

Bioactivity and antibacterial activity of iodine-containing calcium titanate against implant-associated infection

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ARTICLE INFO

Keywords:

Calcium titanate
Iodine
Antibacterial
Implant-related infection
Osteointegration

ABSTRACT

Developing antimicrobial biomaterials is a major challenge in the fields of orthopaedic and dental implants. In this study, we evaluated the bone-bonding ability and antibacterial activity of a novel biomaterial for preventing implant-associated infections. We have previously reported that NaOH heat treatment improved the bone-bonding ability of titanium, which was later modified to release target ions from the calcium titanate surface. Iodine, an essential nutrient, exhibits broad-spectrum antimicrobial activity; hence, we designed a calcium titanate that releases iodine ions (Ca-I-Ti). The material was prepared from a simple solution using heat treatments as well as inexpensive devices and chemical agents. MC3T3-E1 cells seeded on Ca-I-Ti displayed high degrees of bioactivity and viability, and Ca-I-Ti exhibited antibacterial activity against methicillin-susceptible *Staphylococcus aureus*. *In vivo* biomechanical and histological experiments showed that Ca-I-Ti had excellent bone-bonding ability at 8 weeks after implantation. In a subcutaneous infection model in rats, methicillin-susceptible *Staphylococcus aureus* on the implant was reduced by approximately 95% compared to that on commercially pure titanium, indicating that Ca-I-Ti has antibacterial effects *in vivo*. In addition, no local or systemic complications were observed, and active infection in the surrounding tissues was histologically inhibited. Thus, iodine-containing calcium titanate is a safe biomaterial with excellent bioactivity and antibacterial properties, indicating its potential in preventing implant-associated infections.

1. Introduction

Commercially pure titanium (cp-Ti) and titanium alloys are widely utilized in metallic implants because of their biocompatibility, corrosion resistance, and mechanical strength [1,2]. Furthermore, various modifications have been introduced to titanium surfaces to improve their bone-bonding ability [3]. For example, we previously reported surface modification by NaOH heat treatment to establish bioactive surfaces on pure titanium and titanium alloy [4]. Following modification of titanium by NaOH heat treatment, bone can bond directly to the nano- and submicrometer-scale surface roughness [3]. A total hip arthroplasty

prosthesis based on such a treatment was commercialized in 2007 [5]. In recent years, excellent long-term clinical results have been reported with regard to the bone-bonding ability of orthopaedic and dental implants [2,6].

However, implant-associated infections are still a global public health concern and antimicrobial surfaces have been developed to address the issue [1,7–10]. Bacteria can adhere to metal surfaces and secrete extracellular polymeric substances that form capsules, resulting in biofilm formation, which leads to high resistance to antibiotic therapy [10–12]. Most patients with implant-associated infections require multidisciplinary treatments [13,14]. Especially, implant-associated

Abbreviations: ABE, antibacterial efficacy; ALP, Alkaline phosphatase; BIC, bone implant contact; BUN, blood urea nitrogen; Ca-I-Ti, iodine-containing calcium titanate; Ca-Ti, calcium titanate; CFU, colony-forming unit; cp-Ti, commercially pure titanium; Cr, creatinine; FT3, free triiodothyronine; FT4, free thyroxine; H&E, hematoxylin and eosin; qRT-PCR, Quantitative reverse-transcription polymerase chain reaction; SEM, scanning electron microscopy; TSH, thyroid stimulating hormone; XPS, X-ray photoelectron spectroscopy.

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<https://doi.org/10.1016/j.bioadv.2022.212952>

Received 17 December 2021; Received in revised form 2 May 2022; Accepted 23 May 2022

Available online 26 May 2022

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infections in compromised hosts such as diabetes and cancer patients require multiple revision surgeries, and can lead to amputation of limbs or fatal outcomes [15]. Therefore, a single functionalized surface to enhance bone-bonding ability is no longer sufficient, and multiple functionalized surface, including antibacterial activity, is required [10,16]. In our recent studies, multifunctionality including antibacterial activity was introduced into a metal using the ion exchange technique on a titanate surface [17,18]. First, CaCl_2 -heat treatment following soaking in NaOH leads to the formation of a calcium titanate layer with nano- and submicrometer-scale roughness on pure titanium and titanium alloy [17,19,20]. Further studies have demonstrated that various ions (Ag, Sr, and Mg) could be incorporated onto the calcium titanate surface by additional aqueous solution treatment following the above-mentioned NaOH- CaCl_2 heat treatment [18,21–23]. Specifically, soaking in 10 mM ICl_3 solution at 80 °C following NaOH- CaCl_2 heat treatment established iodine-containing and scratch-resistant calcium titanate surfaces with nano- and submicrometer roughness (hereafter named Ca-I-Ti) [24]. In the present study, we evaluate the bone-bonding ability and antibacterial properties of a novel iodine-containing biomaterial prepared using the above-mentioned technique. The sequential process involves heat treatment of a simple solution without requirement of costly devices and chemical agents.

Iodine is used worldwide as an antiseptic with little evidence of adverse effects in clinical practice [25–30].

Therefore, we hypothesized that iodine-containing calcium titanate is a safe and promising candidate for treating implant-associated infections. In the present study, we mainly examined the bioactivity, bone-bonding ability, and safety of iodine-containing calcium titanate *in vitro* and *in vivo*. Additionally, the antibacterial properties of iodine-containing calcium titanate were evaluated in an *in vitro* assay and a subcutaneous implant infection model *in vivo*.

2. Materials and methods

2.1. Surface treatment and characterization of samples

2.1.1. Sample preparation

Commercially pure Ti (ISO5832-2) (cp-Ti; >99.5%; Nilaco Co., Tokyo, Japan) was cut into discs (13 mm diameter $\times$ 1 mm) for all *in vitro* tests and surface characterization analyses, as well as antibacterial activity tests *in vivo*, or into rectangular shapes (15 $\times$ 10 $\times$ 2 mm) for use in biomechanical testing *in vivo* (Fig. 1a). Next, cp-Ti was modified by NaOH- CaCl_2 -heat treatment as described in detail in our previous report [19]. Briefly, the substrates were soaked in 5 M NaOH solution (Kanto Chemical, Tokyo, Japan) at 60 °C for 24 h, and then in 100 mM CaCl_2 (Kanto Chemical) at 40 °C for 24 h with shaking. The substrates were heated to 600 °C for 1 h and immersed in hot water. The Ti group was defined as Ca-Ti (Table 1). After NaOH- CaCl_2 -heat treatment, the substrates were soaked in 10 mM ICl_3 (Kanto Chemical) at 80 °C for 24 h to exchange calcium ions on the metal for iodine ions in the solution [24]. The NaOH- CaCl_2 -heat- ICl_3 -treated Ti was defined as Ca-I-Ti (Table 1). All substrates were washed with acetone, 2-propanol, and ultrapure

Table 1

Summary of the chemical and heat treatment procedures for the materials described in this study.

