Controlled release, antimicrobial activity, and oral mucosa irritation of cetylpyridinium chloride-montmorillonite incorporated in a tissue conditioner

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This study examined the controlled release of cetylpyridinium chloride (CPC) from a tissue conditioner (TC) containing CPC-montmorillonite (CPC-Mont), the associated antimicrobial activity, and oral mucosa irritation. The CPC release test was performed daily for 28 days in three test solutions: distilled water, 0.2 M NaCl, and 0.2 M HCl. The antimicrobial activities for 7, 14, 21, and 28 days against Candida albicans, Staphylococcus aureus, and Streptococcus mutans were assessed according to the JIS Z 2801/ISO 22196 standard. An oral mucosa irritation test was conducted using cheek pouches in five male hamsters according to the ISO 10993-10:2010 standard. The amount of CPC released each day and the cumulative amount released over 28 days (6.12 mg) were less than the daily safe maximum of sore throat medicines (8 mg). Additionally, TC with CPC-Mont could sustain antimicrobial activity against adherent bacteria for 14 days and has no oral mucosa irritation potential.

Keywords: Tissue conditioner, Cetylpyridinium chloride (CPC), Release test, Antimicrobial, Oral mucosa irritation

INTRODUCTION

Tissue conditioners (TC) are commonly used to repair damaged mucosa in denture-bearing areas and to obtain functional impressions1. However, TC harden and degenerate with time and are susceptible to microbial colonization2. Because dentures can function as a reservoir for potential respiratory pathogens3-6, applying a TC further increases the risk of aspiration pneumonia. Since oral care can improve the health of frail individuals who use dentures7, TC maintenance is critical to prevent the accumulation of pathogens. Several studies have assessed the efficacy of incorporating conventional organic antifungal medicines, inorganic antimicrobial agents, and natural and herbal antimiobitics into TC to improve the control and treatment of oral cavity infections8. In particular, the antimicrobial properties, viscoelasticity, cytotoxicity, and effect on saliva of TC preparations containing silver zeolite have been investigated9-10. However, because the antimicrobial silver ions are chlorinated and sulfurated, the TC develops a dark brown hue, and its antimicrobial activity is difficult to control. Thus, cetylpyridinium chloride (CPC)-loaded montmorillonite (Mont) (CPC-Mont) was developed as a new antimicrobial formulation for the controlled release of CPC, which is associated with sustained antimicrobial activity11.

CPC is a cationic surfactant with strong bactericidal and fungicidal properties. The antimicrobial activity of CPC is generally based on the following mechanisms: (1) interaction with the negatively charged components of cellular membranes, (2) leakage of cellular components, (3) disruption of bacterial metabolism, (4) inhibition of cell growth, and (5) cell death12-14. In vitro and in vivo assays have demonstrated that CPC inactivates oral bacteria, consequently reducing dental plaque and gingivitis12-14.

Matsumo et al.,15 first incorporated CPC-Mont in a dental adhesive and demonstrated that CPC-Mont imparts a renewable antimicrobial property to the adhesive without decreasing its bonding potential or increasing its cytotoxicity. Naoe et al.,16 examined the antimicrobial activity and biocompatibility of TC containing CPC-Mont. The prototype antimicrobial TC containing CPC-Mont exerted antimicrobial activity and presented the same mechanical properties and biocompatibility as commercially available TC. Additionally, antimicrobial activity could be restored by recharging the TC with CPC. The mechanism mediating the controlled release of the newly formulated CPC and its associated antimicrobial effects remain unclear. Moreover, in compliance with Good Laboratory Practice, before a clinical trial is conducted, a non-clinical study must confirm whether the CPC released may induce an oral mucosa irritation. Furthermore, the biological safety of the TC containing CPC-Mont should be proved according to the ISO 10993-10:2010 standard17. The antimicrobial TC with CPC-Mont in this study was
formulated by the same powder components and fine adjustment of component ratio of plasticizer and other constituents in the liquid with 7 wt% ethanol of the prototype. And its associated mechanical properties were basically as same as those of the prototype.

