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Title	A MULTICENTER, RANDOMIZED, OPEN-LABEL, PLACEBO-CONTROLLED CLINICAL TRIAL OF THE EFFECT OF CETYLPYRIDINIUM CHLORIDE (CPC) MOUTHWASH AND ON-DEMAND AQUEOUS CHLORINE DIOXIDE MOUTHWASH ON SARS-COV-2 VIRAL TITER IN PATIENTS WITH MILD COVID-19
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ORIGINAL ARTICLE

A MULTICENTER, RANDOMIZED, OPEN-LABEL, PLACEBO-CONTROLLED CLINICAL TRIAL OF THE EFFECT OF CETYLPYRIDINIUM CHLORIDE (CPC) MOUTHWASH AND ON-DEMAND AQUEOUS CHLORINE DIOXIDE MOUTHWASH ON SARS-COV-2 VIRAL TITER IN PATIENTS WITH MILD COVID-19

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ABSTRACT

Objectives
The established effect of cetylpyridinium chloride (CPC) mouthwash on SARS-CoV-2 viral titers is unclear. No clinical trial has examined the impact of on-demand aqueous chlorine dioxide mouthwash on SARS-CoV-2 viral titer.

Methods
In this multicenter, 3-armed, randomized, open-label, placebo-controlled clinical trial involving mildly symptomatic COVID-19 patients, we randomly assigned them to receive 20 mL of 0.05% CPC, 10 mL of 0.01% on-demand aqueous chlorine dioxide, or 20 mL of purified water as a placebo mouthwash in a 1:1:1 ratio. The primary measurement was the SARS-CoV-2 viral titer in saliva, evaluated by a mixed-effects linear regression model.

Results
49 patients received CPC mouthwash (n=16), on-demand aqueous chlorine dioxide mouthwash (n=16), and placebo (n=17) between January 14, 2024, and February 20, 2024. 0.05% CPC mouthwash significantly reduced salivary viral titer at 10 minutes postuse (-0.97 $\log_{10}$ PFU/mL; 95% CI, -1.64 to -0.30; $P = .004$), while no such effect was observed at 30 minutes (difference vs placebo, -0.26 $\log_{10}$ PFU/mL; 95% CI, -0.92 to 0.40; $P = .435$) or 60 minutes (difference vs. placebo, -0.05 $\log_{10}$ PFU/mL; 95% CI, -0.68 to 0.58; $P = .877$). 0.01% on-demand chlorine dioxide mouthwash did not reduce salivary viral titer at 10 minutes, 30 minutes, or 60 minutes compared to placebo.

Conclusions
10 minutes after using a 0.05% CPC mouthwash, the salivary viral titer of SARS-CoV-2 decreased compared to placebo. 0.01% on-demand aqueous chlorine dioxide mouthwash and placebo had no significant difference in SARS-CoV-2 viral titers.

Trial Registration
Japan Registry of Clinical Trials (jRCT): jRCTs031230566.

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KEYWORDS

Cetylpyridinium chloride, COVID-19, Epidemiology, Mouthwash, On-demand aqueous chlorine dioxide solution, Randomized controlled trial

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INTRODUCTION

The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has significantly affected global public health.[1] The oral cavity is involved in COVID-19 infection, suggesting that saliva and the salivary glands are potential sources of COVID-19 infection.[2,3] By reducing viral spread, mouthwashes with virucidal activity could potentially prevent COVID-19 infection.[4,5]

Cetylpyridinium chloride (CPC) is a widely adopted bactericidal agent in mouthwash, sprays, and lozenges.[6] Although recent reviews suggested a reduction in SARS-CoV-2 salivary viral load with CPC mouthwashes,[7,8] our previous randomized controlled trial (RCT) showed no significant reduction in SARS-CoV-2 salivary viral load with the use of 0.05% CPC mouthwash.[9] Therefore, 0.05% CPC mouthwash warrants further investigation for its impact on the viral viability of SARS-CoV-2. Previous laboratory studies and RCTs have shown that CPC mouthwash may also suppress the infectivity of salivary SARS-CoV-2.[5-8,10-13] However, these studies substantially differed with regard to both the mouthwash concentrations they used and in their participant numbers, and the effects of CPC mouthwash on SARS-CoV-2 viral titer are accordingly not well established.

A new mouthwash, MA-T (Matching Transformation System), has been developed, which addresses the limitations of ordinary chlorine dioxide solutions.[14] This product features an on-demand aqueous chlorine dioxide solution. In the presence of viruses or live bacteria, on-demand aqueous chlorine dioxide generates free radicals and exhibits strong microbicidal activity through catalytic action in the respiratory system.[14] Previous studies propose that on-demand aqueous chlorine dioxide may be effective as a disinfectant mouthwash against SARS-CoV-2,[15] however, its impact on viral titer is undetermined. To enhance the accuracy of detecting viral infectivity in saliva, further RCT of the efficacy of CPC mouthwash and on-demand aqueous chlorine dioxide mouthwash on the viral viability of SARS-CoV-2 is crucial.