Substrate	1st treatment	2nd treatment	3rd treatment	4th treatment
cp-Ti	Untreated			
Ca-Ti	5 mol/l NaOH; 60 °C; 24 h	100 mmol/l CaCl_2 ; 40 °C; 24 h	600 °C; 1 h	H_2O ; 80 °C; 24 h
Ca-I-Ti	5 mol/l NaOH; 60 °C; 24 h	100 mmol/l CaCl_2 ; 40 °C; 24 h	600 °C; 1 h	10 mmol/l ICl_3 ; 80 °C; 24 h

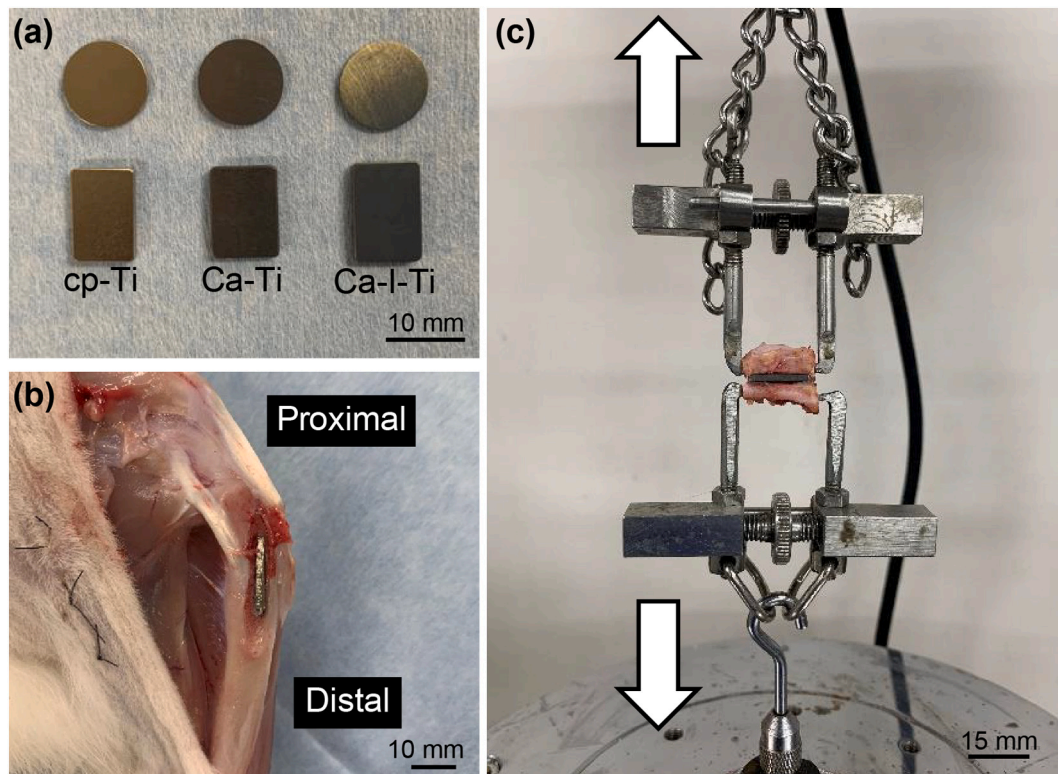


Fig. 1. Appearance of substrates of cp-Ti (untreated), Ca-Ti, and Ca-I-Ti, and photograph of biomechanical test. (a) Appearance of the disc and rectangular shape substrates. (b) Photograph of rabbit tibia with implanted rectangular substrate. (c) Detaching test to evaluate bone-bonding ability of the substrate.

water for 30 s in an ultrasonic cleaner and dried. All substrates were sterilized using ethylene oxide gas.

2.1.2. Surface characterization

Surface topography and cross-section were analyzed under a field-emission scanning electron microscope (FE-SEM, S-4300, Hitachi Co., Tokyo, Japan) and under an atomic scanning electron microscope (AFM, Bruker, Dimension Icon, USA). Surface elemental composition was determined using X-ray photoelectron spectroscopy (XPS, PHI-5000 Versaprobe II, ULVAC-PHI, Kanagawa, Japan) with a monochromatic Al K α source at a 45° take-off angle. The homogeneity of the coating was verified by energy dispersive X-ray (EDX, EMAX-7000, Horiba Ltd., Kyoto, Japan) mapping analysis. Crystalline phases were evaluated using TF-XRD (Model RNT-2500, Rigaku Co., Tokyo, Japan). In addition, the structures of the surface layers were analyzed under Fourier transform confocal laser Raman spectroscopy (FT-Raman, LabRAM HR800, Horiba Jobin Yvon, France). SEM, XRD, and elemental composition data of the Ca-I-Ti were adapted from our previous report [24].

2.2. In vitro bioactivity and cytotoxicity test

2.2.1. Cell culture

Osteoblast-like MC3T3-E1 cells were used for all bioactivity and cytotoxicity tests *in vitro*. In all tests using the cells, the cells were incubated in α -minimal essential medium (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin, and 10% fetal bovine serum (Gibco) at 37 °C in 5% CO₂. After achieving 80% confluence, cells were used for subsequent experiments. All experiments were conducted at least three times with three or four samples in each group (cp-Ti, Ca-Ti, Ca-I-Ti).

2.2.2. Cell proliferation

Cell proliferation and viability were analyzed using a Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany), which quantifies the dye intensity of orange formazan compounds. Briefly, MC3T3-E1 cells were seeded at a density of 2×10^4 cells/well onto each substrate in 24-well plates. After culturing the cells for 1, 3, and 7 days, 500 μ L of activated-XTT solution was added to the samples and incubated for 3 h at 37 °C. The absorbance of 150 μ L of the transferred aliquots on a 96-well plate was measured at 405 and 620 nm to quantify cell proliferation and viability.

2.2.3. Cell adhesion on scanning electron microscopy

MC3T3-E1 cells (1×10^4 cells/well) were seeded and cultured on the substrates in 24-well plates. After 24 h of culture, each sample was fixed in 2.5% glutaraldehyde at 4 °C for 2 h on ice. The samples were then dehydrated with gradually increasing concentrations of ethanol for 10 min each, and dried with hexamethyldisilazane. All samples were covered by a thin Pt-Pd film and examined under scanning electron microscopy (SEM; S-4700; Hitachi Ltd., Tokyo, Japan).

2.2.4. Fluorescent immunostaining to evaluate of cell adhesion

MC3T3-E1 cells (1×10^4 cells/well) were seeded and cultured on the substrates in 24-well plates. After 4 and 24 h of culture, the cells were fixed in 4% paraformaldehyde for 12 min, followed by incubation in 0.1% Triton X-100 for 10 min. After blocking with 4% fetal bovine serum, the samples were stained with rhodamine-phalloidin (Invitrogen, Carlsbad, CA, USA), to mark the cell cytoskeleton, and with DAPI (Invitrogen) to mark the nucleus. The cells were examined under a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan).

2.2.5. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was evaluated using LabAssay™ ALP (Wako Pure Chemical Industries, Osaka, Japan). MC3T3-E1 cells (2×10^4 cells/well) on the substrates were incubated in 24-well plates for 7 days. Then, the cells on the substrates were rinsed with normal saline and lysed with 1% NP-40 (Iwai Kagaku, Tokyo, Japan). After reacting

the samples with 6.7 mM *p*-nitrophenyl phosphate for 30 min, the optical density of *p*-nitrophenol at 405 nm was analyzed, and ALP activity was calculated using a standard curve. The ALP levels were normalized to the total protein content, and the relative levels, to the control group (cp-Ti), were calculated.

2.2.6. Gene expression assay

MC3T3-E1 cells (2×10^4 cells/well) on the substrates were incubated in 24-well plates for 7 days. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed to quantify osteogenic genes (*Alp*, osteocalcin (*Ocn*), osteopontin (*Opn*)), a cell adhesion-related gene (*integrin β 1*), and an extracellular matrix-related gene (*Col1a1*). RNA was extracted from the cells, and cDNA was synthesized using reverse transcriptase in triplicate using a StepOnePlus system (Applied Biosystems, Foster City, CA, USA) with the primers listed in Table 2. An internal housekeeping gene (*Gapdh*) was also measured to normalize the expression levels of mRNA, and the relative levels were calculated compared to the control group (cp-Ti).