Therefore, this study investigated the relationship between the controlled release of CPC from a CPC-Mont-containing TC and the associated antimicrobial activity and oral mucosa irritation. It was hypothesized that the release mount of CPC from TC with CPC-Mont would induce the antimicrobial activity but exhibit no oral mucosa irritation.

MATERIALS AND METHODS

Antimicrobial agent formulation

The structure of the CPC-Mont (MedicalCrafton, Okayama, Japan) formulation is shown in Fig. 1. The Mont layer and interlayer space were 0.97 and 3.08 nm thick, respectively. The CPC molecules were 2.31 nm long and arranged in a bilayer at a 42° incline in the interlayer space. According to thermal analysis, CPC (CP+ cation+CPC molecule) constituted 54.65% of the CPC-Mont preparation. The particle size of the CPC-Mont was <150 µm (100 mesh).

Table 1 Initial CPC amount per unit area of samples (powder, 4.50 g; liquid, 3.00 g; powder:liquid ratio, 1.5) released into different test solutions

| Test solution | Area of sample (cm²) | Initial CPC*/area (mg/cm²) | Code |
|---------------|----------------------|---------------------------|------|
| Distilled water | 36.9 | 1.33 | Water |
| 0.2 M NaCl | 37.1 | 1.33 | NaCl |
| 0.2 M HCl | 39.8 | 1.24 | HCl |
| mean±SD | 37.9±1.6 | 1.30±0.05 | — |

*The initial CPC amount in CPC-Mont was 49.185 mg (=4.5 g×2 wt%×54.65%).

CPC, cetylpyridinium chloride; Mont, montmorillonite; SD, standard deviation

TC

The TC (Nissin Dental Products, Kyoto, Japan) was formulated as previously reported. The powder used to formulate the TC consisted of 77 wt% poly(butyl methacrylate) (molecular weight: 142.20 g/mol; particle size: 42–62 µm), 19 wt% poly(ethyl methacrylate) (molecular weight: 114.14 g/mol; particle size: 28–42 µm), 2 wt% CPC-Mont, and 2 wt% other constituents. The liquid consisted of 90 wt% dibutyl sebacate, 7 wt% ethanol, and 3 wt% other constituents. In the case that the TC was prepared with a powder-to-liquid ratio of 1.5, the TC satisfied the following standard specifications: type of shore A0 hardness, type B (extra soft; ≤30 at 2 h, ≤60 at 7 days); class of consistency, class 1 (medium flow; 25≤diameter<60 mm); and requirement of detail reproduction, line b (20 µm) according to the International Organization for Standardization (ISO) 10139-1:2018 standard for short-term denture-lining materials.

CPC release test

The sample for the CPC release test was comprised a mixture of 4.5 and 3.0 g of the powder and liquid, respectively (powder-to-liquid ratio=1.5). The shape of sample was the disk with a thickness of 2 mm, and the surface area of the sample was measured for the calculation of the CPC release amount per unit area. One sample was tested per each of the following test solutions. Distilled water, 0.2 M NaCl, and 0.2 M HCl (pH=0.7) were used as test solutions. The initial CPC amount (mg) per unit area (cm²) in each sample was calculated from the amount of CPC in CPC-Mont (4.5 g×2 wt%×54.65%=49.185 mg; Table 1).

The sample was adjusted to the bottom of a Petri dish, and 10 mL of distilled water was poured into the dish. The dish with the test solution was constantly shaken (BR-23FP, TAITEC, Koshigaya, Japan) for 24 h at 37°C. The solution was then collected in a polypropylene tube. This process was performed daily for 28 days.

The CPC content in each sample was determined by measuring the absorbance of the sample at 259 nm using a UV-visible spectrophotometer (UV-3100(PC) S, SHIMADZU, Kyoto, Japan). A calibration curve was...
prepared with working solutions containing 2.5, 5.0, 7.5, 10, and 20 mg/L CPC in distilled water, and the derived coefficient of determination (R²) was >0.999. The UV absorbance of CPC (at 259 nm) was analyzed using Origin Pro (OriginLab, Northampton, MA, USA), and the amount of CPC (mg) in each solution was derived from the calibration curve.

