Journal of Prosthodontic Research 64 (2020) 436–443

Contents lists available at ScienceDirect

Journal of Prosthodontic Research

journal homepage: www.elsevier.com/locate/jpor

Original article

Development of tissue conditioner containing cetylpyridinium chloride montmorillonite as new antimicrobial agent: Pilot study on antimicrobial activity and biocompatibility

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ARTICLE INFO

Article history:
Received 7 April 2019
Revised 26 November 2019
Accepted 3 December 2019
Available online 13 February 2020

Keywords:
Antimicrobial activity
Biocompatibility
Cetylpyridinium chloride
Mechanical properties
Tissue conditioner

ABSTRACT

Purpose: The mechanical properties, antimicrobial activity, and biocompatibility of a novel antimicrobial tissue conditioner containing cetylpyridinium chloride with montmorillonite (CPC-Mont) were evaluated.

Methods: To examine the mechanical properties of the novel material, hardness, consistency, and penetration tests were conducted. Antimicrobial activity against *Candida albicans (C. albicans)* and *Staphylococcus aureus (S. aureus)* was evaluated. Cell viabilities of fibroblasts and epithelial cells using eluates from materials were measured to evaluate cytotoxicity. In addition, to assess tissue response, animal experiments were conducted.

Results: The hardness test results were similar to those of other commercially available materials. The novel tissue conditioner showed good antimicrobial activity against *C. albicans* and *S. aureus* compared with other materials. This effect was sustained for a week for *C. albicans.* In the case of *S. aureus*, microbial growth was suppressed for up to 3 weeks. Cell viability of the novel material for the eluate at 1 day was significantly less than those of other material for both cells. However, the cell viability at 7 days showed no significant difference. Animal experiments demonstrated that inflammatory responses around materials were not observed on the oral mucosa as other material.

Conclusion: Within the limitations of this *in vitro* and *in vivo* study, the results suggest that the newly developed tissue conditioner containing CPC-Mont has not only excellent antimicrobial properties, but also the same mechanical properties and biocompatibility as tissue conditioners on the market.

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https://doi.org/10.1016/j.jpor.2019.12.002
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1. Introduction

Tissue conditioners have commonly been used for the prosthetic treatment of dentures in tissue conditioning, dynamic impression, and relining of the immediate dentures [1]. They are intended to be in direct contact with the oral mucosa; this means that they must be non-irritating, non-toxic, and inhibit bacterial and fungal colonization [2,3].

Aspiration pneumonia in older patients is a most serious problem, which may be life-threatening [4,5], or at least may result in a decline in the individual's quality of life. It has also been reported that aspiration pneumonia is related to periodontal disease, dental caries, and poor oral hygiene [6]. Recently, O'Donnell et al. suggested that dentures can act as a reservoir for potential respiratory pathogens in the oral cavity, thus increasing the risk of developing aspiration pneumonia [7]. In fact, it has been shown that microorganisms, including *Candida albicans (C. albicans), Staphylococcus aureus (S. aureus)*, and *Streptococcus mutans (S. mutans)*, adhere to the inner surface of complete dentures [8,9]. In particular, *C. albicans* and *S. aureus* are significant in causing aspiration pneumonia [10,11]. Therefore, it is quite important to prevent older persons from contracting aspiration pneumonia by reducing the number of microbes attached to dentures. The proliferation of microbes is enhanced by the surface roughness of acrylic resins on dentures [12–14]. Further complicating matters, when tissue conditioner is repeatedly applied to the denture for long periods, because of its porosity and softness, it might act as an even more damaging reservoir for microorganisms compared with acrylic resin, as it allows microbial adherence, accumulation, and penetration, or may become so degraded that plaque deposits will form on it and microbes are found to colonize its surface.

To reduce the number of microbes colonizing tissue conditioners, attempts have been made to add antimicrobial agents, such as oil and silver [15–17], but it has been reported that these produce harmful effects, such as metal allergies [18–20]. Biocompatibility is a very important property for a tissue conditioner, as is antimicrobial activity. However, few animal experiments have been conducted. Therefore, antimicrobial tissue conditioners have been subjected to extensive research, but have not yet reached a stage at which they are ready for commercialization [15–21].

Cetylpyridinium chloride (CPC) is a highly safe antibacterial substance, and commonly used as an antimicrobial agent in both food and oral care; this suggests that it could play a role in the oral care of older people. The CPC molecule binds to the negatively charged surface of the bacterial cell membrane [22]. The nonpolar region of the molecule, which has similar traits to membrane phospholipids, penetrates the cell membrane of the bacteria, altering it and generating an imbalance in osmotic regulation, resulting in the loss of cytoplasmic material and, ultimately, cell death [22,23]. However, it was found that the antimicrobial effect does not persist with CPC alone; some kind of carrier are necessary to sustain its antimicrobial activity. Montmorillonite is a highly safe material, often used in human trials [24]. It is a very soft phyllosilicate group of minerals that forms layered clay and has been extensively used in catalytic processes to facilitate cation exchange; it has also attracted attention from researchers for its adsorption qualities [25]. Montmorillonite can intercalate cationic organic surfactants between the layers [26]. By using this property, CPC intercalated to montmorillonite would be able to exhibit sustainable antibacterial effects. Thus, we developed the new tissue conditioner containing CPC-Mont (TC-CPC) as a highly novel antimicrobial tissue conditioner.