2.3. In vitro antibacterial activity tests

2.3.1. Preparation of bacterial suspension

All antibacterial assays were performed using methicillin-susceptible *Staphylococcus aureus* (MSSA: ATCC 6538P). A monoclonal of the pathogen was dipped into 10 ml of tryptic soy broth medium and cultured in an incubator at 37 °C and 200 rpm. The final bacterial concentration in the culture medium was adjusted with sterile phosphate-buffered saline (PBS) to approximately 1.5×10^6 colony-forming units (CFU)/ml using a pre-made calibration curve at an optical density of 595 nm.

2.3.2. CFU count assay

The bacterial suspension (6.0×10^5 CFU/well) was seeded onto each disc substrate in a 24-well plate. The substrates were completely immersed and cultured for 12 and 24 h at 37 °C with 5% CO₂. After incubation, the samples were gently rinsed three times with sterile PBS to remove planktonic bacteria and then soaked in 5 ml of sterile PBS in a test tube. Adhesive bacteria were removed by vortex mixing for 2 min and sonication for 1 min. The bacteria in the vortex suspension were cultured on tryptic soy broth agar plates and incubated for 24 h at 37 °C. Viable bacteria were quantified by plate counting, and antibacterial efficacy (ABE) was calculated using the following formula [31]: ABE = (B – A)/B $\times$ 100%, where A is the number of viable bacteria in the Ca-Ti or Ca-I-Ti group and B is the number of viable bacteria in the cp-Ti group.

2.3.3. LIVE/DEAD staining

After seeding the bacterial suspension (1.5×10^6 CFU/well) onto each disc substrate in a 24-well plate, the substrates were cultured for 24 h and 7 days. After 7 days of culture, the medium was replaced with fresh tryptic soy broth every other day. After cultivation, the samples

Table 2

Target genes and primer sequences used in RT-qPCR.

Gene	Primer sequence (F, forward; R, reverse; 5'–3')
<i>Gapdh</i>	F: TGTCGCTCGTGGATCTGAC R: CCTGCTTCACCACCTTCTTG
<i>Alp</i>	F: ACTCAGGGCAATGAGGTAC R: CACCCGAGTGGTAGTCACAA
<i>Ocn</i>	F: AGACTCCGGCGCTACCTT R: CTCGTCAACAGCAGGTTAAG
<i>Opn</i>	F: GGAGGAAACAGCCAAGG R: TGCCAGAATCAGTCACITTCAC
<i>Integrin β1</i>	F: TTGGGATGATGTCGGGAC R: AATGTTTCAGTGCAGAGCC
<i>Col1a1</i>	F: CTCCTGGCAAGATGGAGAT R: AATCCACGAGCACCTGA

were stained with the Live/Dead BacLight Bacterial Viability Kits L7012 (Invitrogen) according to the manufacturer's protocol and cultured for 1 h at 37 °C in the dark. After gently rinsing with sterile PBS to remove excess stain, live (green) and dead (red) bacteria on the metals were confirmed using a fluorescence microscope (BX-X710; Keyence, Japan).

2.3.4. Crystal violet assay

The procedure described in Section 2.3.3 was used to seed and culture the bacteria on the substrates for 7 days. Biofilms grown on the substrates were stained with 0.1% crystal violet for 10 min to determine biomass density. After gentle rinsing with sterile PBS to remove excess stain, dye on the samples was dissolved in 2 ml of 30% glacial acetic acid. The absorbance was measured at 595 nm using a microplate reader. Optical density was calculated by subtracting the absorbance measured for 30% glacial acetic acid.

2.3.5. SEM observation

The procedure described in Section 2.3.3 was used to seed and culture the bacteria on the substrates for 7 days. The samples were fixed, dehydrated, and dried as described in Section 2.2.3. The samples were covered with a thin Pt-Pd film and examined under SEM.

2.4. Bone-bonding ability in vivo and blood test

2.4.1. Experimental animals

This experiment was approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, Japan (approval number: Med Kyo 21,252). Adult male Japanese white rabbits (weight 2.7–3.2 kg) were used in the experiments. Rectangle-shaped substrates were implanted in each leg of all rabbits (Fig. 1a & 1b). Biomechanical tests and histological analysis of bone-bonding ability were performed in each group at 4, 8, and 16 weeks after implantation. At each time point, nine rabbits (18 legs) were biomechanically evaluated, and five rabbits (nine legs) were histologically analyzed.

2.4.2. Surgical procedure

The substrates were implanted as described in our previous studies [32,33]. The rabbits were anesthetized by intravenous administration of sodium pentobarbital (40 mg/kg), maintenance with isoflurane inhalation, and local anesthesia with 1% lidocaine. A 4-cm longitudinal skin incision was made on the medial side of the knee. The fascia and periosteum were carefully excised and separated. A box slit (16 × 2 mm) was created using a dental burr parallel to the longitudinal axis of the tibia. After washing with normal saline to remove bone fragments, the substrates were fitted through the box slit. The periosteum and fascia were sutured layer by layer, and the skin was carefully closed.

2.4.3. Biomechanical testing

A detaching test was conducted on the resected bone at the implantation site to evaluate the strength of bone bonding. The protocol for the detaching test has been described in our previous studies [18,34,35]. Traction was applied perpendicularly to the implant surface at a cross-head speed of 35 mm/min using an Instron-type autograph (Model 1101; Aikoh Engineering, Osaka, Japan). The resected bone was held using a specially designed hook system, and the failure load was recorded as the load at which the implant and bone interface were detached (Fig. 1c).

2.4.4. Histological examination

Specimens, including implants, were fixed in 10% phosphate-buffered formalin, dehydrated in graded concentrations of ethanol, and embedded in polyester resin. Sections (500 µm) were prepared perpendicularly to the implant axis and ground to 60–80 µm with a grinding-sliding device (ML-182; MARUTO INSTRUMENT, Tokyo, Japan). The samples were stained with Van Gieson's picrofuchsin and Stevenel's blue and analyzed under a transmitted light microscope

(Eclipse 80i; Nikon, Tokyo, Japan). The ratio of bone directly in contact with the substrate to the substrate perimeter was quantified as the bone implant contact (BIC) ratio using a two-dimensional image processing software (ImageJ; National Institutes of Health, Bethesda, USA). The values were averaged from three areas (proximal, middle, and distal third) of each specimen.

2.4.5. X-ray photoelectron spectroscopy analysis after removal of implants

To analyze residual iodine on the surface of Ca-I-Ti after implantation, the depth profiles of the XPS spectra up to 600 nm were analyzed with Ar etching at 4 and 16 weeks after implantation. The profile before implantation was treated as the control. The protocol for XPS analysis has been described in Section 2.1.2.

2.4.6. Blood test

As described in Section 2.4.2, rectangle-shaped implants in the cp-Ti and Ca-I-Ti groups were implanted into rabbits. Implants in the same group were implanted in both legs of the rabbits. The following kidney function and thyroid hormone levels in the blood were tested: creatinine (Cr), blood urea nitrogen (BUN), thyroid stimulating hormone (TSH), free triiodothyronine (FT3), and (FT4). TSH was considered 0 if its level was below the detection sensitivity. Blood sampling was performed at pre-implantation and at 1 week and 1, 3, and 6 months after implantation. Centrifuged serum was analyzed using a chemistry analyzer (7180; Hitachi High-Technologies, Tokyo, Japan) and an immunochemistry analyzer (Cobas 8000 e801 module; Roche Diagnostics).

