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博 士 論 文

Development of the tissue conditioner containing CPC-Montmorillonite as a new antimicrobial agent. A pilot study on antimicrobial activity and biocompatibility.
(新規抗菌材 CPC-Montmorillonite 含有粘膜調整材の抗菌性及び生体適合性)

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北海道大学

大学院歯学研究科口腔医学専攻

直　江　　翼

Title: Development of the tissue conditioner containing CPC-Montmorillonite as a new antimicrobial agent. A pilot study on antimicrobial activity and biocompatibility.

Antimicrobial and biocompatibility of tissue conditioner containing CPC-Montmorillonite as a new antimicrobial agent

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Key Word: tissue conditioner. cetylpyridinium chloride. mechanical properties. antimicrobial activity. biocompatibility.

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Abstract

Purpose: The aim of this study was to evaluate the mechanical properties, antimicrobial activity and biocompatibility of a novel antimicrobial tissue conditioner containing cetylpyridinium chloride.

Materials and Methods: To examine the mechanical properties of the novel developed material, measurements according to JIS-T6519 and hardness test were carried out. Antimicrobial activity against *C. albicans* and *S. aureus* was evaluated. The cell viabilities of fibroblasts and epithelial cells using eluates from materials were measured for cytotoxicity. Also, to assess tissue response, animal experiments were carried out. As control, tissue conditioners which are available on the market were used.

Results: On penetration test of JIS-T6519, results of the novel material failed to satisfy the standard. For hardness test, the results were kept on border of other manufactures. In antimicrobial test, the novel tissue conditioner showed antimicrobial activity against *C. albicans* and *S. aureus* compared to control materials. This effect was sustained for a week at *C. albicans.* In the case of *S. aureus*, the growth of the microbe was suppressed up to 3 weeks. Cell viability of the novel material for the eluate at 1 day was significantly lower than those of control materials for both cells. But the cell viability at 7 days showed no significantly difference. Animal experiments showed that

inflammatory responses around materials were not observed on the oral mucosa as control material.

Conclusion: Within the limitation of this *in vitro* and *in vivo* study, the results suggest that the new developed tissue conditioner containing cetylpyridinium chloride has not only more excellent antimicrobial property, but also same mechanical property and biocompatibility as tissue conditioners on the market.

Key Words: tissue conditioner. cetylpyridinium chloride. mechanical properties. antimicrobial activity. biocompatibility.

1 Introduction

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

Aspiration pneumonia in aged patients is one of the most serious problem, which may be mortal[4,5] or at least may result in decline in the individual's quality of life. It has been also 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 adhere to the inner surface of complete dentures, including *C. albicans*, *S. aureus* and *S. mutans*[8, 9]. Especially, *C. albicans* and *S. aureus* are crucial in aspiration pneumonia[10, 11]. Therefore, it is quite important to prevent aged persons from causing the pneumonia by reducing number of microbes attached to dentures. The proliferation of microbes is known to be induced by the surface roughness of acrylic resins on dentures [12-14]. To make matters worse, when tissue conditioner is kept on having put on the denture for a long time, because of with their porosity and softness, it

might act as a worse reservoir for microorganisms compared to acrylic resin that allow microbial adherence, accumulation and penetration, or become to degraded to have deposition of plaque and found to colonize on the surface.

To reduce the number of microbes on tissue conditioners, attempts were made to add antimicrobial agents such as oil and silver [15-17], but it has been reported that they have harmful effects like metal allergy[18-20]. Biocompatibility is very important property for tissue conditioner as well as antimicrobial activity. However, there are few studies on animal experiments. Therefore, antimicrobial tissue conditioner is subjected to extensive research, but it has not reached 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, suggesting that it could play a role in oral care of the older people. It is known that CPC molecules bind 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, therefore, altering it and generating an imbalance in the osmotic regulation, resulting in loss of cytoplasmic material and ultimately cell death[22, 23]. However, it was found that antimicrobial effect does not persist with CPC alone, some kind of carrier should be necessary to sustain its antimicrobial activity.

Montmorillonite is a highly safe materials and often use as human trials[24]. It is a very soft phyllosilicate group of minerals as clay and has been extensively used in catalytic processes by cation exchange and also known as a material attracting attention for adsorption[25]. By using this property, CPC that adhered to the surface of montmorillonite would be able to exhibit sustainable antibacterial effects. Thus, we developed cetylpyridinium chloride with montmorillonite [TC-CPC] as the antimicrobial tissue conditioner with high novelty.

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

2 Materials and methods

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

2-1 Physical hardness test

2-1.1 Consistency Test

The apparatus and method used were as specified by JIS-T6519 (Japanese Industrial Standard) which based on ISO 10139-1. A 2 ml of material put onto a glass

plate. After mixing for 30 sec, the sample was compressed by vertically applying another glass plate, having a mass of 100 g and a thickness not less than 2 mm. The plates and the sample were then maintained at temperature at 37 °C. At 120 sec after mixing the major load (1000 g) was applied vertically, so that the total load (major and minor) was 1100 g. This load was maintained for 60 sec. At 8 min after mixing, the maximum and minimum diameters of the resulting disc of material were measured. The average of the two measurements was calculated.