**Antimicrobial test**

1. Sample preparation

Samples with and without CPC-Mont (denoted as CPC-Mont (+) and CPC-Mont (−), respectively) were prepared in the form of disks (diameter, 20 mm; thickness, 1.6 mm). At 2 h after their preparation, the disks were immersed in 50 mL of sterilized PBS at 37°C for 7, 14, 21, and 28 days, and the PBS was renewed every 7 days to assess the relationship with the release of CPC from the samples. Three disks were prepared for each immersion condition. Samples that were not immersed in PBS were used as positive controls.

2. Test microorganisms

The test microbes were *Candida albicans* IFM40009 (ATCC 48130), as an opportunistic oral pathogen; *Staphylococcus aureus* FDA209P (ATCC 6538P), as an opportunistic pathogen of the nasal cavity; and *Streptococcus mutans* 109c, as a causative agent of *Streptococcus mutans* an opportunistic pathogen of the nasal cavity; and *Candida albicans* FDA209P (ATCC 6538P), as an opportunistic pathogen of the nasal cavity. The test microbes were cultured in Sabouraud dextrose broth; nutrient broth; and *Streptococcus mutans* 109c, as a causative agent of *Streptococcus mutans* an opportunistic pathogen of the nasal cavity; and *Candida albicans* FDA209P (ATCC 6538P), as an opportunistic pathogen of the nasal cavity. The test microbes were cultured in Sabouraud dextrose broth; nutrient broth; and *Streptococcus mutans* 109c, as a causative agent of *Streptococcus mutans* an opportunistic pathogen of the nasal cavity; and *Candida albicans* FDA209P (ATCC 6538P), as an opportunistic pathogen of the nasal cavity. The test microbes were cultured in Sabouraud dextrose broth; nutrient broth; and *Streptococcus mutans* 109c, as a causative agent of *Streptococcus mutans* an opportunistic pathogen of the nasal cavity; and *Candida albicans* FDA209P (ATCC 6538P), as an opportunistic pathogen of the nasal cavity. The test microbes were cultured in Sabouraud dextrose broth; nutrient broth; and *Streptococcus mutans* 109c, as a causative agent of *Streptococcus mutans* an opportunistic pathogen of the nasal cavity; and *Candida albicans* FDA209P (ATCC 6538P), as an opportunistic pathogen of the nasal cavity. The test microbes were cultured in Sabouraud dextrose broth; nutrient broth; and *Streptococcus mutans* 109c, as a causative agent of *Streptococcus mutans* an opportunistic pathogen of the nasal cavity; and *Candida albicans* FDA209P (ATCC 6538P), as an opportunistic pathogen of the nasal cavity. The test microbes were cultured in Sabouraud dextrose broth; nutrient broth; and *Streptococcus mutans* 109c, as a causative agent of *Streptococcus mutans* an opport

3. Antimicrobial assay

The sample (CPC-Mont (+), CPC-Mont (−), or non-immersed in PBS as positive control) was immersed in the microbial suspension (5 mL), which was cultured with the sample at 37°C on a shaker (Multi Shaker MMS, Tokyo Rikakikai, Tokyo, Japan) (40 rpm) for 2 h. The microbial suspension culture (suspension A) without the sample was collected and used to evaluate the antimicrobial activity of CPC on the suspended microbes. The sample was then washed thrice with 6 mL of sterilized PBS to eliminate non-adherent microbes and treated with 6 mL of sterilized PBS containing 0.1% Triton X-100 for 20 s on a laboratory vibrator (Microplate mixer MPX-96A, AS ONE, Osaka, Japan) to remove adherent microbes. The sterilized PBS with 0.1% Triton X-100 (suspension B) was used to assess the antimicrobial activity of CPC against the adherent microbes. Ten-fold dilutions of suspensions A and B were prepared, and each dilution (0.1 mL) was spread thrice on a selective medium (Candida GS agar for *C. albicans*, Staphylococcus no. 110 agar for *S. aureus*, and Mitis-Salivarius agar for *S. mutans*). After 3 days of incubation at 37°C under aerobic conditions, the antimicrobial activity was reported as colony-forming units (CFU).