2.5. Antibacterial activity in vivo

2.5.1. Animals and surgical procedure

This animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, Japan (approval number: Med Kyo 21,254). Male rats (Wister/ST, 12 weeks old, 350–380 g) were used according to the institution's regulations on animal experiments for bacterial infection. A subcutaneous implant infection model was used to validate the antibacterial activity *in vivo* [36,37]. Disc substrates were soaked in an MSSA suspension (6.0×10^5 CFU/well) for 1 h to create an infected implant [37–39]. Considering the low *in vitro* antibacterial activity of Ca-Ti, we excluded the Ca-Ti group from this section and only compared the two groups (cp-Ti and Ca-I-Ti) for reasons pertaining to animal welfare. The backs of the rats were shaved, and 20-mm skin incisions were made bilaterally at the level of the scapula. The infected substrates were implanted subcutaneously, and the skin was closed. As a control at implantation, we confirmed the number of viable bacteria on the substrates after 1 h of soaking in MSSA suspension, as described in Section 2.3.2.

2.5.2. Observation of implantation site, CFU count, and histological evaluation

The rats were euthanized at 7 days post-surgery. The general appearance of the implantation site was assessed and the implant was aseptically retrieved and transferred into 5 ml sterile PBS. Using the method described in Section 2.3.2, the collected sample was vortexed and sonicated, and the bacteria in the vortex suspension were counted. Subsequently, the soft tissue capsule surrounding the implant was resected for histological evaluation. The resected soft tissue was treated with 4% paraformaldehyde and embedded in paraffin. The paraffin-embedded tissue was sectioned and stained with hematoxylin and eosin (H&E) and subjected to Gram staining for pathological evaluation.

2.6. Statistical analysis

All data are expressed as mean ± standard deviation. Differences among groups were analyzed using one-way analysis of variance and Tukey's multiple comparison tests. Student's *t*-test was used to compare the two groups. Statistical significance was set at $p < 0.05$. JMP software

(version 14.0.0, SAS Institute, Cary, NC, USA) was used for statistical analyses.

3. Results

3.1. Surface characterization

Fig. S1 presents the SEM images for comparison of the cp-Ti, Ca-Ti, and Ca-I-Ti treatments. A fine network structure was confirmed on the Ca-Ti and Ca-I-Ti treatments. Cross section images showed approximately 1- μ m thick treated layer on the surfaces of the Ca-Ti and Ca-I-Ti treatments. The three-dimensional AFM revealed evident changes *via* visual and quantitative analysis (Fig. S2a). Surface roughness with

spikes-like structures was formed in the Ca-Ti and Ca-I-Ti treatments. Ra values of the cp-Ti, Ca-Ti, and Ca-I-Ti groups were 20 ± 1.6 nm, 46.3 ± 8.2 nm, and 45.3 ± 4.5 nm, respectively (Fig. S2b). The chemical compositions and XPS full spectra of the samples are presented in Table S1 and Fig. S3, respectively. The spectra peaks of Ti, O, and C were confirmed in the three sample groups. In addition, the spectra peak of Ca 2p was observed in the Ca-Ti and Ca-I-Ti groups, and the new spectrum peak of I 3d at 615–640 eV was observed only in the Ca-I-Ti group. No chlorine was detected in the substrates. The EDX colour mapping images are shown in Fig. S4. EDX colour mapping indicated the homogeneity of the coating in the Ca-I-Ti treatments. XRD spectra present the additional peaks in the Ca-Ti and the Ca-I-Ti compared to in the cp-Ti treatment (Fig. S5). Such additional peaks in the Ca-Ti and Ca-I-Ti groups indicate

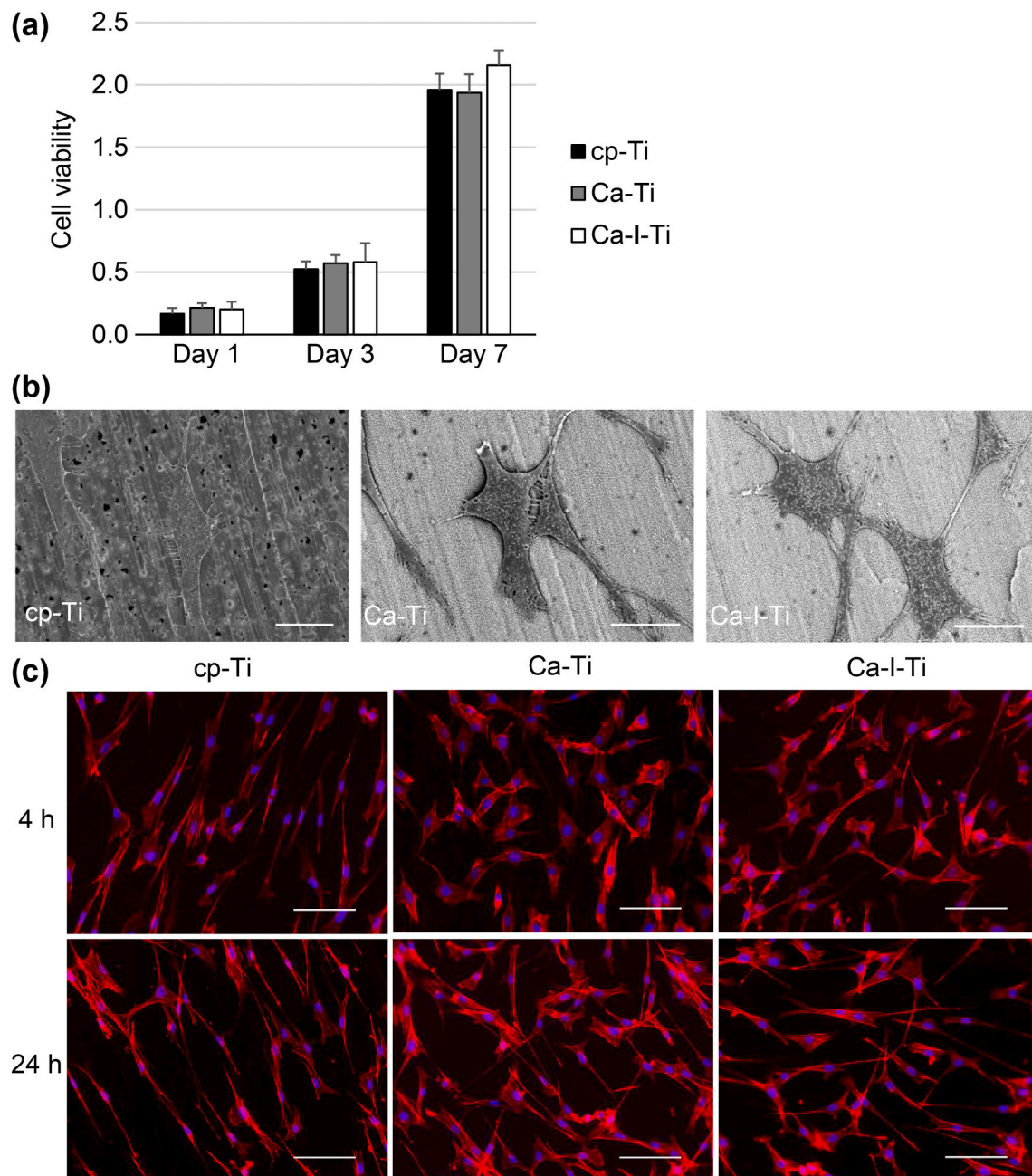


Fig. 2. Cell proliferation, viability, and attachment of MC3T3-E1 cells on each substrate group. (a) Results of cell proliferation and viability in three groups determined using XTT assay ($n = 4$). (b) Cell attachment of each Ti substrate observed using SEM, bar: 25 μ m. (c) Cell attachment of each Ti substrate on fluorescent immunostaining images (cytoskeletons are stained as red and nuclei are stained as blue), bar: 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that calcium titanates exist in both group (JCPDS Powder Diffraction Data Files 00-026-0333, 01-072-1134, 00-025-1450), and the slight shift in the peak between Ca-Ti and Ca-I-Ti groups indicate that iodine is incorporated into the crystal structure of calcium titanate. Fig. S6 shows the FT-Raman spectra of the samples, and the Ca-Ti and Ca-I-Ti treatments has additional peaks of titanium (II) oxides (anatase and rutile), calcium titanates. The results are consistent with the results of XRD analysis.