The addition of CPC-Mont has made it possible to develop new tissue conditioner with antimicrobial property. It is hypothesized that CPC and montmorillonite are both safe materials and TC-CPC is also safe for clinical use. However, because such materials are in prolonged contact with the oral mucosa, the mechanical properties and biocompatibility of TC-CPC must be considered in connection with clinical use.

The purpose of this study was to evaluate the mechanical properties and antimicrobial activity of TC-CPC, as well as its biocompatibility *in vitro* and *in vivo*.

2. Materials and methods

Details of the materials tested are listed in Table 1, which also includes the powder-to-liquid ratios used. Specimens used for experiments were prepared according to the manufacturer's instructions.

2.1. Physical tests

2.1.1. Hardness tests

A specimen for hardness test with 20 mm in diameter and 3 mm in thickness was prepared. Three specimens for each material were stored at 25 °C (room temperature) for 2 h and consecutively in deionized water at 37 °C for 2 h. A hardness value was measured by a universal testing machine (Testing machine EZ, Shimaz, Kyoto, Japan) with a cylindrical plunger (20 mm in diameter and 8 mm in height). The test condition specified in a crosshead speed of 1 mm/sec, an initial clearance of 5 mm, a displacement of 10% of the specimen, and a holding time of 60 s. The initial maximum stress value (N) was recorded as the hardness value. The mean was calculated by use of the value of three specimens. The data were analyzed using one-way ANOVA and Tukey's post hoc test for multiple comparisons ($p < 0.05$).

2.1.2. Consistency test and penetration test

The apparatus and method used were as specified by JIS-T6519 (Japanese Industrial Standard), which is based on ISO 10139-1 [27].

Consistency test: A syringe was used to dispense 2.0 ± 0.1 mL of material onto a glass plate. 30 ± 1 s after mixing, the specimen was compressed by vertically applying another glass plate termed the minor load, having a mass of 100 ± 1 g and a thickness not less than 2 mm. The plates and the specimen were then maintained at temperature at 37 ± 1 °C. 120 ± 1 s after mixing the major load of 1000 ± 5 g was applied vertically, so that the total load was 1100 ± 6 g. This load was maintained for 60 ± 1 s after which it was removed, leaving the minor load in place. 8 ± 0.5 min after mixing, the maximum and minimum diameters of the resulting disk of material were measured to an accuracy of ±0.5 mm. The average of the two measurements was calculated. To satisfy the standard specification the value should be between 25 mm and 75 mm. The mean was calculated by use of the value of four specimens.

Penetration test: The apparatus comprises a cylindrical penetrator of 1 mm in diameter which is fixed on a vertical rod (Japan Mecc, Tokyo, Japan). The vertical rod plus the penetrator has a mass of 50 ± 5 g. There is a locking device which fixes the penetrator at any vertical position and is used to measure the depth of penetration. A specimen was placed in a metal ring (30 ± 1 mm internal diameter and 3 ± 1 mm high) on the PMMA flat plate (50 mm x 50 mm x 4 mm). There was a slight overflow. A polyethylene film (110 mm x 110 mm x 0.025 mm) was used to cover the specimen. A glass plate (50 mm x 50 mm x 60 mm) was then placed on top and a weight of 2.0 ± 0.1 kg was applied. 10 min after mixing, the load, the glass plate and film were removed and the specimen was stored in a water bath at 37.0 ± 1.0 °C. 115 ± 0.5 min after mixing, the specimen was removed from the water bath and placed in the penetrometer. The penetrator was brought just into contact with surface of the specimen and locked in position. The rod from the gage (ABS Digimatic

Table 1
Tissue conditioners investigated.

Brand name	Manufacturer	Lot.No.	Code	Powder	Liquid		P/L ratio by weight
					plasticizer	Ethanol (wt.%)	
Experimental tissue conditioner	–	–	TC–CPC	Poly butyl methacrylate (PBMA) Poly ethyl methacrylate (PEMA) Cetylpyridinium chloride(CPC)-montmorillonite (2 wt.%)	Dibutyl sebacate (DBS)	7	1.5
Experimental tissue conditioner without CPC	–	–	TC	Poly butyl methacrylate (PBMA) Poly ethyl methacrylate (PEMA)	Dibutyl sebacate (DBS)	7	1.5
Fictioner	Nissin	5A8400500	NSTC	Poly butyl methacrylate (PBMA) Poly ethyl methacrylate (PEMA)	Dibutyl sebacate (DBS) Butyl phthalyl butyl glycolate (BPBG)	0	2.0
Tissue conditioner	GC	1507031	GCTC	Poly ethyl methacrylate (PEMA)	Dibutyl sebacate (DBS)	14	1.2

Indicator ID-S1012X, Mitsutoyo, Kawasaki, Japan) was brought into contact with the vertical rod and adjusted to zero. 120 ± 0.5 min after mixing, the vertical rod was released for 1.5 ± 0.5 s, allowing the penetrator to penetrate the specimen and it was then locked in position. The gauge was used to measure the depth of penetration three times and the average of the depth was calculated. The test was carried out at three measurement points (5 mm or over from the margin of specimen, and distance of 5 mm or over between the measurement). The mean value of three measurement points was recorded as value A. A had to be 1.8 mm or less to satisfy the standard specification. The specimen was stored again in water bath. The test was repeated after seven days and the mean value was recorded as value B. B had to be 0.18 mm or more to satisfy the standard specification. The penetration ratio was calculated as A / B had to be 5.0 or less. The mean was calculated by use of the value of three specimens.