4. Assessment of the antimicrobial activity

The antimicrobial activity of CPC was assessed according to the Japanese Industrial Standards (JIS) Z 2801:2012 (antibacterial products —test for antibacterial activity and efficacy)/ISO 22196:2011 standard. The derived activity values indicated that the proportion of microbes on the surface of the CPC-Mont (+) sample was ≤1/100 (antimicrobial activity value, log (CFU) was 2.0 or more) times that on the surface of the CPC-Mont (−) sample.

**Oral mucosa irritation test**

This test was conducted according to Ordinance No. 37 of the Ministry of Health, Labour and Welfare, and the ISO 10993-10:2010 standard for biological evaluation of medical devices —Part 10: tests for irritation and skin sensitization. The animal experiments with the care to minimize pain and discomfort were carried out in accordance with the animal research ethical code of the Japan Food Research Laboratories (JFRL), Chitose Laboratory.

Five 11-week-old male hamsters with body weights of 109.3–133.6 g at the effective date and 110.1–139.4 g at the euthanasia date (after 14 days) (Slc:Syrian, Japan SLC, Shizuoka, Japan) were used as test animals. The hamsters were kept in a plastic cage (Clean S: 225 mm wide×338 mm depth×140 mm height; Clea Japan, Tokyo, Japan) housed at a controlled temperature of 22.5–23.5°C, relative humidity of 45.5–58.3%, and lighting time of 12 h with a cage exchange of twice/week. The hamsters were fed a rodent diet (a pellet with a diameter of 12 mm, lot. 160407 and 160509; CRF-1, Oriental yeast, Tokyo, Japan), which was freely given with free intake of tap water from a self-filling bowl.

1. Experimental design

The CPC-Mont (+) sample (diameter, approximately 15 mm; thickness, approximately 1 mm) was continuously applied to one unilateral cheek pouch of the hamster (application site) for 14 days, and the other cheek pouch was sham-operated (control site). After 14 days, five hamsters were euthanized by inhalation of anesthesia with isoflurane (Fujifilm Wako Pure Chemical, Tokyo, Japan), and bleeding from the ventral aorta and bilateral cheek pouches were removed. After removal and reversal of the cheek pouches, macroscopic evaluation was conducted using a grading system for erythema and eschar formation: numerical grading 0, no erythema; 1, very slight erythema (barely perceptible); 2, well-defined erythema; 3, moderate erythema; and 4, severe erythema (beet-redness) to eschar formation preventing grading of erythema. After macroscopic evaluation, formalin fixed cheek pouches with 10% neutral buffered formalin (Sigma-Aldrich Japan, Tokyo, Japan) were paraffin-embedded in longitudinal and perpendicular directions to the pouch, hematoxylin and eosin (HE) staining (Cosmo bio, Tokyo, Japan) was performed for the tissue sections, and histological evaluation was conducted using a grading system for microscopic examinations.
(numerical grading 0–4 for the epithelium, leucocyte infiltration (magnification: ×400), vascular congestion, and edema). Irritation was assessed using the irritation index (average grade 0, none; 1–4, minimal; 5–8, mild; 9–11, moderate; and 12–16, severe) based on the average microscopic grade of the application or control groups.

**Statistical analysis**

The mean and cumulative amounts of CPC released into the three test solutions per day and over 28 days, respectively, were statistically analyzed using analysis of covariance (ANCOVA) and Tukey’s test at a probability level of 0.05. BellCurve for Excel statistical analysis software (Social Survey Research Information, Tokyo, Japan) was used for the analyses.