3.2. In vitro bioactivity and cytotoxicity

3.2.1. Cell proliferation and viability

Cell proliferation and viability in each substrate group were evaluated using the XTT assay (Fig. 2a). MC3T3-E1 cells in all groups proliferated over time. There was no significant difference in cell viability among the three groups at 1, 3, and 7 days. Ca-I-Ti showed no cytotoxicity compared to in the other substrate groups.

3.2.2. Cell morphology according to SEM

The morphology of MC3T3-E1 cells cultured on the substrates for 24 h is shown in Fig. 2b.

The cells on Ca-Ti and Ca-I-Ti were well-spread and showed thick and polygonal cytoplasm with pseudopodia. In contrast, cells on cp-Ti were thin and vertically long, although the cells spread.

3.2.3. Immunofluorescence imaging

Immunofluorescence imaging showed that the cells had spread well on Ca-Ti and Ca-I-Ti at 4 h after seeding (Fig. 2c). The cells were connected to each other via elongated pseudopodia and showed polygonal changes after 24 h of incubation. Thin and sharp cells were observed on cp-Ti at 4 h after seeding and showed poor cell-to-cell connections even after 24 h of incubation. The morphological characteristics of the cells observed by fluorescent immunostaining were comparable to those observed under SEM.

3.2.4. ALP activity and qRT-PCR analysis

ALP activity and expression of the five genes were compared among the groups. The average ALP activity in the Ca-Ti and Ca-I-Ti groups was significantly higher than that in the cp-Ti group (Fig. 3). The expression

of *Alp* after 7 days of incubation showed a similar trend in ALP activity. With regard to the expression of *Ocn*, the Ca-Ti group showed significantly higher levels of expression than the cp-Ti group. Also, in the case of *Opn*, the Ca-Ti group showed significantly higher levels of expression than the other groups, whereas there was no significant difference in *Integrin β 1* and *Col1a1* levels of expression among the groups.

3.3. In vitro antibacterial activity

3.3.1. CFU count assay

The numbers of viable bacteria after 12 h of incubation on the substrates (cp-Ti, Ca-Ti, and Ca-I-Ti) were 2.9 ± 1.4 , 2.5 ± 0.7 , and 0.79 ± 0.18 (CFU $\times 10^4$), respectively; after 24 h of incubation on the substrates (cp-Ti, Ca-Ti, and Ca-I-Ti), the values were 7.3 ± 4.5 , 6.1 ± 4.1 , and 0.13 ± 0.18 (CFU $\times 10^4$), respectively (Fig. 4a and b). The ABE value (%) of Ca-I-Ti after 24 h of incubation was 97.9 ± 2.9 , which was significantly higher than that in the other groups. The ABE value of Ca-Ti after 24 h of incubation was $-1.9 \pm 56.3\%$.

3.3.2. LIVE/DEAD staining

After 24 h of incubation, fluorescence microscopy images showed that cp-Ti and Ca-Ti were covered with numerous and scattered viable bacteria (Fig. 4c). In contrast, a few living bacteria were observed on Ca-I-Ti. After 7 days of incubation, the living bacteria spread and formed a membrane structure on cp-Ti, and strong luminescence along the rough structure was observed on Ca-Ti. Although a small number of solitary colonies was scattered on Ca-I-Ti, bacterial growth was inhibited compared to in the other groups.

3.3.3. Crystal violet staining for quantifying biomass density

Biomass on the disc substrates after 7 days of incubation was quantitatively measured using crystal violet staining. The mean optical densities of dissolved crystal violet in the cp-Ti, Ca-Ti, and Ca-I-Ti groups were 2.2 ± 0.16 , 2.6 ± 0.49 , and 0.85 ± 0.09 , respectively (Fig. 4d). The optical density in the Ca-I-Ti group was significantly lower than those in the cp-Ti and Ca-Ti groups. In addition, the density of biomass on Ca-I-Ti was $\sim 60\%$ lower than that in the other groups.

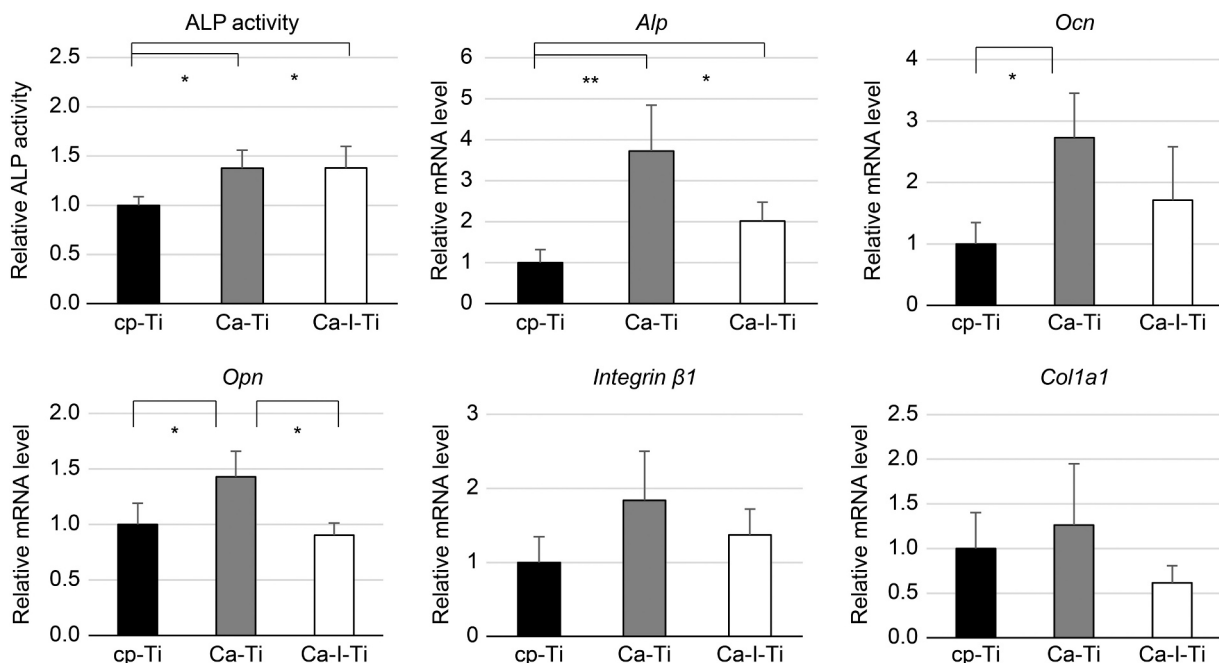


Fig. 3. ALP activity and relative gene expression level of MC3T3-E1 cells incubated in each group ($n = 4$). $*p < 0.05$ and $**p < 0.01$.