2.2. Antimicrobial test

2.2.1. Culture of organisms

C. albicans IFM40009 (ATCC48130) was cultured in Sabouraud dextrose broth and *S. aureus* FDA209P (ATCC 6538P) was cultured in nutrient broth. The organisms were cultured under aerobic condition at 37 °C for 24–48 h, at which point the growth was in the mid-logarithmic phase. Aliquots of the microbe cultures were collected and stored at −80 °C, and their optical density as observed at a wavelength of 600 nm was adjusted to 0.1 when used.

2.2.2. Detection of viable organisms

The number of viable organisms was determined by counting colonies on an agar plate, and the viable counts were expressed using the colony-forming unit. The agar plates used were selective media; *Staphylococcus* no. 10 agar (Eiken Chemical Co., Tokyo, Japan) for *S. aureus*, and *Candida* GS agar (Eiken) for *C. albicans*. The numbers of organisms adhered to tissue conditioners were determined as follows. The tissue conditioner samples were incubated with broth containing organisms, and then the broth was removed, washed three times with sterilized phosphate-buffered saline (PBS) and treated with 0.1% (v/v) Triton X-100 in sterilized PBS for 10 min on a vibrator. The numbers of floating-microbes in the broth were determined by plating serial 10-fold dilutions of the broth containing microbes onto each of selective agar plates. The numbers of colonies formed on the plates were treated as viable counts of floating-microbes as described.

2.2.3. Assay of antimicrobial activity of CPC-Mont

To confirm the antimicrobial activity of CPC-Mont in the tissue conditioner, two tissue conditioners were used, named TC-CPC (tissue conditioner with CPC-Mont) and TC (tissue conditioner without CPC-Mont), as listed in Table 1. Two sets of these two tissue conditioners were prepared; one set was used soon after the preparation and another set was used after incubation for 1 week in 50 ml of sterilized PBS at 37 °C to examine the sustainability of CPC-Mont in the tissue conditioner. The samples were incubated with broth containing organisms, and their viable counts were determined as described.

2.2.4. Sustainability of antimicrobial activity of TC-CPC

Samples of TC-CPC, GCTC, and NSTC (defined in Table 1) were put in 50 ml tubes filled with sterilized PBS and incubated at 37 °C. The PBS in the tubes was exchanged once a week for up to 4 weeks. The numbers of viable organisms in the broth were determined as described.

2.2.5. Determination of CPC recharging effect on TC-CPC

Since montmorillonite absorbs some antibiotics, owing to its structure [28], attempts were made to recharge CPC into the tissue conditioner after releasing CPC. The novel TC-CPC was prepared and incubated in 500 ml of PBS to promote release of CPC. The PBS was exchanged once a week; after incubation for 2 weeks, the tissue conditioner was put in 2% CPC solution dissolved in PBS for recharging or PBS as a control. The numbers of viable organisms in the broth or adhered to the samples were determined as described to determine the effect of recharging CPC to the tissue conditioners.

2.2.6. Statistical analysis

Student's *t*-test for multiple observations was used for statistical analysis. Statistical significance was set at $p < 0.05$.

2.3. Cytotoxicity test

2.3.1. Preparation of eluates of specimens

The culture medium for NIH-3T3 mouse fibroblast cells was supplemented with 10% calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The culture medium for Ca9-22 human gingival carcinoma cells was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. For the preparation of eluates, each specimen (TC–CPC and GCTC) was

rinsed with 70% ethanol and PBS followed by soaking in 2 mL cell culture medium in a 12-well plate in a humidified incubator at 37 °C with 5% CO_2 and 95% air for 1 and 7 days [29]. After 1 day, collected medium extracts were kept as the eluate at 1 day. After incubation for 7 days, the specimen was further incubated in the changed medium for 1 day, and collected medium extracts were kept as the eluate at 7 days. The surface area to volume ratio was 0.88 cm^2/mL; this was set according to the ISO standard 10,993-5 (0.5–6.0 cm^2/mL) [30].

2.3.2. Cytotoxicity assay

Cell viability was estimated using a colorimetric assay, utilizing the tetrazolium salt 4-[3-(4-*Iodophenyl*)−2-(4-*nitrophenyl*)−2H-5-*tetrazolio*]−1,3-benzene disulfonate (WST-1) (Takara Bio Inc., Otsu Shiga, Japan) according to the manufacturer's instructions [31]. Samples of NIH-3T3 mouse fibroblast cells and Ca9-22 human gingival carcinoma cells were seeded at 1×10^5 cells/mL in a 96-well plate and incubated for 24 h. The medium was replaced with 100 µL of the eluates obtained 1 and 7 days after immersion. The cells were then incubated for 1 day. The WST-1 colorimetric assay was conducted by adding 10 µL of WST-1 (5 mg/mL) into a well to make a final concentration of 0.5 mg/mL and then incubating the plate. Each assay was run in triplicate. The optical density was measured at a wavelength of 450 nm using a microplate reader (Thermo, Vantaa, Finland) after 24 h of incubation. Cell viability was measured by dividing the optical density of the treated specimen by the optical density of the untreated specimen.