2-1.2 Penetration Test

The penetrometer apparatus and method used were as specified by JIS-T6519. The cylindrical penetrator has a mass of 50 g. A sample of approximately 20 ml was placed in a metal ring (30 mm in diameter and 3 mm in height) on the flat plate made of polymer sheet. A polymer sheet was used to cover the material. A glass plate was then placed on top and a weight of 2000±100 g was applied. At 10 minutes after mixing, the glass plate and polymer sheet were removed, and the sample was stored in a water bath at 37 °C. At 115 min after mixing the specimen was removed from the water bath and placed in the penetrometer. The cylinder was released for 1.5 sec after 120 min, allowing the penetrator to penetrate the specimen. The test was carried out three times on each specimen using a different area each time. The mean of the three measurements

was recorded as value A. The specimen was stored again in water at 37 °C and the test was repeated after seven days and the mean value was recorded as value B. The penetration ratio was calculated as A/B and the result was expressed to one decimal place.

2-1.3 Hardness Test

The apparatus (Testing machine EZ, Shimaz Japan) with cylindrical plunger (20 mm in diameter and 8 mm in height) was used. A specimen was shaped in 20 mm internal diameter and 8 mm height. The specimen was stored on room temperature 2 hours (Dry-2h) or in deionized water 2 hours (Wet-2h). The plunger was brought 5 mm apart of the specimen and locked. At 120 min after mixing the plunger was released for 60 sec, a cross-head speed of 1 mm/sec allowing the plunger to measure the hardness.

The test was carried out three times on each specimen using a different specimen each time. The mean of the three measurements was recorded. The values of mean ± S.D. (mm) and the hardness (N) were calculated.

2-2 Antimicrobial test

2-2.1 Culture of organisms

C. albicans IFM40009 (ATCC48130) was grown in Sabouraud dextrose broth,

and *S. aureus* FDA209P (ATCC 6538P) was grown in nutrient broth. The organisms were cultured under aerobic condition at 37 °C for 24-48 h, at which point the growth were in the mid-logarithmic phase. Their aliquots were made and stored at -80 °C, and their optical density at 600 nm (OD600) wasadjusted 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 (CFU). The agar plates used were selective media, such as, 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 3 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.

2-2.3 Assay of antimicrobial activity of CPC

To confirm the antimicrobial activity of CPC in the tissue conditioner, two tissue conditioners were used. One is TC-CPC and another is TC (Table 1). Two sets of these two tissue conditioners were prepared, and one set was used soon after the preparation

and another set was used after a week-incubation in 50 ml of sterilized PBS at 37 °C to examine the sustainability of CPC 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

TC-CPC, GCTC and NSTC samples were put in a 50 ml tube filled with sterilized PBS and incubated at 37 °C. PBS in the tubes was exchanged once a week up to 4 weeks. The numbers of viable organisms in the broth were examined as described.

2-2.5 Determination of CPC recharging effect on TC-CPC

Since montmorillonite has a sorption property to some antibiotics due to its structure[29], attempts were made to recharge CPC into the tissue conditioner after releasing CPC. TC-CPC tissue conditioner was prepared and incubated in 500 ml of PBS to promote release of CPC. PBS was exchanged once a week, and after 2 weeks incubation, 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 examined 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 a P value of less than 0.05.

2-3 Cytotoxicity test

2-3.1 Preparation of eluates of specimens

Culture medium for NIH-3T3 was supplemented with 10% calf serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. And that for Ca9-22 was supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. For the preparation of eluates, each specimen (TC-CPC tissue conditioner and GCTC as control) was rinsed with 70% ethanol and phosphate-buffered saline (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[26]. After 1 day, collected medium extracts were kept as the eluate at 1 day. After 7 days incubation, specimen was furthermore 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 which was set according to the ISO standards (0.5-6.0 cm^2/mL)[27].

2-3.2 Cytotoxicity assay

Cell viability was estimated by a colorimetric assay using 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 manufacturer's

instructions[28]. 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 each other. The medium was replaced with 100 μL of the eluates obtained the 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 450 nm using a micro-plate reader (Thermo, Vantaa, Finland) after 24 hours of incubation. Cell viability was measured by dividing the optical density value of the treated specimen by that of the untreated one.

2-3.3 Statistical analysis

Analysis of variance (2-way 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

The samples were formed to spherical shape with a diameter of 3 mm, and were rinsed with 70% ethanol and PBS. GCTC was used as control. Twelve 10 weeks old male Wistar rats were used in this study. The specimens were implanted in the pockets formed in the subcutaneous tissue of the dosal region, and set on the lower lip mucosa

by suturing mucosa as covering them. Rats were sacrificed at 1 and 14 days after surgery, on the lower lip mucosa and in the subcutaneous tissue experiments, respectively. The specimens were harvested with the surrounding tissue, and then fixed using 10% neutral buffered formaldehyde and embedded in paraffin. Hematoxylin and eosin-stained specimens were observed by 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

3-1.1 Consistency Test

The value of consistency test showed 34 mm. It showed that TC-CPC satisfy the JIS-T6519 standard specification.