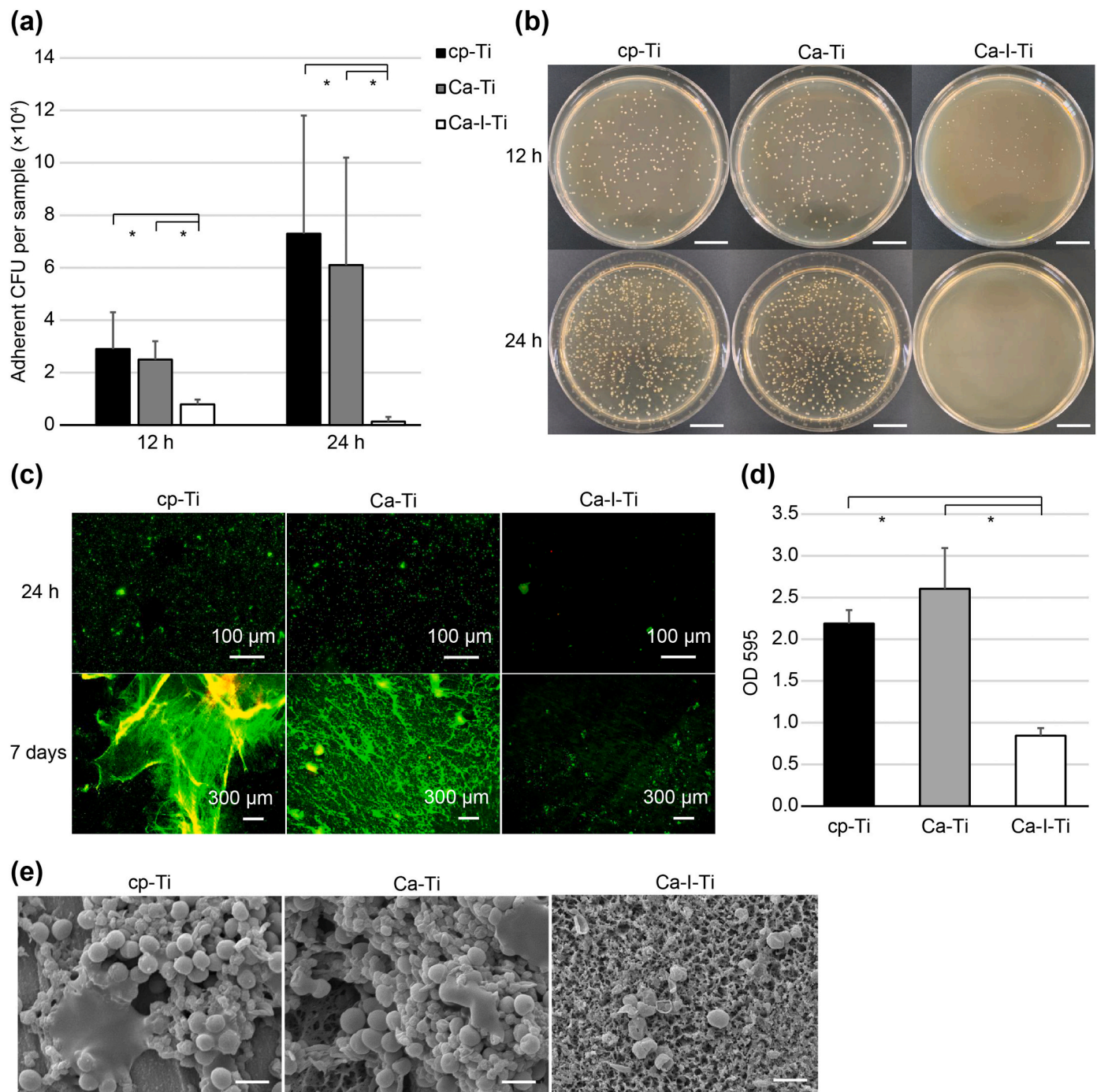


Fig. 4. Antibacterial activity of Ca-I-Ti substrate. (a) Number of viable adherent bacteria on each substrate after incubation for 12 and 24 h ($n = 6$). (b) Representative images of incubated colonies of vortex suspension, bar: 2 cm. (c) Viable bacteria stained with live/dead assay on different substrates. (d) Biomass density quantified by optical density of crystal violet assay ($n = 4$). (e) SEM images of bacterial morphology on each substrate, bar: 1 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3.4. SEM observation of bacterial colonies

SEM observation of the metal surface at 7 days after incubation revealed numerous bacteria in the cp-Ti and Ca-Ti groups (Fig. 4e). In the cp-Ti and Ca-Ti groups, the bacteria colonized and formed a slime-like structure as a thick and multilayered biofilm. In the Ca-I-Ti group, a small number of bacteria was observed, and the bacteria were ruptured and shriveled.

3.4. In vivo bone-bonding ability and blood test

Implantation was performed uneventfully in all animals. No delay in wound healing due to inflammatory reactions was observed and no deaths occurred after implantation.

3.4.1. Biomechanical testing

Bone-bonding strength was measured using a detaching test (Fig. 5a). At 4 weeks, there was no significant difference in the failure loads among groups. At 8 and 16 weeks, Ca-Ti and Ca-I-Ti showed