2.3.3. Statistical analysis

Two-way analysis of variance (ANOVA) and Bonferroni's test for multiple comparisons were used to statistically analyze the mean values of cell viabilities ($p < 0.001$).

2.4. Animal experiments

Spherical samples with a diameter of 3 mm were formed, and were rinsed with 70% ethanol and PBS. Twelve 10-week-old male Wistar rats were used in this study. The specimens (TC-CPC and GCTC) were implanted in pockets formed in the subcutaneous tissue of the dorsal region, and set on the lower lip mucosa by suturing mucosa to cover them. Rats were sacrificed at 1 and 14 days, respectively, after surgery for the lower lip mucosa and the subcutaneous tissue experiments. The specimens were harvested with the surrounding tissue, fixed using 10% neutral buffered formaldehyde, and embedded in paraffin. Specimens stained with hematoxylin and eosin were observed using light microscopy. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, Hokkaido University (Animal testing approval number: 15-0132).

3. Results

3.1. Physical hardness test

3.1.1. Hardness test

Fig. 1 shows the hardness of tissue conditioners kept at room temperature for 2 h. It was found that there was significance between the results of TC-CPC and that of GCTC, also TC-CPC and NSTC (as defined in Table 1). Fig. 2 also shows these conditioners after immersion in deionized water for 2 h. It was found that there was no significance between the results for TC-CPC and GCTC, while there was significance between TC-CPC and NSTC.

3.1.2. Consistency test and penetration test

In the consistency test, the value of consistency test showed 47.6 ± 1.1 mm. It was showed that TC–CPC satisfies the JIS-T6519 standard specification (Table 2).

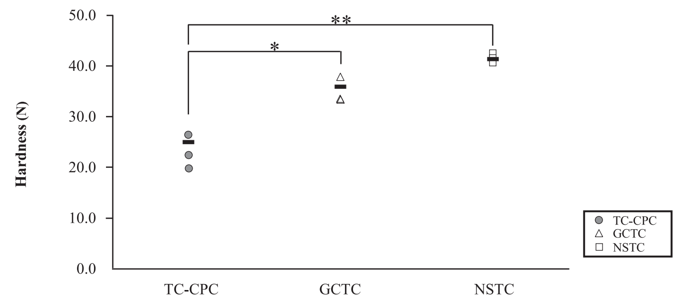

Fig. 1. Physical hardness (N) of tissue conditioners stored at room temperature for 2 h. The TC-CPC sample remained soft, as compared with the other materials. $^{**}p < 0.01$; $^{*}p < 0.05$. Thick black line shows average level in each group.

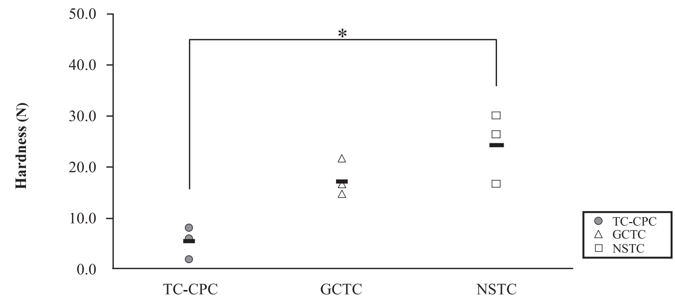

Fig. 2. Physical hardness (N) of tissue conditioners immersed in deionized water for 2 h. The TC-CPC sample maintained softness, as compared with the other materials. $^{**}p < 0.01$; $^{*}p < 0.05$. Thick black line shows average level in each group.

In the penetration test, the value A was 3.1 ± 0.0 mm, and value B was 2.9 ± 0.1 mm. Therefore, the value of penetration ratio (A/B) was 1.1 ± 0.0. Since A must be 1.8 mm or less, the degree of penetration after 120 min exceeded the provision of JIS-T6519 (Table 2). Conversely, the degree of penetration after 7 days and the penetration ratio were satisfied, as B had to be 0.18 mm or more and A/B had to be 5 or less (Table 2).

3.2. Antimicrobial test

3.2.1. Antimicrobial activity of CPC-Mont in the tissue conditioner

It was found that the addition of CPC-Mont to the tissue conditioner significantly decreased the quantities of both adhered and free-floating *C. albicans* (Fig. 3(a)). This effect was sustained for a week at this condition (Fig. 3(a)). Similar results were obtained for *Staphylococcus aureus* (Fig. 3(b)).

3.2.2. Comparison of antimicrobial activities among various tissue conditioners

It was found that no adhered *C. albicans* was detected on TC–CPC after incubation for 2 h, whereas more than 10^3 colony-forming units of *C. albicans* were detected on GCTC and NSTC (Fig. 4(a)). The quantity of free-floating *C. albicans* in the broth incubated with TC-CPC was significantly decreased after incubation for 2 h, whereas no significant decrease in the quantities of *C. albicans* were observed in the broths incubated with GCTC or NSTC (Fig. 4(b)). Similar results were obtained in the case of *S. aureus* (Fig. 4(c) and (d)). Although there is no description of antimicrobial activity of GCTC, to our knowledge, GCTC showed antimicrobial effects in this experiment.