3-1.2 Penetration Test

In the penetration test, the value A was 2.18 mm, and value B was 2.03 mm. Therefore, the value of penetration ratio (A/B) was 1.07. Since A must be 1.8 mm or less, the degree of penetration after 120 minutes exceeded the provision of JIS. On the other hand, the degree of penetration after 7 days and penetration ratio were satisfied to border, as B had to be 0.18 mm or more and A/B had to be 5 or less.

3-1.3 Hardness Test

Figure 1 showed the hardness of tissue conditioners put on room temperature at 2 hours. It was found that there was no significance among results of TC-CPC, DPTC and SFTC. Figure 2 also showed those in deionized water at 2 hours. It was found that there was no significance among results of TC-CPC, GCTC and SFTC.

3-2 Antimicrobial test

3-2.1Antimicrobial activity of CPC in the tissue conditioner

It was found that addition of TC-CPC to tissue conditioner significantly decreased the numbers of both adhered and free-floating *C. albicans* (Fig. 3A). This effect was sustained for a week at this condition (Fig. 3A). Similar results were obtained in the case with *S. aureus* (Fig. 3B).

3-2.2 Comparison of antimicrobial activities among various tissue conditioners

It was found that no adhered *C. albicans* on TC-CPC tissue conditioner was detected after 2 hours incubation, whereas more than 10^3 CFU of *C. albicans* on GCTC and NSTC were detected (Fig. 4A). The number of free-floating *C. albicans* in the broth incubated with TC-CPC tissue conditioner was significantly decreased after 2 hours incubation, whereas no significant decrease of the numbers of *C. albicans* were observed in the broth oursincubated with GCTC or NSTC (Fig. 4B). Similar results were obtained in the case of *S. aureus* (Fig. 4C and D). Although there is no description

of antimicrobial activity of GCTC, GCTC showed antimicrobial effects in this experiments.

3-2.3 Sustainability of antimicrobial activity of TC-CPC tissue conditioner

It was shown that growth of *C. albicans* was significantly suppressed by TC-CPC tissue conditioner up to 3 weeks compared with NSTC (Fig. 5A). GCTC also significantly suppressed the growth of *C. albicans* up to 4 weeks compared with NSTC, and interestingly, GCTC showed the strongest antimicrobial activity from 1 week to 4 weeks (Fig. 5A). In the case of *S. aureus*, TC-CPC significantly suppressed the growth of the microbe up to 3 weeks (Fig. 5B). GCTC was also found to possess significant antimicrobial activity on *S. aureus*, although the effect of TC-CPC tissue conditioner was rather potent (Fig. 5B).

3-2.4 CPC recharging into TC-CPC tissue conditioner

More than 10^4 CFU of *C. albicans* were found to adhere to the sample of TC-CPC incubated in PBS for 2 weeks (Fig. 6A). Similar results were obtained in the case of free-floating *C. albicans* (Fig. 6A) and in the case of free-floating *S. aureus* (Fig. 6B), whereas less than 10 CFU of *S. aureus* were found to adhere to the sample (Fig. 6B). It was found that the CPC-released TC-CPC tissue conditioner 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. 6A). Similar results were obtained using *S. aureus* (Fig. 6B).

3-3 Cytotoxicity test

The cell viabilities (%) on the experimental and control specimens eluates at 1 and 7 days are shown in Figure 7. The cell viability of the TC-CPC of the using eluates at 1 day was significantly lower than that of control on both NIH-3T3 and Ca9-22. But the cell viability of the TC-CPC using eluates at 7 days showed no significant difference compared to control on both cells. Furthermore, in the TC-CPC group, the cell viability at 1day was significantly lower than that at 7 days. It showed that TC-CPC had more strong cytotoxicity than control at 1 day but it decreased to the same level as control at 7 days.

3-4 Animal experiment

At 1day on the rat mucosa, the epithelial layers of oral mucosa under TC-CPC and GCTC were found to be flat, while the normal mucosa was observed the convex-concave pattern. However, the inflammatory response could not be recognized on the surface of mucosa same as normal mucosa in both specimens shown in Figure 8. This result suggested that the pressure appears by wrapping up the specimen in the case of materials.

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

4 Discussion

In this study, CPC was used as an antimicrobial agent in combination with Mont. CPC is known to possess a broad antimicrobial spectrum, with rapid killing of gram-positive pathogens and yeast[30]. Mont was also used for the purpose of the carrier of TC-CPC is a kind of mineral and known as safety materials used for soap and bath articles[31].

The results from antimicrobial test showed that addition of TC-CPC confers antimicrobial activity to tissue conditioner. Furthermore, TC-CPC tissue conditioner possesses strong antimicrobial activity against both adhered and free-floating microbes compared to GCTC or NSTC. Antimicrobial activity of TC-CPC tissue conditioner showed the strongest just after the preparation. And the level of antimicrobial activity is decreased by the time after 1 week. This result suggested that CPC would to be released from tissue conditioner by time.