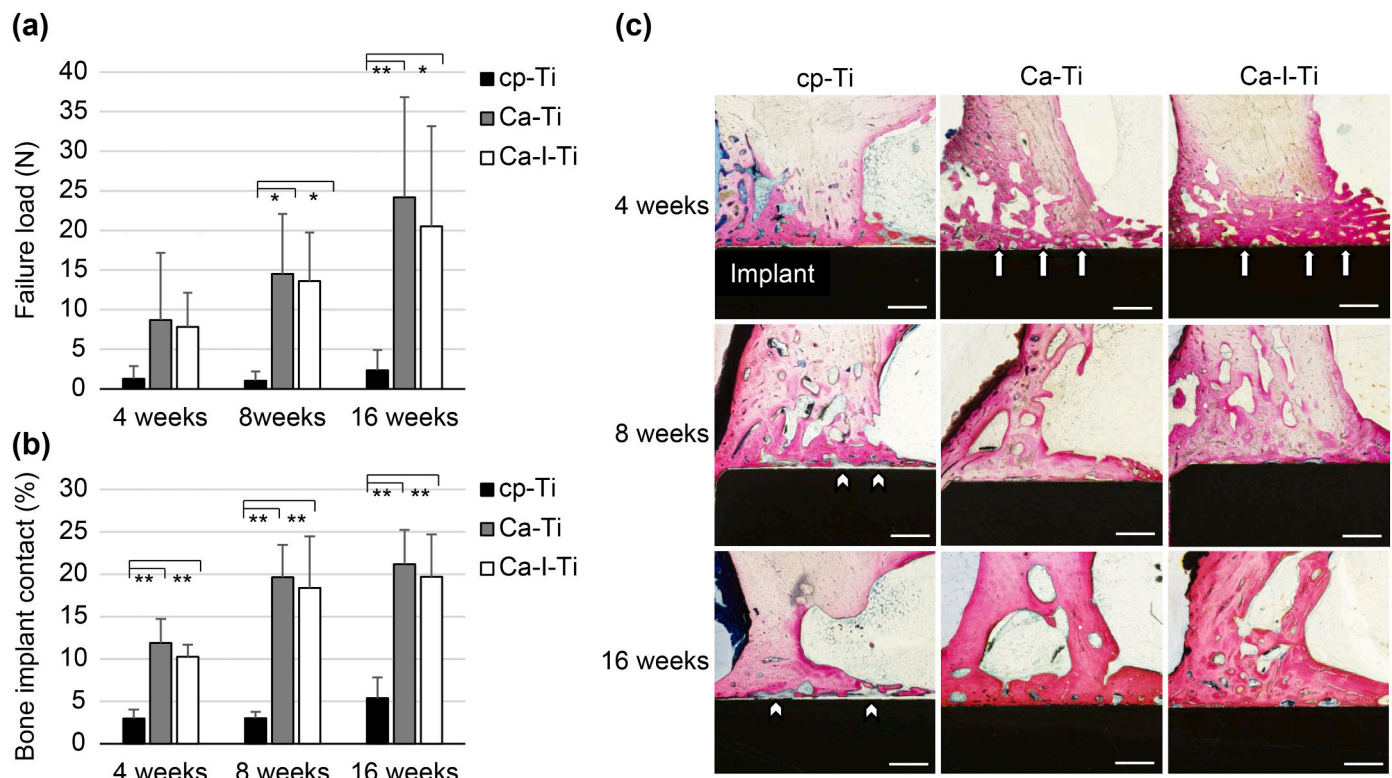


Fig. 5. Biomechanical test, histological images, and bone contact implant contact (BIC) ratio. (a) Failure load in each group at different periods (cp-Ti; $n = 3$, Ca-Ti and Ca-I-Ti; $n = 6$). (b) Bone implant contact ratio at 4–16 weeks after implantation ($n = 4$); $**p < 0.01$. (c) Histological sections stained with Stevenel's blue and Van Gieson's picrofuchsin (bar: 500 μm). White arrow indicates ongrowth of immature bone to substrate surfaces. White arrowhead indicates soft tissue (blue) interrupting osseointegration to the substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly higher failure loads than cp-Ti. At all time-points, there was no significant difference in the failure loads between Ca-Ti and Ca-I-Ti. The bonding strengths of Ca-Ti and Ca-I-Ti increased and were sustained at 16 weeks.

3.4.2. Histology for bone-bonding ability

Fig. 5b shows the BIC ratio determined through histological analysis. The BIC ratios of the Ca-Ti and Ca-I-Ti groups were significantly higher than that of the cp-Ti group after 4 weeks and were similar between Ca-Ti and Ca-I-Ti until 16 weeks. Representative histological samples are shown in Fig. 5c. The growth of immature bone contacting the substrates was observed in the Ca-Ti and Ca-I-Ti groups at 4 weeks. Massive mature bone was formed and directly bonded with the substrate at 16 weeks after implantation in both groups. In the cp-Ti group, soft tissues intervened in the growing bone even at 16 weeks.

3.4.3. XPS analysis after implant removal

The depth profiles of the XPS spectra before implantation, at 4 and 16 weeks after implantation, are presented in Fig. S7. The loaded iodine gradually decreased with implantation time. The decrease was particularly conspicuous at the outermost surface. Iodine levels remained low at 16 weeks. A similar trend was observed for the removed substrates.

3.4.4. Blood test

During the pre-implantation period, there was no difference in baseline Cr, BUN, TSH, FT3, and FT4 levels between the cp-Ti and Ca-I-Ti groups (Fig. S8). Regarding kidney function, the values of serum Cr and BUN from the pre-implantation period to 6 months after implantation did not exceed the reference intervals described in previous studies [40,41]. The Ca-I-Ti group showed no obvious renal dysfunction. Thyroid hormone (TSH, FT3, and FT4) levels did not significantly differ between the cp-Ti and Ca-I-Ti groups at any time point.

3.5. In vivo antibacterial activity

3.5.1. Appearance of implantation site and CFU count assay

The antibacterial activity of Ca-I-Ti was evaluated using a subcutaneous implant infection model (Fig. 6a). In the cp-Ti group, pus and thick granulation tissue were observed at 7 days after implantation (Fig. 6a). In contrast, in the Ca-I-Ti group, pus was not observed in any cases, and granulation tissue adhered to the metal surface, indicating antibacterial activity. The numbers of bacteria on the substrate were 5.7 ± 0.35 and 0.09 ± 0.06 (CFU $\times 10^4/\text{ml}$) in the cp-Ti, and Ca-I-Ti groups, respectively (Fig. 6b). The ABE value was $95.3 \pm 6.9\%$. There was no significant difference between the cp-Ti and Ca-I-Ti groups in the number of viable bacteria after soaking in the MSSA suspension for 1 h (baseline) (Fig. 6b).

3.5.2. Histology analysis of antibacterial activity

The soft tissue around the substrate was subjected to H&E and Gram staining as shown in Fig. 6c. Smooth granulation and fibrous tissue were formed in the Ca-I-Ti group; the fibrous tissue connection was undisturbed. In contrast, the connection of fibrous tissue in the cp-Ti group was disrupted and irregular because of the active infection. A few solitary gram-positive *S. aureus* were observed in the Ca-I-Ti group, whereas a large number of free gram-positive *S. aureus* were present, and the macrophages phagocytized bacteria in the cp-Ti group (Fig. 6c).

4. Discussion

In the present study, we verified the bioactivity, cytotoxicity, and antibacterial activity of Ca-I-Ti *in vitro* and *in vivo*. In *in vitro* bioactivity studies, SEM and immunofluorescence imaging indicated that Ca-I-Ti maintains a high degree of bioactivity even when iodine ions are incorporated on the surface. As for the ALP activity and gene expression