**RESULTS**

**Controlled release of CPC**

The amount (mg/cm²) of CPC released over 28 days is shown in Fig. 2. According to the sustained release properties of CPC in the three test solutions, the highest release was observed on day 1 (distilled water, 0.019 mg/cm²; NaCl, 0.019 mg/cm²; and HCl, 0.011 mg/cm²), and the amount of CPC released gradually decreased after day 2. The mean amount of CPC released into the three test solutions on day 1 (0.016±0.005 mg/cm²) was significantly higher than that released on day 2 (0.008±0.003 mg/cm²; p<0.01). There were no significant differences in the mean amounts of CPC released daily from days 2 to 28 (p>0.05). The cumulative CPC amount (mg/cm²) released over 28 days versus the square root of time (day¹/²) followed Higuchi’s law (p<0.001) as depicted in Fig. 3. The amount of CPC released into 0.2 M HCl was lower than that released into water or 0.2 M NaCl (p<0.01) at near-neutral pH. The cumulative amounts of CPC released (mg/cm²) over 7, 14, 21, and 28 days were estimated (Table 2) to examine the controlled release mechanism and determine if the amounts were within safety limits.

**Antimicrobial activity**

The *C. albicans*, *S. aureus*, and *S. mutans* CFUs on the sample disks after immersion in PBS for 7, 14, 21, and 28 days, and those on the non-immersed sample disks are shown in Table 3. None of the CPC-Mont (+) samples showed antimicrobial activity against suspended *C. albicans* cells, according to the JIS Z 2801/ISO 22196 standard. The non-immersed samples and samples immersed for 7 days [7 day-CPC-Mont (+)] showed noticeable

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**Table 2** Cumulative amount of CPC (mg/cm²) released over 7, 14, 21, and 28 days

| Code | Cumulative release amount (mg/cm²) |
|------|-----------------------------------|
|      | 7 days | 14 days | 21 days | 28 days |
| Water| 0.058  | 0.081   | 0.099   | 0.112   |
| NaCl | 0.052  | 0.076   | 0.095   | 0.111   |
| HCl  | 0.033  | 0.053   | 0.069   | 0.083   |
| mean±SD | 0.048±0.013 | 0.070±0.015 | 0.087±0.016 | 0.102±0.016 |
| % of initial CPC/area (1.30 mg/cm²) | 3.69    | 5.39    | 6.74    | 7.85    |

CPC, cetylpyridinium chloride; SD, standard deviation
Antimicrobial activity against adherent *C. albicans* cells, but the samples immersed for 14 days [14-day-CPC-Mont (+)] presented a slightly lower antimicrobial activity against adherent *C. albicans* [4.5×10^6 CFU of CPC-Mont (−)/1/100=4.8×10^6 CFU of CPC-Mont (+)].

In the *S. aureus* and *S. mutans* test series, the non-immersed and 7-day-CPC-Mont (+) sample disks displayed antimicrobial activity against both suspended and adherent bacteria, whereas the 14-day-CPC-Mont (+) sample was only active against adherent bacteria.

Assessment of oral mucosa irritation

The macroscopic evaluation verified no abnormality in both the application and control groups based on grade (0, no erythema). The histological evaluation demonstrated no abnormality on the epithelium, leucocyte infiltration, vascular congestion, and edema in both application and control groups based on grade (0). No abnormal findings were detected in the histological images of the mucosal epithelium and submucosa in both the application and control sites (Fig. 4). Therefore, the CPC-Mont (+) sample was judged to be a non-irritant based on the irritation index (0, none).

**DISCUSSION**

The hypothesis that the relevant mount of CPC release was required for the antimicrobial activity and the release mount did not exhibit oral mucosa irritation was accepted.

In the CPC release test, the reasons for selecting 0.2 M NaCl and 0.2 M HCl as the test solutions were described as follows. Because the montmorillonite (Mont) is an ion exchanger, it was necessary to verify the effect of the ionic strength of the solutions in a strong acidity-neutral region on the amount of CPC released. CPC-Mont was synthesized from the exchange of Na in Na-Mont for CPC. For Na-Mont, 0.2 M HCl exchanged almost all of Na⁺ for H⁺ for several hours. The CP⁺ ions in CPC bind electrostatically to the negatively charged aluminosilicate layers of Mont for the structure of CPC-Mont. The CP⁺ layer in CPC-Mont was formed via electrostatic interactions and functions as a carrier for CPC molecules through hydrophobic interactions (Fig. 1). Upon contact with water (H₂O), the controlled release of CPC from CPC-Mont might involve releasing CPC molecules intercalated in CPC-Mont by hydrophobic interactions. Therefore, the CPC molecule was dissociated as CP⁺ and Cl⁻ by water, and CP⁺ exhibits antimicrobial activity against the microbes.