The antimicrobial activity is exerted by binding of positive charge of CPC to negatively charged microbial surfaces[32], and this property of CPC was also utilized for

recharging into Mont, because the adsorption by Mont occurs by cation exchange[33]. Utilization of Mont with CPC may be one of the best combinations in tissue conditioners, since they showed significantly good antimicrobial activity (Fig. 3-6).

The results of cytotoxicity tests with fibroblast and epithelial cells showed that TC-CPC had a strong cytotoxicity on the eluates at 1day after immersion. But the cytotoxicity of TC-CPC was at the same level as control at 7 days. These phenomena were likely to the results of antimicrobial activity tests. The cytotoxicity seems to be associated with antimicrobial activity[34]. It is suggested that CPC which is an antibacterial ingredient of TC-CPC is released for approximately one week. It is thought that the cytotoxicity became weak because antimicrobial properties of TC-CPC decreased. There was a significant difference between at 1and 7 days in control group. It showed that the eluates from control materials were damage to delicate Ca9-22 especially early time.

The results from animal experiment 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 same to control materials. Therefore, TC-CPC was considered to be not prophlogistic as other commercial materials and useful in clinical situation.

TC-CPC failed the penetration test after 2 hours while other standard satisfied JIS-T6519:2005 [31]. However, at clinical use, tissue conditioners frequently were mixed by changing P/L ratio by manufacturer's instructions. TC-CPC could be satisfied the penetration test, when P/L ratio was changed. Therefore, we attended on physical hardness of tissue conditioner. As a result, TC-CPC showed no significant difference to other commercial tissue conditioners both dry and wet conditions. TC-CPC seems to have the same hardness as a commercial product. TC-CPC might satisfy mechanical properties in clinical situations.

Although the results obtained from in *vitro* and *vivo* studies cannot be directly extrapolated to clinical situations, this study demonstrated that TC-CPC might be one of the important player to prevent aged patients from aspiration pneumonia with excellent antimicrobial activity biocompatibility. Clinical trials would be needed for its practical use.

Conclusion

Novel tissue conditioner containing CPC was developed in this study. It was demonstrated that novel material has excellent antimicrobial activity and similar mechanical property and biocompatibility to commercial materials.

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Table.1

Brand name	Manufacturer	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)	7.0	1.5
Experimental tissue conditioner without CPC	–	TC	Poly(butyl methacrylate) (PBMA) Poly(ethyl methacrylate) (PEMA)	Dibutyl sebacate (DBS)	7.0	1.5
Fictioner	Nissin	NSTC	Poly(butyl methacrylate) (PBMA) Poly(ethyl methacrylate) (PEMA)	Dibutyl sebacate (DBS) Butyl phthalyl butyl glycolate (BPBG)	0.0	2.0
Tissue conditioner	GC	GCTC	Poly(ethyl methacrylate) (PEMA)	Dibutyl sebacate (DBS)	14.0	1.2
Tissue Care	TOKUYAMA DENTAL	TDTC	Methacrylate-based polymer others	Acrylate-based polymer	8.0	1.9
TISSUE CONDITIONER Ⅱ	SHOFU	SFTC	Poly(ethyl methacrylate) (PEMA) others	Benzyl benzoate others	13.0	1.2
Visco-gel	Dentsply	DPTC	Poly(ethyl methacrylate) (PEMA)	Phthalyl butyl glycolate Dibutyl phthalate	4.9	1.5

Fig.1

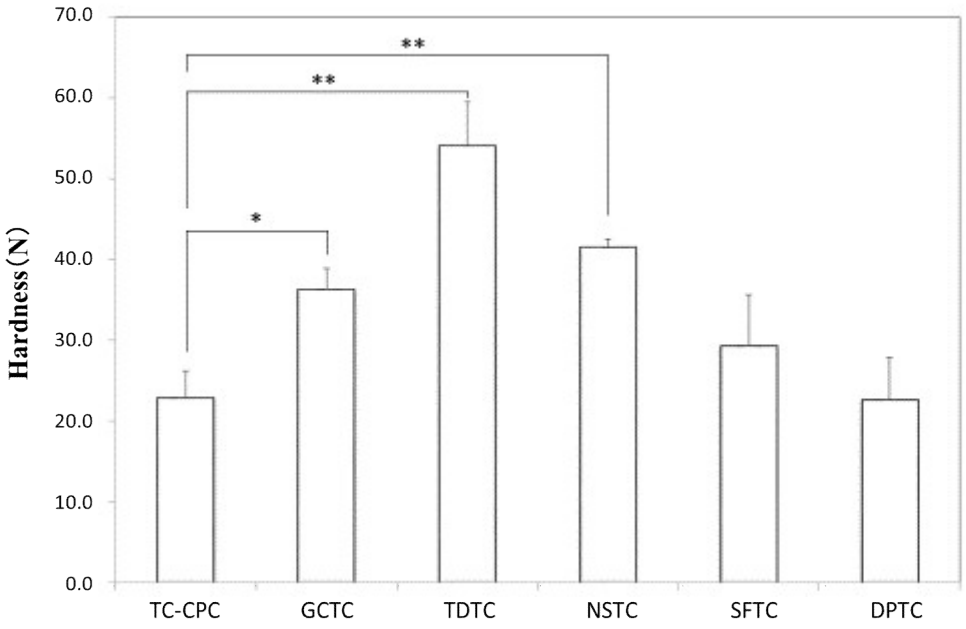

70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Hardness (N)
**
**
*
TC-CPC
GCTC
TDTC
NSTC
SFTC
DPTC