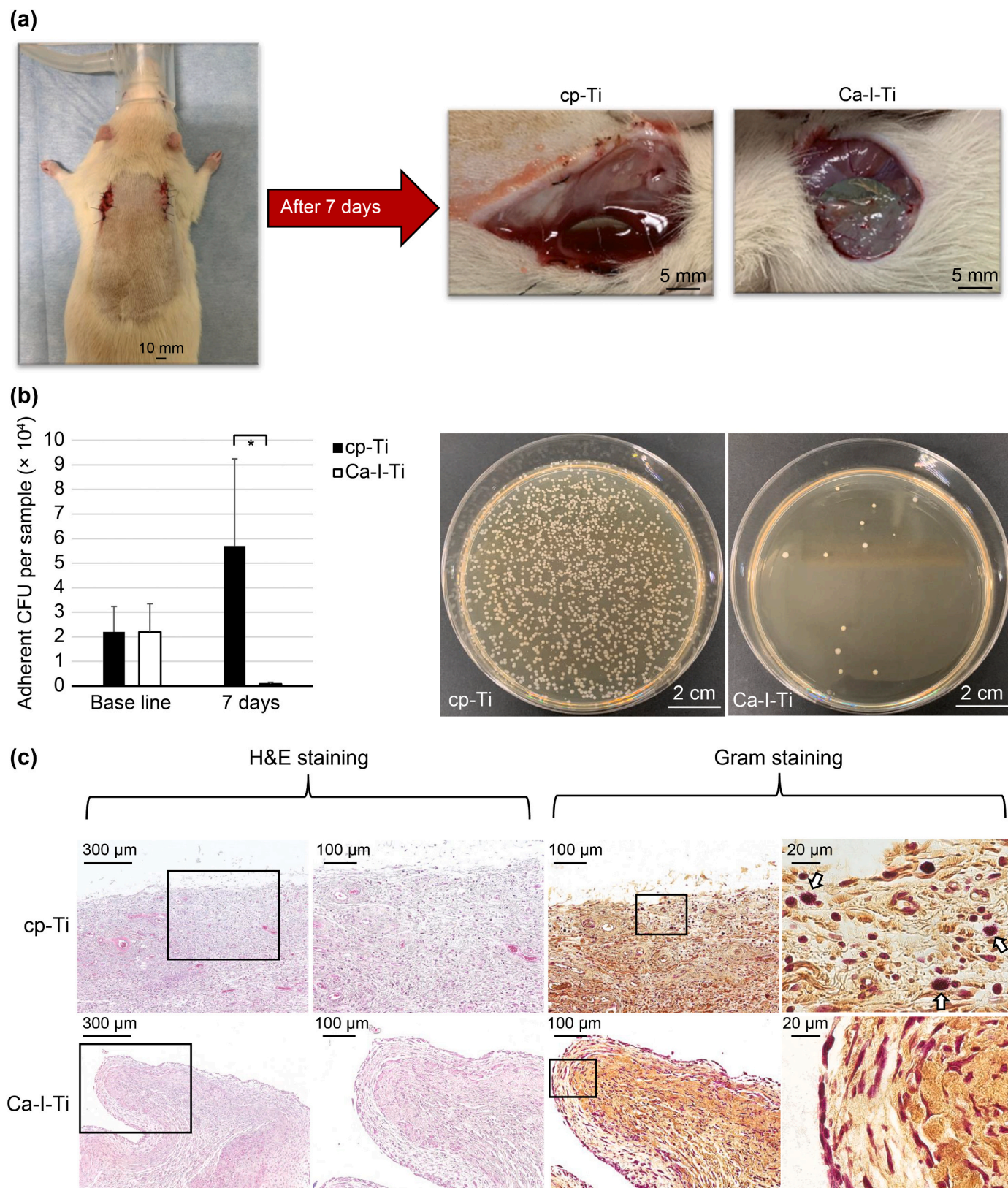


Fig. 6. Antibacterial activity evaluated using subcutaneous infection model. (a) General appearance after implantation and surgical site at 7 days after implantation. (b) Number of viable adherent bacteria on each substrate at 7 days after implantation and representative images of incubated colonies of vortex suspension ($n = 6$). (c) Soft tissue on each substrate stained with hematoxylin and eosin (H&E) and Gram's staining. The left two columns show H&E-stained images and right two columns show Gram-stained images. White arrow indicates macrophages phagocytizing bacteria. Black frame indicates the same area of the magnified image.

level of *Alp*, Ca-I-Ti and Ca-Ti showed significantly higher activity and expression levels than the cp-Ti, whereas the gene expression levels of *Ocn* and *Opn* in the Ca-Ti group were higher than those in the other groups. Previous studies have suggested that a small number of Ca^{2+} are released at the surface of Ca-Ti [19,20,42]. Ca^{2+} activates the calcium-sensing receptor in bone cells to accelerate the activity of osteoclasts and osteoblasts, resulting in increased expression of *Ocn* and *Opn* mRNAs [43,44]. Therefore, the Ca-Ti group may exhibit highly express genes involved in osteogenic differentiation. However, the results of bone-bonding strength and histological evaluation were comparable between the Ca-Ti and Ca-I-Ti groups until 16 weeks. Partial ion exchange between calcium and iodine ions did not negatively affect bone-bonding ability *in vivo*.

Although a previous study suggested the high scratch resistance of Ca-I-Ti [24], the present study revealed that NaOH-CaCl₂-heat-ICl₃ treatment produces submicrometer- and nano-scale roughness on the titanium surface. The bone-bonding ability of Ca-I-Ti can be attributed to its topography and chemical composition.

Next, we evaluated the cytotoxicity and adverse effects of Ca-I-Ti *in vivo*. There is little evidence available that iodine, which has been used as an antiseptic since 1839, has adverse clinical effects [27,29,30,45,46]. However, several studies have suggested that iodine causes low cytotoxicity in fibroblasts, keratinocytes, and other cell lines [28,47]. The XTT assay indicated that Ca-I-Ti had no significant adverse effect on the proliferation and viability of MC3T3-E1 cells. Previous studies have reported that the cell viability of human fibroblasts, osteoblasts, and myoblasts sharply decrease at concentrations of 0.1% PVP-I [48,49]. Other studies have revealed that the concentration of free iodine in PVP-I is 24 ppm in 0.1% solution, 13 ppm in 0.01% solution, and less than 5 ppm in 0.001% solution [50,51]. The ion release test in our previous study confirmed that Ca-I-Ti slowly released a total of 5.6 ppm of free iodine over 90 days [24]. The safety of Ca-I-Ti demonstrated in this study is consistent with previous findings. There were no cases of obvious wound inflammation or death in the Ca-I-Ti group, and the serum levels of kidney function markers and thyroid hormone remained within the reference intervals during a 6-month follow-up [40,41]. Only at 1 month postoperatively, Cr in the cp-Ti group was significantly higher than that in Ca-I-Ti but showed recovery over time, likely because of transient postoperative stress and dehydration [40,41].

Finally, we evaluated the antibacterial activity of Ca-I-Ti. The mechanism of action of iodine remains controversial [25]. Recent studies have suggested that free iodine ions enter bacteria through membrane channels (porins) and induce oxidation of key groups of proteins, nucleotides, and fatty acids [25,29,45]. The CFU count assay *in vitro* indicated that Ca-I-Ti reduced the number of adhesive bacteria by 97.9% compared to cp-Ti after 24 h. After 7 days of culture, biofilms were significantly reduced in the Ca-I-Ti group. Importantly, the antimicrobial activity of Ca-I-Ti was maintained *in vivo*. At 7 days after implantation, the number of adhesive bacteria on Ca-I-Ti was reduced to approximately 1.6% of that in the cp-Ti group, while the number of adhesive bacteria on the Ca-I-Ti decreased to approximately 5.3% of the baseline level. In addition, XPS analysis of the removed implants revealed that iodine remained, albeit attenuated, even at 16 weeks after implantation. This result may be useful for clinical applications because Ca-I-Ti may exhibit a continuous antibacterial effect on bacterial adhesion and biofilm formation. Iodine-containing calcium titanate ensures the bioactivity of the implant, and such treatment may provide sustained antimicrobial activity without obvious toxicity *in vivo*.

The present study had some limitations. First, the surface area of the experimental implants compared to the weight of the experimental animals was smaller than that used in clinical application. To precisely assess the safety and efficacy of Ca-I-Ti for clinical use, a larger implant should be used in larger experimental animals. Second, this study was the first step in verifying the antibacterial properties of iodine-containing titanate. The long-term antibacterial effect and toxicity of Ca-I-Ti have not been directly evaluated. Although iodine in Ca-I-Ti was

mainly released during the early phase and remained in minute quantities, long-term observation is required. Third, we did not test the antimicrobial activity against refractory infections such as osteomyelitis, which is a difficult-to-treat infectious disease characterized by bone destruction due to repeated inflammation. This study confirmed the promising antimicrobial activity of Ca-I-Ti; hence, validation using a refractory infection model is required in further studies.

5. Conclusions

Iodine-containing calcium titanate showed high bioactivity. *In vivo*, sustainable bone-bonding ability over the long-term was confirmed through mechanical and histological experiments. Analysis of antibacterial ability using MSSA revealed that iodine-containing calcium titanate may have a potent antibacterial effect, with no obvious toxicity. Similar observations were made *in vivo* in a subcutaneous implant infection model. Therefore, the proposed biomaterial is a promising candidate for treating implant-associated infections.

CRedit authorship contribution statement

Norimasa Ikeda: Conceptualization, Investigation, Writing - Original Draft; **Shunsuke Fujibayashi:** Conceptualization, Supervision; **Seiji Yamaguchi:** Conceptualization, Methodology; **Koji Goto:** Writing - Review & Editing, Supervision; **Bungo Otsuki:** Methodology, Writing - Review & Editing; **Toshiyuki Kawai:** Methodology, Writing - Review & Editing; **Takayoshi Shimizu:** Methodology, Writing - Review & Editing; **Yaichiro Okuzu:** Methodology, Writing - Review & Editing; **Kazutaka Masamoto:** Writing - Review & Editing; **Yu Shimizu:** Methodology, Investigation; **Yusuke Takaoka:** Writing - Review & Editing; **Shuichi Matsuda:** Supervision.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Mr. Kohda and Ms. Furuta for SEM observation and technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioadv.2022.212952>.

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