3.2.3. Sustainability of antimicrobial activity of TC-CPC

It was shown that growth of *C. albicans* was significantly suppressed by TC-CPC for up to 3 weeks compared with NSTC (Fig. 5(a)). In addition, GCTC significantly suppressed the growth

Table 2
The data of consistency test and penetration test.

Item		JIS (ISO) standard	Data
Consistency		25–75 mm (±15%)	47.6 ± 1.1 mm
Penetration	After 2 h	1.8 mm or less	3.1 ± 0.0 mm (Nonstandard)
depth	After 7 days	0.18 mm or more	2.9 ± 0.1 mm
Penetration ratio		5.0 or less	1.1 ± 0.0

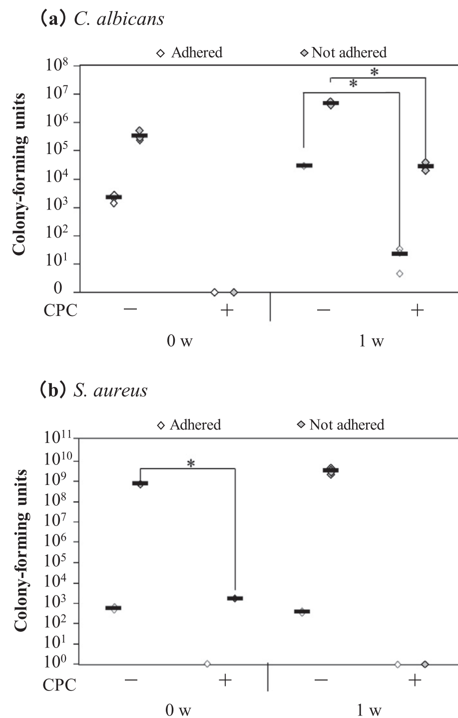

Fig. 3. Antimicrobial activity of CPC in the tissue conditioner: number of viable counts of (a) *C. albicans* and (b) *S. aureus* adhered to tissue conditioners or free-floating in broth incubated with tissue conditioner with CPC (+) or without CPC (−). The tissue conditioners were used soon after preparation (0 w) or allowed to release CPC for a week (1 w). Results are expressed as mean ± standard deviation of triplicate assays of a representative experiment. * $p < 0.05$. Thick black line shows average level in each group.

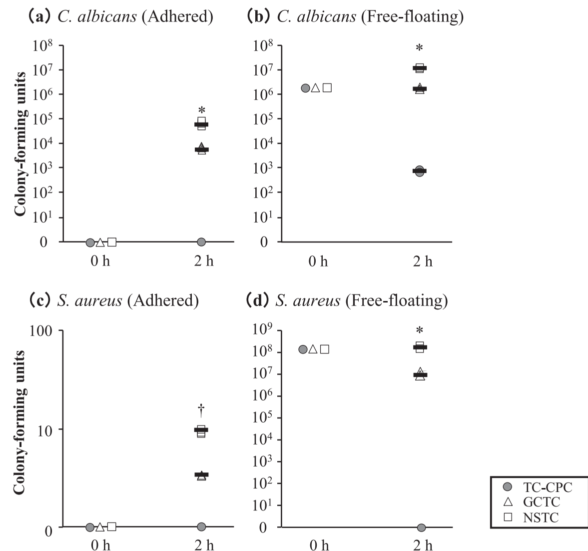

Fig. 4. Number of viable counts of *C. albicans* (a, b) and *S. aureus* (c, d) adhered to tissue conditioners or free-floating in the broth incubated with TC-CPC, GCTC, or NSTC incubated for 2 h. The tissue conditioners were used soon after preparation. Results are expressed as the mean ± standard deviation of triplicate assays of a representative experiment. * Statistically significant differences among TC-CPC, GCTC, or NSTC; † statistically significant difference between TC-CPC and GCTC or NSTC ($p < 0.05$). Thick black line shows average level in each group.

of *C. albicans* for up to 4 weeks compared with NSTC, and, interestingly, GCTC showed the strongest antimicrobial activity from 1 week to 4 weeks (Fig. 5(a)). In the case of *S. aureus*, TC-CPC significantly suppressed the growth of the microbe for up to 3 weeks (Fig. 5(b)). It was also found that GCTC possessed significant antimicrobial activity against *S. aureus*, although the effect of TC-CPC was rather more potent (Fig. 5(b)).

3.2.4. Recharging CPC into TC-CPC

More than 10^4 colony-forming units of *C. albicans* were found to adhere to the sample of TC-CPC incubated in PBS for 2 weeks (Fig. 6(a)). Similar results were obtained in the case of free-floating *C. albicans* (Fig. 6(a)) and in the case of free-floating *S. aureus* (Fig. 6(b)), whereas fewer than 10 colony-forming units of *S. aureus* were found to adhere to the sample (Fig. 6(b)). It was found that the CPC-released TC-CPC recovered antimicrobial activity against *C. albicans* when incubated with CPC solution, since CPC-recharged TC-CPC showed significant antimicrobial activity compared with CPC-released TC-CPC (Fig. 6(a)). Similar results were obtained using *S. aureus* (Fig. 6(b)).