Fig.2

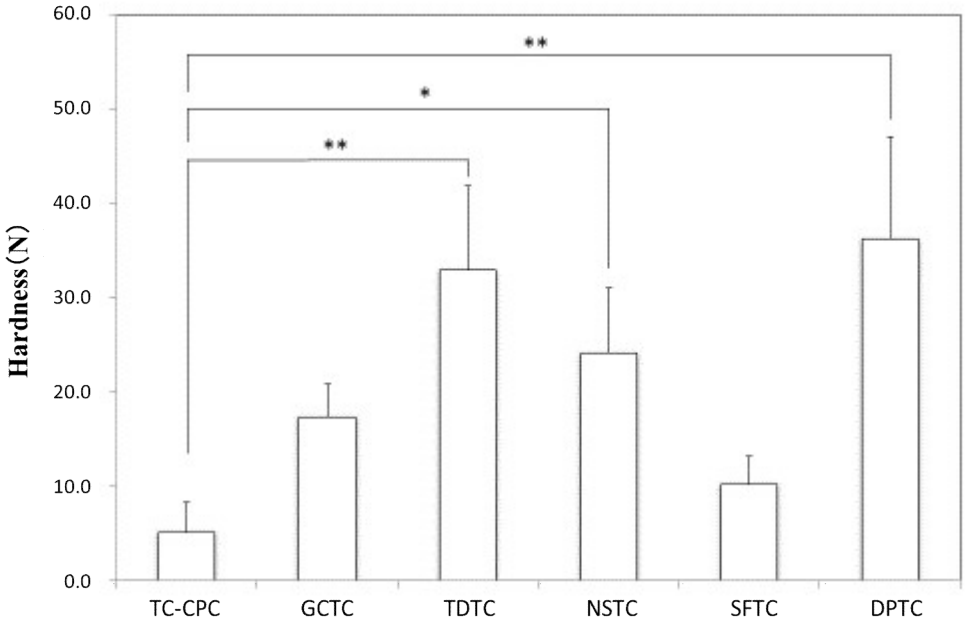

60.0
50.0
40.0
30.0
20.0
10.0
0.0
Hardness (N)
**
*
**
TC-CPC
GCTC
TDTC
NSTC
SFTC
DPTC

Fig.3

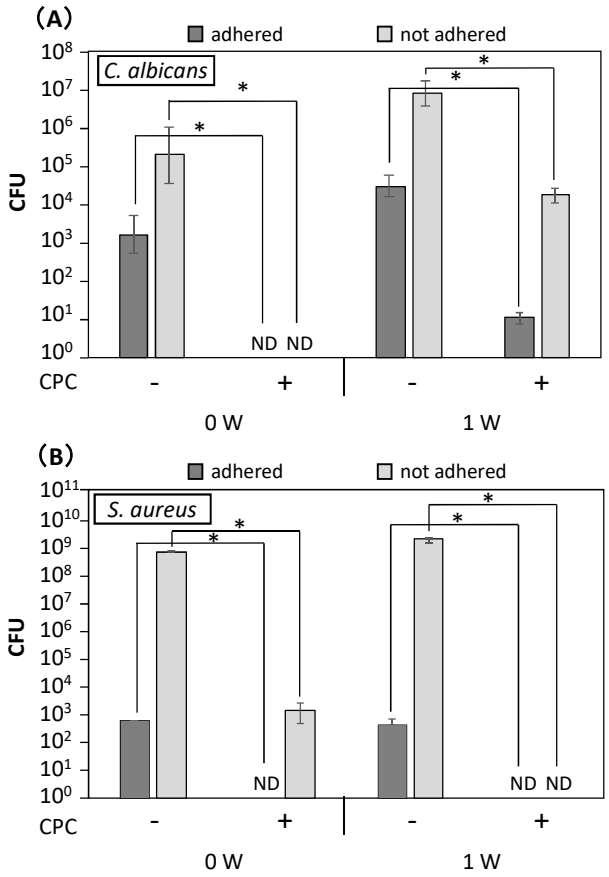

(A)
adhered
not adhered
C. albicans
CFU
ND ND
CPC
-
+
-
+
0 W
1 W
(B)
adhered
not adhered
S. aureus
CFU
ND
ND ND
CPC
-
+
-
+
0 W
1 W