In the oral environment of eating, the TC with CPC-Mont was exposed to the salinity of foods and the acidic drinking waters. Thus, 0.2 M NaCl and 0.2 M HCl solutions were selected as the effect of salinity and strong acidity, respectively. 0.2 M NaCl solution was accepted.

### Table 3 Viable cells (CFU) of each microbe in samples immersed in PBS for 7, 14, 21, and 28 days, or not immersed

| Microorganisms     | Behavior | Samples Not immersed | 7 days      | 14 days      | 21 days      | 28 days      |
|--------------------|----------|----------------------|-------------|-------------|-------------|-------------|
| *Candida albicans* | Suspended| CPC-Mont (−) (4.9±2.7)×10⁶/(3.8±1.1)×10⁶ | (3.8±0.5)×10⁶ | (3.7±1.1)×10⁶ | (3.9±0.4)×10⁶ |
|                    |          | CPC-Mont (+) (5.4±4.4)×10⁶/(1.3±0.7)×10⁶ | (2.6±0.9)×10⁶ | (2.8±1.2)×10⁶ | (5.0±2.7)×10⁶ |
|                    | Adherent | CPC-Mont (−) (4.7±0.8)×10⁶/(4.2±0.6)×10⁶ | (4.5±1.1)×10⁶ | (4.4±1.1)×10⁶ | (4.5±0.9)×10⁶ |
|                    |          | CPC-Mont (+) (1.6±0.5)×10⁷/(3.5±0.8)×10⁷ | (4.8±0.9)×10⁷ | (1.6±0.3)×10⁷ | (2.3±0.1)×10⁷ |
| *Staphylococcus aureus* | Suspended | CPC-Mont (−) (1.6±0.4)×10⁴/(1.5±0.2)×10⁻⁴ | (1.4±0.5)×10⁻⁴ | (1.5±0.3)×10⁻⁴ | (1.6±0.2)×10⁻⁴ |
|                    |          | CPC-Mont (+) Undetected/(6.0±6.0)×10⁴ | (1.0±0.2)×10⁴ | (5.9±3.6)×10⁴ |
|                    | Adherent | CPC-Mont (−) (5.7±2.5)×10⁶/(6.2±2.8)×10⁶ | (7.7±4.0)×10⁶ | (7.6±2.0)×10⁶ | (8.0±4.3)×10⁶ |
|                    |          | CPC-Mont (+) Undetected/(3.0±5.2)×10⁶ | (2.1±0.9)×10⁶ |
| *Streptococcus mutans* | Suspended | CPC-Mont (−) (6.1±2.4)×10⁶/(4.8±1.4)×10⁶ | (5.0±2.4)×10⁶ | (5.8±0.8)×10⁶ | (5.4±0.5)×10⁶ |
|                    |          | CPC-Mont (+) Undetected/(1.0±0.5)×10⁻⁴ | (2.1±0.5)×10⁻⁴ | (1.6±0.5)×10⁻⁴ |
|                    | Adherent | CPC-Mont (−) (1.5±1.4)×10⁷/(1.5±1.4)×10⁷ | (1.6±1.4)×10⁷ | (1.5±1.5)×10⁷ |
|                    |          | CPC-Mont (+) Undetected/(3.2±2.4)×10⁷ | (5.2±3.8)×10⁷ |

Underbar: The value for CPC-Mont (+) is ≤1/100 times of that of CPC-Mont (−), according to JIS Z 2801/ISO 22196.

CPC, cetylpyridinium chloride; Mont, montmorillonite; SD, standard deviation; CFU, colony forming units

**Fig. 4** Histological images of the mucosal epithelium and submucosa in both application (A) and control (B) sites (magnification: ×55).

No abnormal findings were detected in both images.
was elucidated in detail, the CPC release test was performed daily for 28 days.

