained constant under these conditions [17]. Apatite-forming ability and bone-bonding ability are correlated [8, 27]. Consistent with the findings of a previous study of surface apatite formation on SBF [18], here, we found that ACaHW treatment resulted in more sustained and steady growth of BICs than AH treatment, as shown by the BIC and NBIC values for the mesh group from 2 to 4 weeks (Fig. 6b, c). Our results are also consistent with previous in vitro results reported by Kizuki [17]. Stable bonding ability between implants and tissues is important for clinical application, and products that are more reliable would provide more consistent clinical efficacy. ACaHW-treated products might replace existing AH-treated products in the future, thus improving the reliability of these products.

AH-treated artificial joints have demonstrated short-term and long-term efficacy in clinical practice, and such products have been used in many patients since 2007 [15, 16]. In the present study, differences in bone-bonding strength between the AH- and ACaHW-treatment groups were not reflected in the plate group (Fig. 3b). Perhaps a larger sample size than that used in this study ($n = 6$ for the detaching test) might reveal differences between results obtained using similar chemical treatments; thus, the use of more animals is less desirable. This study provides convincing evidence that ACaHW treatment results in bone-bonding strength similar to that of AH treatment at 4 and 8 weeks. Furthermore, osseointegration resulting from ACaHW treatment was found equal to or greater than that resulting from AH, as demonstrated by the histological studies of the mesh group. Due to these advantages, ACaHW treatment has the potential to replace AH treatment and provide better and more stable early clinical effects.

5 Conclusions

In this study, a mesh structure was shown to increase early bone-bonding ability, and ACaHW treatment provided higher osseointegration than AH treatment at 4 weeks. Moreover, we showed that ACaHW and AH treatments resulted in similar bone-bonding strengths at 4 and 8 weeks. We propose that the treatment of artificial joints using a mesh structure with ACaHW might replace existing AH treatment to provide stronger and more stable clinical effects in future applications.

Acknowledgments The authors would like to thank Teijin-Nakashima Medical Co., Ltd., Japan, for supplying all the implants used in this study. We also thank Keiko Okamoto-Furuta and Haruyasu Kohda (Division of Electron Microscopic Study, Center for Anatomical Studies, Graduate School of Medicine, Kyoto University).

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