Fig.4

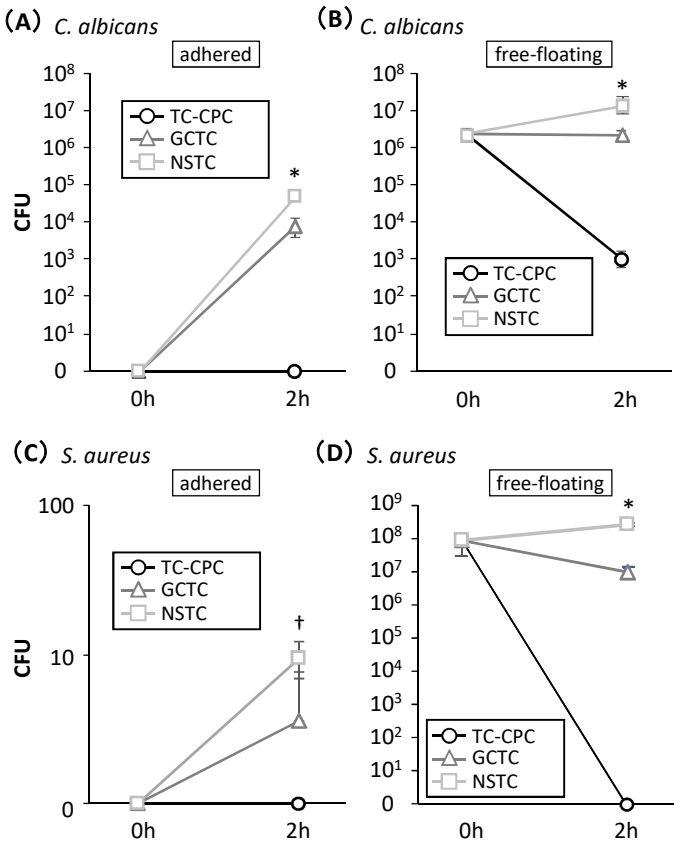

(A) C. albicans
adhered
CFU
TC-CPC
GCTC
NSTC
*
0h
2h
(B) C. albicans
free-floating
*
TC-CPC
GCTC
NSTC
0h
2h
(C) S. aureus
adhered
CFU
TC-CPC
GCTC
NSTC
†
0h
2h
(D) S. aureus
free-floating
*
TC-CPC
GCTC
NSTC
0h
2h

Fig.5

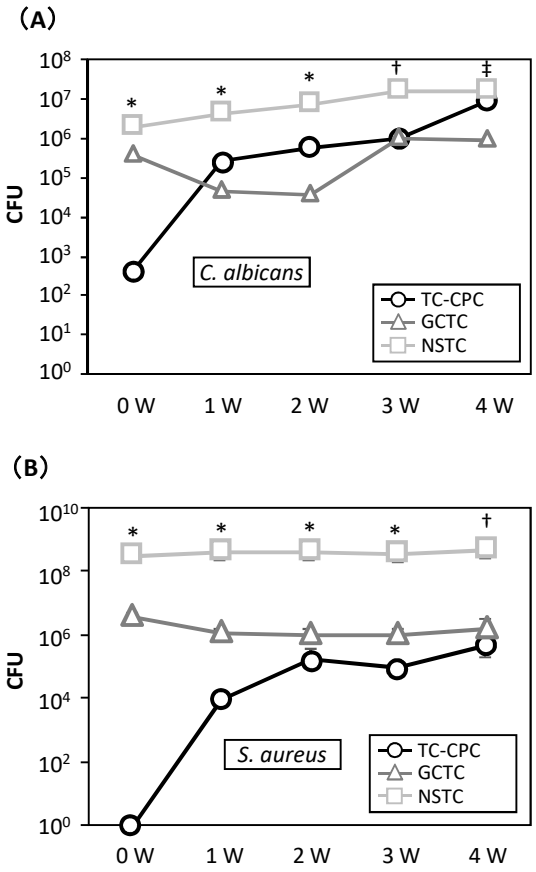

(A)
CFU
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
*
†
‡
C. albicans
TC-CPC
GCTC
NSTC
0 W
1 W
2 W
3 W
4 W
(B)
CFU
10^{10}
10^8
10^6
10^4
10^2
10^0
*
*
*
*
†
S. aureus
TC-CPC
GCTC
NSTC
0 W
1 W
2 W
3 W
4 W