The highest rate of CPC release was observed on day 1 (Fig. 2) because water molecules might have diffused into the Mont layers following contact. However, the cumulative amount of CPC released over 28 days in all test solutions (Fig. 3) was linearly proportional to the square root of time (day\(^{1/2}\); \(p<0.001\)) according to Higuchi's law\(^{24,25}\). The controlled release of CPC into water and NaCl solution at near-neutral pH was similar (\(p=0.83\)), whereas CPC release into 0.2 M HCl was inhibited (\(p<0.01\)). The inhibition of CPC release into the HCl solution occurred early, and the amount of CPC released into the three test solutions was similar after seven days. Thus, CP\(^{+}\) of CPC molecules exchange intercalated in CPC-Mont by hydrophobic interactions for the cations in the NaCl or HCl solutions was not induced too much. Therefore, it was thought that the influence of the ionic strength of the solutions in the strong acidity-neutral region on the amount of CPC released was very small.

In the antimicrobial test, the object solution of CPC release was PBS. The CPC release test against the PBS was not performed because CPC release test results available for forecasting the amount of CPC released in the PBS were considered as follows: (a) the minimal effect of the ionic strength of the solutions in the strong acidity-neutral region, (b) the absence of salinity effect, (c) the minimal exchange of Cl\(^-\) for HPO\(_4^{2-}\) and H\(_2\)PO\(_4^{-}\) in the PBS through anion-exchange process\(^{11}\). Therefore, the amount of CPC released in the PBS was considered as follows: (a) the minimal effect of the ionic strength of the solutions in the strong acidity-neutral region, (b) the absence of salinity effect, (c) the minimal exchange of Cl\(^-\) for HPO\(_4^{2-}\) and H\(_2\)PO\(_4^{-}\) in the PBS through anion-exchange process\(^{11}\). Therefore, the amount of CPC released in the PBS would mean the amount of CPC released into the PBS. Moreover, the amounts of CPC released on days 7, 14, 21, and 28 were used to consider the antimicrobial activities against the microbes. The sample was immersed in 50 mL of PBS in the antimicrobial test, an adequate quantity that will not saturate the amount of CPC released from the sample for 7 days. The evaluation of antimicrobial activities on days 7, 14, 21, and 28 revealed sustainable antimicrobial activities. The sample on days 7, 14, 21, and 28 lost the cumulative amount of CPC for 6, 13, 21, and 27 days, respectively. Moreover, the release amount of CPC could only sustain antimicrobial activity against adherent bacteria for 14 days (Table 3). In clinical practice, the usage of TC is for days or up to two weeks. Thus, the oral mucosa irritation test was performed for 14 days.

The possibility of releasing CPC from the superficial, internal, or both sides of the TC was considered. Because the Higuchi plot indicated linear regression, and 7.85% of the initial CPC was released over 28 days (Table 2), it was assumed that CPC release from CPC-Mont occurred on the surface of the TC. The TC was initially loaded with the required CPC amount, and the possible CPC release from the internal CPC-Mont cannot be neglected. The release of CPC from TC bulk would require the elution of the plasticizer from the TC or the absorption of water through the plasticizer to facilitate contact between the internal CPC-Mont and water. Because the TC formulation ensured that the plasticizer could hardly elute\(^{16}\), only minimal CPC was released from CPC-Mont on the TC surface. CPC-Mont must be uniformly dispersed on the material surface.

The involvement of the following three mechanisms should be considered for the potential antimicrobial activity of CPC released from the TC: (1) intake of CPC into the oral mucosa, (2) inhibition of microbial proliferation on the material surface or inside, and (3) diffusion of CPC into the oral cavity, resulting in the antimicrobial effect of CPC on oral flora and the absorption of CPC into the digestive tract (Fig. 5). Mechanism (1) depends on the oral mucosa, which is composed of a stratified squamous epithelium with a structure similar to the internal structure of the skin. Drug absorption via the oral mucosa is generally a passive diffusion process that occurs according to the pH-partition theory\(^{26,27}\). The absorption of a drug depends on its state of dissociation (ionic or molecular form), and ionic drugs, such as CP\(^{+}\), are hardly absorbed by the oral mucosa\(^{29}\). Moreover, the TC containing CPC-Mont had no mucosal irritation potential in the hamster cheek pouches (Fig. 4). Therefore, mechanism (1) can be discarded. The amount of CPC released in this study

![Fig. 5: Three conceivable mechanisms of action of CPC released from the TC. Mont, montmorillonite](image-url)
mainly corresponds to mechanism (2), and mechanism (3) can be disregarded considering the minimal amount of CPC released.