3.3. Cytotoxicity test

The cell viabilities (%) of the TC-CPC and GCTC specimen eluates at 1 and 7 days are shown in Fig. 7. The cell viability of the TC-CPC of the eluates at 1 day was significantly less than that of the GCTC on both NIH-3T3 mouse fibroblast cells and Ca9-22 human gingival carcinoma cells. However, the cell viability of the TC-CPC using eluates at 7 days showed no significant difference compared with the GCTC for both organisms. Furthermore, in the TC-CPC group, the cell viability at 1 day was significantly less than that at 7 days. This shows that TC-CPC had stronger cytotoxicity than GCTC at 1 day but the cytotoxicity decreased to the same level as the GCTC at 7 days.

3.4. Animal experiment

At 1 day on the rat mucosa, the epithelial layers of oral mucosa under TC-CPC and GCTC samples were found to be flat, while the normal mucosa was had a convex–concave pattern. However, an inflammatory response could not be recognized on the surface of the mucosa, which appeared identical to normal mucosa in both specimens, as shown in Fig. 8.

In the subcutaneous tissue, slight inflammatory responses, such as some dilated capillaries, mesenchymal cells, and thin collagen fibers, were observed around the specimen of TC-CPC and GCTC at 2 weeks (Fig. 9).

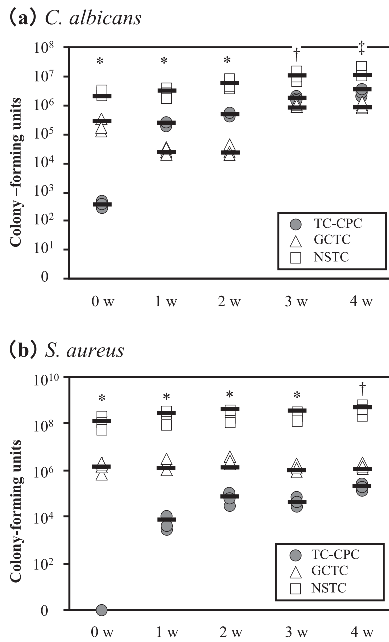

Fig. 5. Sustainability of antimicrobial activity of TC-CPC: number of viable counts of free-floating cells in the broth incubated with TC-CPC, GCTC, or NSTC. The tissue conditioners were used soon after preparation or allowed to release CPC for 1 to 4 weeks. The results are expressed as the mean ± standard deviation of triplicate assays of a representative experiment. (a) *C. albicans*. * Statistically significant differences among TC-CPC, GCTC, or NSTC; † statistically significant difference between TC-CPC and GCTC or NSTC; ‡ statistically significant difference between GCTC and TC-CPC or NSTC ($p < 0.05$). (b) *Staphylococcus aureus*. * Statistically significant differences among TC-CPC, GCTC, or NSTC; † statistically significant difference between NSTC and TC-CPC or GCTC ($p < 0.05$) Thick black line shows average level in each group.

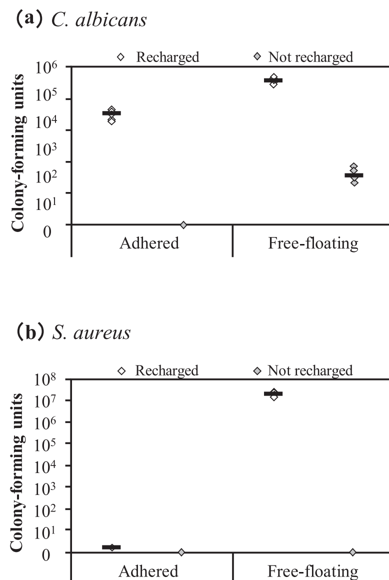

Fig. 6. Determination of CPC recharging effect on TC-CPC. Number of viable counts of (a) *C. albicans* and (b) *Staphylococcus aureus* adhered to tissue conditioners or free-floating in the broth incubated with TC-CPC. The sample of TC-CPC was allowed to release CPC for 2 weeks and then TC-CPC was recharged or not recharged with CPC. Results are expressed as the mean ± standard deviation of triplicate assays of a representative experiment. * statistically significant differences ($p < 0.05$). Thick black line shows average level in each group.

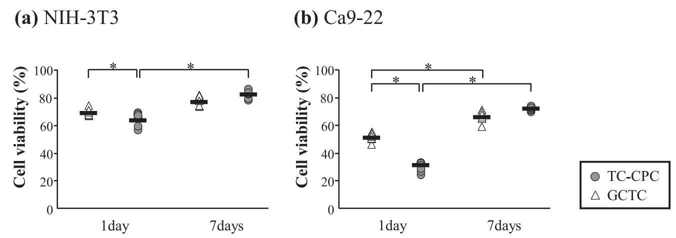

Fig. 7. Cell viability (%) of experimental and control specimens with specimens incubated for 1 and 7 days. The cell viability of the TC-CPC using eluates with specimens incubated for 1 day is significantly less than that of the GCTC. The cell viability of the TC-CPC using eluates with specimens incubated for 7 days shows no significant difference between NIH-3T3 and Ca9-22 cells. * $p < 0.001$. Thick black line shows average level in each group.