Fig.6

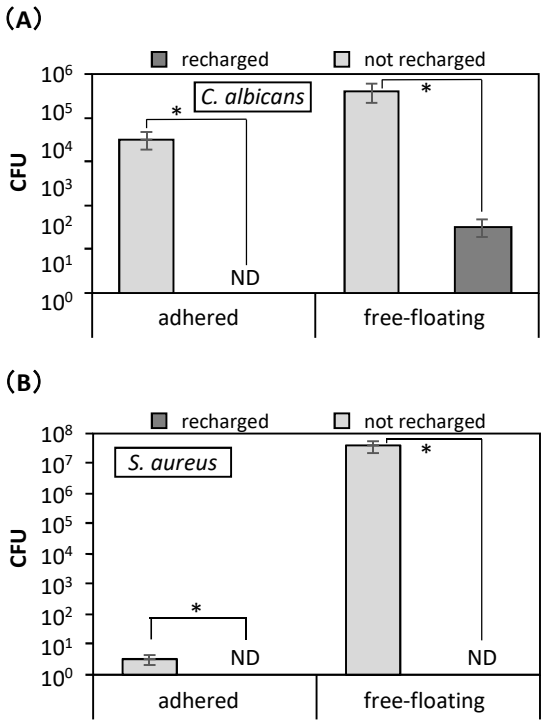
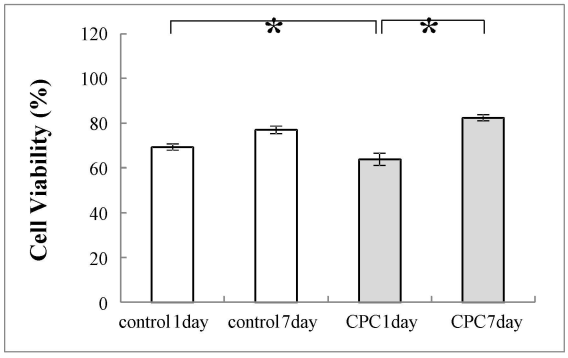

(A)
recharged
not recharged
C. albicans
CFU
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
ND
adhered
free-floating
(B)
recharged
not recharged
S. aureus
CFU
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
ND
ND
adhered
free-floating

Fig.7

(a)

NIH3T3 *P<0.001



(b)

Ca9-22 *P<0.001

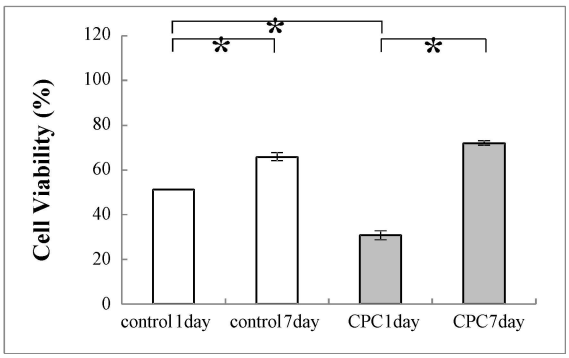
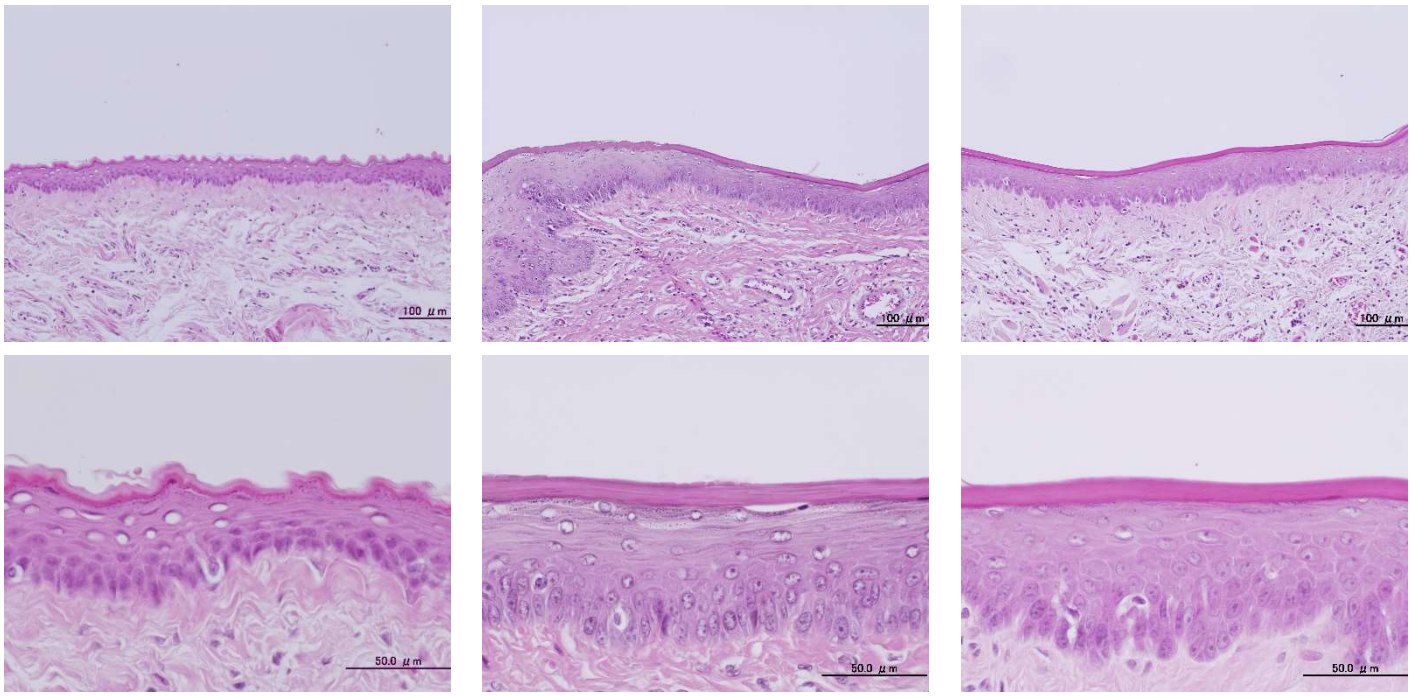