It is necessary to ensure the biological safety of the released CPC. Per the dosage forms of CPC Troche 2 mg “IWAKI” (2.00 mg/piece: 1 piece per dose, 3 to 4 times daily; IWAKI SEIYAKU, Tokyo, Japan) and Vicks Medicated Drops (0.46 mg/piece: 3 pieces per dose, 4–6 times daily; Taisho Pharmaceutical, Tokyo, Japan), used as medicines for sore throat, the daily safe maximum CPC dosage is ~8 mg. Assuming that a patient wears both upper and lower complete dentures relined with the TC containing CPC-Mont, the released amount of CPC based on the maximum denture base area of approximately 60 cm² (upper: 40.1±5.4 cm²; lower: 20.0±3.8 cm²) would be 0.96 mg on day 1. The cumulative amount would be 6.12 mg for 28 days. Hence, the amount of CPC released was below the daily safe maximum of 8 mg.

According to the JIS Z 2801/ISO 22196 standard, the TC containing CPC-Mont should sustain antimicrobial activity against the adherent forms of *C. albicans*, *S. aureus*, and *S. mutans* for 14 days. The antimicrobial activity was associated with 0.004 mg/cm² CPC released on day 7, and 0.003 mg/cm² released on day 14. Thus, the amount of CPC released based on the sample area of 6.28 cm² was 0.03 mg on day 7 and 0.02 mg on day 14. Therefore, the amount of CPC released on days 7 and 14 in 5 mL of the microbial suspension was 6 and 4 µg/mL, respectively. Sreenivasan et al. reported that the minimum inhibitory concentrations (MICs) of CPC against *C. albicans*, *S. aureus*, and *S. mutans* were 7.5, 3.75, and 15 µg/mL, respectively. However, the MIC of 15 µg/mL against *S. mutans* is considerably higher than the 0.06–0.48 µg/mL reported by Yeon and Young and 2.0 µg/mL reported by Kitagawa. In our study, the CPC amounts of 6 and 4 µg/mL released on days 7 and 14, respectively, are adequate to exert antimicrobial activity against adherent *S. aureus* and *S. mutans*. However, the CPC amount released on day 14 was not necessarily sufficient to affect adherent *C. albicans* cells. As the TC with CPC-Mont did not always show antimicrobial activity against the suspended microbes, its ability to prevent the proliferation of surface microbes could be attributed to the antimicrobial agent in the TC, according to the JIS Z 2801/ISO 22196 standard.

In the future, oral candidiasis treatment with a TC containing a high CPC-Mont concentration should be investigated. The antimicrobial TC used in this study could contribute to good oral hygiene maintenance in conjunction with oral candidiasis treatment. However, the TC did not prevent the proliferation of surface *C. albicans*. Alternatively, topical drugs applied to the affected area for treating limited infections have some mild adverse effects due to limited absorption and do not interact with other drugs. Oral ketoconazole is a systemic drug with a potential for hepatotoxicity in addition to drug interactions and adrenal insufficiency.

As topical and systemic drugs have a safe administration period, the cessation of a drug would lead to the recurrence of oral candidiasis due to denture use. Therefore, a high concentration of CPC-Mont in the TC could be useful for treating oral candidiasis due to the high safe maximum daily released CPC dosage.

This study found that the amount of CPC released each day and the cumulative amount released over 28 days was less than or equal to the daily safe maximum of sore throat medicines. Additionally, the antimicrobial activity of the CPC-Mont-containing TC corresponded to the controlled release of CPC. Therefore, TC with CPC-Mont could sustain antimicrobial activity against adherent bacteria and has no oral mucosa irritation potential.

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