4. Discussion

In this study, CPC was used as an antimicrobial agent in combination with montmorillonite. It is known that CPC possesses a broad antimicrobial spectrum, with rapid killing of gram-positive pathogens and yeast [32]. Montmorillonite, which is a mineral, was also used for the purpose of a carrier of TC-CPC, as it is known for its safety in materials used for soap and bath articles [33].

Results of antimicrobial tests showed that the addition of CPC-Mont confers antimicrobial activity to tissue conditioner. Furthermore, the TC-CPC possesses strong antimicrobial activity against both adhered and free-floating microbes, as compared with GCTC or NSTC. The antimicrobial activity of TC-CPC was found to be the strongest just after the preparation. Moreover, the level of antimicrobial activity is decreased after 1 week. This result suggests that CPC would be released from tissue conditioner over time.

The TC-CPC exhibited excellent antibacterial activity. The added amount CPC-Mont is a major factor influencing the antibacterial activity. In our previous studies [34,35,39,40], we demonstrated that a silver-zeolite (Zeomic AJ10N, Sinanen Zeomic, Nagoya, Japan) as an inorganic antimicrobial agent was applied to the tissue conditioning materials, and the tissue conditioner containing 2 wt%-silver–zeolite is a possible candidate for a novel antimicrobial tissue conditioner. Ueshige et al. [34] reported that 2, 5, 10 wt%- silver-zeolite were incorporated into the powder of five commercially available tissue conditioners, and the application of 2 wt%-silver-zeolite could exert the smallest influence on their viscoelastic properties. The average particle size of the silver-zeolite is 0.6–2.2 µm. The cetylpyridinium chloride montmorillonite (CPC-Mont) is about the size of the silver-zeolite, and both crystals of montmorillonite and zeolite consist of aluminosilicate even though they are different crystal structures. From the above reasons, we assumed that the dispersion of CPC-Mont into the powder of the tissue conditioner would be almost equal to that of silver-zeolite. Therefore, in this study, the dosage of CPC-Mont was set to 2 wt%, and the assumption was verified.

In this study, it was set to 2wt% with reference to the value applied as another antimicrobial materials [34,35], and sufficient antimicrobial activity was confirmed by this addition amount.

The antimicrobial activity is exerted by binding of the positive charge of CPC to negatively charged microbial surfaces [36]; this property of CPC was also utilized for recharging into montmorillonite, because the adsorption by montmorillonite occurs by cation exchange [37]. Utilization of montmorillonite with CPC may be one of the best combinations in tissue conditioners, since this combination showed significantly good antimicrobial activity (Figs. 3–6).

The results of cytotoxicity tests using fibroblast and epithelial cells showed that TC-CPC had a strong cytotoxicity on the eluates at 1 day after immersion. However, the cytotoxicity of TC-CPC was at the same level as the GCTC at 7 days. These findings are sim-

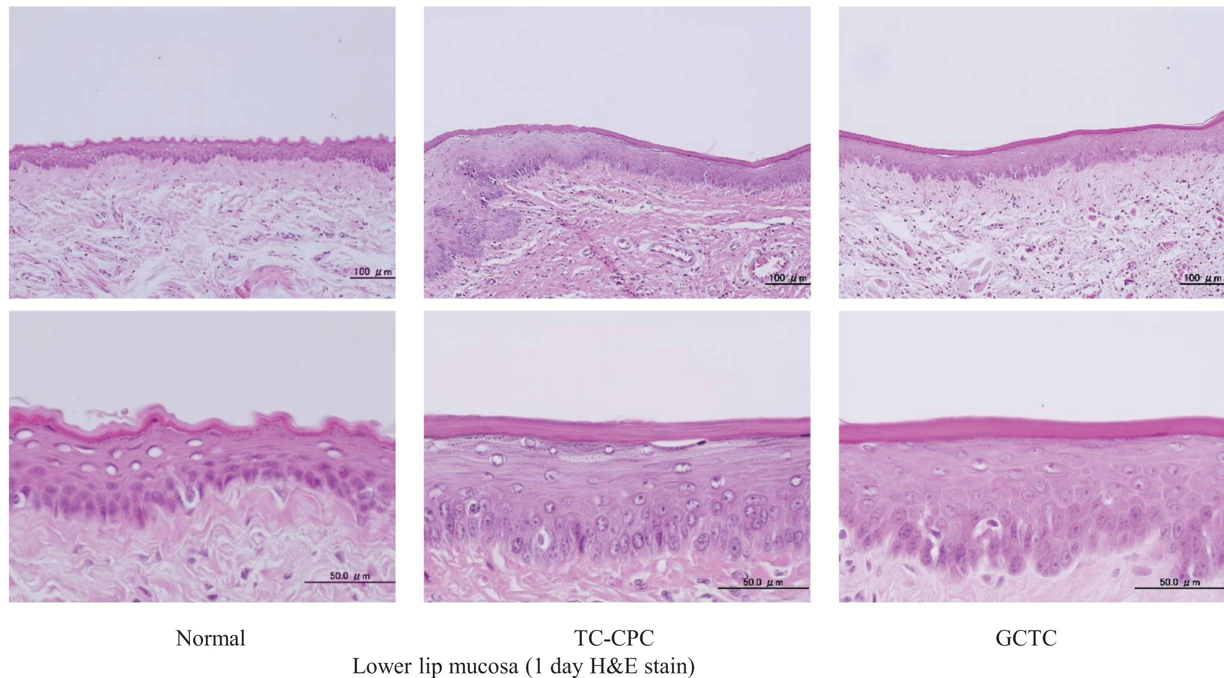

Fig. 8. Materials pasted on the rat lower lip mucosa after 1 day. The epithelial layers of mucous membrane under TC-CPC and GCTC were found to be flat, while the normal mucosa had a convex–concave pattern. The inflammatory response could not be recognized on the surface of the mucosa, which appeared identical to normal mucosa in all specimens.