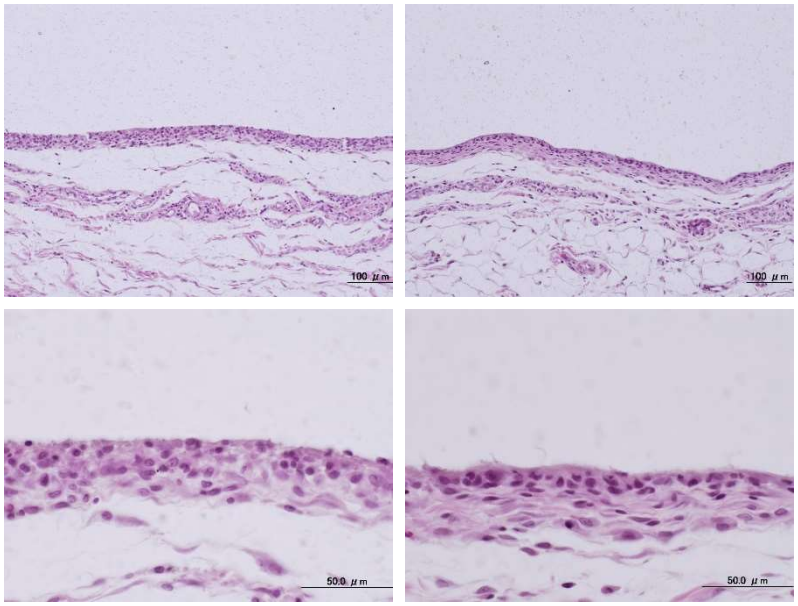
Fig.8



Normal TC-CPC GCTC

lower lip mucosa(1 day H.E stain)

Fig.9



TC-CPC GCTC

subcutaneous dosal region(14 day H.E stain)

Table1
Tissue conditioners investigated

Fig.1
Physical hardness test results stored on room temperature 2 hours.
The amount of tissue conditioner's physical hardness (N) stored on room temperature 2 hours condition.TC-CPC was maintaining the softness compared to other materials.
* Indicates statistically significant differences (**P < 0.01;* P < 0.05)

Fig.2
Physical hardness test results in deionized water 2 hours.
The amount of tissue conditioner's physical hardness (N) in deionized water 2 hours condition. TC-CPC was maintaining the softness compared to other materials
* Indicates statistically significant differences (**P < 0.01;* P < 0.05)

Fig.3
Antimicrobial activity of CPC in the tissue conditioner
Number of viable counts of *C. albicans* (A) and *S. aureus* (B) adhered to tissue conditioners or free-floating in the broth incubated with CPC-containing tissue conditioner (+) or with no CPC-containing tissue conditioner (-). The tissue conditioners were used soon after preparation (0 W) or allowed to release CPC for a week (1 W). The results are expressed as the mean ± SD of triplicate assays of a representative experiment. * Indicates statistically significant differences ($p < 0.05$).
ND indicates not detected.

Fig.4
After 2 hours incubation microbes in the broth and adhered
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 hours. The tissue conditioners were used soon after preparation. The results are expressed as the mean ± SD of triplicate assays of a representative experiment. * Indicates statistically significant differences among TC-CPC, GCTC or NSTC, and † indicates statistically significant difference between TC-CPC and GCTC or NSTC ($p < 0.05$).
.

Fig.5
Sustainability of antimicrobial activity of TC-CPC
Number of viable counts of free-floating *C. albicans* (A) and *S. aureus* (B) 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 ± SD of triplicate assays of a representative experiment. * Indicates statistically significant differences among TC-CPC, GCTC or NSTC, † indicates statistically significant difference between TC-CPC and GCTC or NSTC, and ‡ indicates statistically significant difference between GCTC and TC-CPC or NSTC (A) ($p < 0.05$). * Indicates statistically significant differences among TC-CPC, GCTC or NSTC, and † indicates statistically significant difference between NSTC and TC-CPC or GCTC (B) ($p < 0.05$)

Fig.6
Determination of CPC recharging effect on TC-CPC
Number of viable counts of *C. albicans* (A) and *S. aureus* (B) adhered to tissue conditioners or free-floating in the broth incubated with TC-CPC. TC-CPC was allowed to release CPC for 2 weeks and then TC-CPC was recharged or not recharged with CPC. The results are expressed as the mean ± SD of triplicate assays of a representative experiment. * Indicates statistically significant differences ($p < 0.05$).
ND indicates not detected.

Fig.7
The cell viability (%) of the 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 significantly lower than control one.
The cell viability of the TC-CPC using eluates with specimens incubated for 7 days show no significant difference between NIH3T3 and Ca9-22.

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

Fig.9
Materials implanted in the pockets formed in the subcutaneous tissue of the dosal 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.