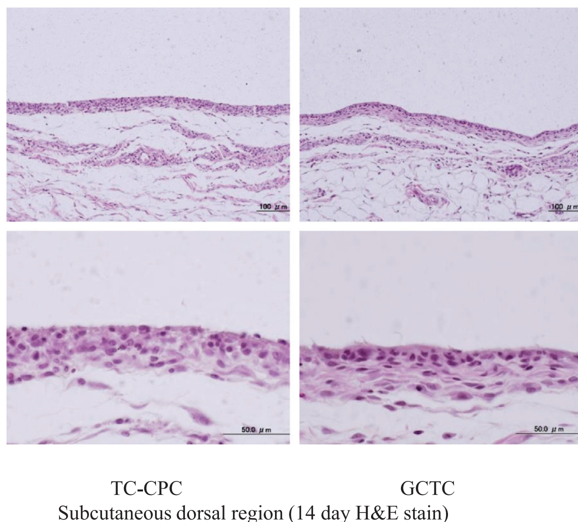

Fig. 9. Materials implanted in the pockets formed in the subcutaneous tissue of the dorsal region after 2 weeks. In the subcutaneous tissue, some dilated capillaries, mesenchymal cells, and thin collagen fibers, were observed around the specimen of TC-CPC and GCTC at 2 weeks.

ilar to the results of antimicrobial activity tests. The cytotoxicity seems to be associated with antimicrobial activity [38]. It is suggested that CPC, which is an antibacterial ingredient of TC-CPC, is released for approximately 1 week. It is thought that the cytotoxicity became weaker because the antimicrobial properties of TC-CPC decreased. There was a significant difference between the results at 1 and 7 days in the GCTC group. This showed that the eluates from GCTC were damaging to delicate Ca9-22 cells, especially at early times.

The results from animal experiments showed that contact of the specimens to the lower lip mucosa did not occur any inflammatory change. Also, the inflammatory response of TC-CPC in the subcutaneous tissue was almost the same as that of GCTC. In the case of materials, the inflammation appears to enclose the specimen.

Therefore, TC-CPC was considered to be not prophlogistic, like other commercial material, and is considered likely to be useful in clinical situations.

The composition of TC-CPC was originally designed for the purpose of maintaining softness, not using a commercial one. It is considered that the result of the physical property test reflects the composition design. The antibacterial performance was due to the controlled release of CPC from CPC-Mont on the surface of the tissue conditioner. Therefore, in order to prove the biological safety of CPC released as an antibacterial component, it was verified by a cytotoxicity test and an animal experiment. As a result, it has been proved that the biological safety is secured at the controlled release amount of CPC which exhibits the antibacterial effect.

The disadvantage of the tissue conditioner developed in this study is the cell viability of the TC-CPC of elutes at 1 day was significantly less than of the GCTC on both cells. It showed that TC-CPC have toxic one than GCTC in 1 day *in vitro* tests. On the other hand, TC-CPC using elutes at 7days showed no significant difference compared with GCTC. In animal experiment of *in vivo* tests, there were no significantly difference inflammatory response between TC-CPC and GCTC.

The TC-CPC samples failed the penetration test after 2 h while the other requirements of standard JIS-T6519: 2005 were satisfied [33]. However, in clinical use, tissue conditioners were frequently mixed by changing the powder-to-liquid ratio according to the manufacturer's instructions. Thus, TC-CPC could satisfy the penetration test, if the powder-to-liquid ratio were changed. Therefore, we investigated the physical hardness of the tissue conditioner. As a result, TC-CPC showed no significant difference from other commercial tissue conditioners under both dry and wet conditions. It seems that TC-CPC has the same hardness as commercially available products; therefore, it deduced that TC-CPC might satisfy mechanical properties in clinical situations. In addition, antimicrobial activity was recovered by the recharging CPC to TC-CPC. There-

fore, this material would be useful on the durability in clinical situation.

Although the results obtained from *in-vitro* and *in-vivo* studies cannot be directly extrapolated to clinical situations, this study demonstrates that TC-CPC might play an important role in preventing older patients from contracting aspiration pneumonia, as it has excellent antimicrobial activity and biocompatibility in addition to mechanical properties. Clinical trials would be required for its practical use.

5. Conclusion

A novel tissue conditioner containing CPC was developed in this study. It was demonstrated that the novel material has excellent antimicrobial activity and similar mechanical properties and biocompatibility to commercially available materials.

Funding

This study was supported by a Grant-in-Aid for Development of Medical Devices through Collaboration between Medicine and Industry (no. 27-027) from the Japan Agency for Medical Research and Development (2015-2016).

Declaration of Competing Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jpor.2019.12.002.

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