Here, we conducted a randomized, multicenter, open-label, placebo-controlled clinical trial to evaluate the impact of CPC mouthwash and on-demand aqueous chlorine dioxide mouthwash on SARS-CoV-2 viral titer in the saliva of mildly symptomatic COVID-19 patients. At the moment, it seems like most clinical studies were based on a PCR-based measurement of viral load before and after mouthwash, which gives no relevant data as it only measures RNA fragments but gives no indication of the activity and infectivity of these fragments. On the contrary, the quantitative plaque assay performed in this study gives robust data on viral infectivity in the samples.

METHODS

Overview of the Trial

This randomized, multicenter open-label, placebo-controlled clinical trial aimed to evaluate the effectiveness of the use of mouthwashes on SARS-CoV-2 viral titers among mildly symptomatic adult patients with COVID-19. This trial was approved by the Certified Review Board of Medical Corporation Tokushukai (No. CRB23-01) on December 12, 2023, and registered in the Japan Registry of Clinical Trials (jRCT) (No. jRCTs031230566) on January 12, 2024. This study complied with local regulations, the Declaration of Helsinki, and Good Clinical Practice Guidelines. Patient enrollment was performed from January 14, 2024, to February 20, 2024, at 3 clinical sites in Osaka, Japan. The participants all signed written informed consent.

Patients

Eligibility requirements were ≥18 years old; COVID-19 infection confirmed by nucleic-acid amplification testing (antigen tests, reverse transcription-polymerase chain reaction [RT-PCR], or loop-mediated isothermal amplification [LAMP]) with mild symptoms of COVID-19 ≤3 days following onset; and no clinical contraindication to mouthwash. Following the COVID-19 Medical Practice Guidelines,[16] mild COVID-19 was defined as patients with percutaneous arterial oxygen saturation (SpO_2) of 96% or higher, with mild clinical symptoms, and without signs of pneumonia. Individuals with COVID-19 history, pending hospitalization, pregnancy, breastfeeding, mouthwash usage, antiviral or immunosuppressive medicines intake (molnupiravir, remdesivir, nirmatrelvir/ritonavir, sotrovimab, casirivimab/imdevimab, anti-interleukin-6 receptor antibodies, corticosteroids, or Janus kinase inhibitors), or mouthwash usage since COVID-19 onset were excluded from the study. The patients' data included sex, age, body mass index (BMI), underlying medical conditions, smoking history, vaccination history, date of onset, date of diagnosis, and SpO_2 levels, which were all managed through REDCap at Osaka University.[17,18]

Intervention

Eligible patients were allocated via a randomization module in REDCap[17,18] at a 1:1:1 ratio to 20 mL of purified water as placebo for 30 s, 20 mL of 0.05% CPC mouthwash for 30 s, or 9 mL of 0.01% on-demand aqueous chlorine dioxide mouthwash for 30 s. Patients rinsed with an allocated placebo or mouthwash. The amount and duration of use of each mouthwash were determined according to product specifications. Randomization was stratified according to sex. The randomization list was hidden from investigators, laboratory staff, and study monitors until the study database was finalized. Earth Corporation (Tokyo, Japan) provided CPC and

on-demand aqueous chlorine dioxide mouthwashes, and Hikari Pharmaceutical Corporation (Tokyo, Japan) supplied the placebo.

Outcomes

The primary endpoint was the SARS-CoV-2 viral titer in unstimulated saliva samples, as measured by quantitative plaque assay,[19] collected after the individuals abstained for 30 minutes or more from eating, drinking, and oral hygiene activities. Patients rinsed their mouths with mouthwash or placebo immediately after providing a baseline saliva sample while being supervised by a researcher. The unstimulated whole saliva samples were collected from each participant by a splitting method, and were taken at 10, 30, and 60 minutes. At the Japan Textile Products Quality and Technology Center (QTEC), individual sterile tubes containing the samples were stored at -40°C for up to 24 hours before testing.

SARS-CoV-2 Plaque Assay

Plaque assay was performed as described previously.[20-25] Briefly, three days before infection, VeroE6/TMPRSS2 cells (JCRB1819) (JCRB Cell Bank, Japan) were added to a 6-well plate and infected with SARS-CoV-2 at 37°C with 5% CO_2 for 1.5 hours. Mounting Minimum Essential Medium Eagle (MP Biomedicals, LLC, Cat# 1010122) containing 2% FBS (Nichirei Biosciences Inc., Cat# 174012), 0.75% agar (Ina Food Industry Co., Ltd., Type TC-5) and 0.01% Deae Dextran (pK Chemicals, Cat# 17-0350-01) was overlaid, followed by incubation at 37°C with 5% CO_2. At 3-5 d.p.i., the cells were fixed with 1% glutaraldehyde solution for 1 hour. The agar medium was removed, and the fixed cells were stained with staining solution (0.0375% methylene blue [Nacalai Tesque, Cat# 22412-14] in water) for 1 hour. The number of plaques was counted after the stained cells were washed with tap water and dried.

Sample Size

We assumed that using CPC or on-demand aqueous chlorine dioxide mouthwash would decrease salivary viral titer compared to using a placebo. A parallel, 3-group design (consisting of a control group and two mouthwash groups) was used to assess if there was a difference between the means of each mouthwash group and the control group mean (H0: $\delta = 0$ versus H1: $\delta \neq 0$, $\delta = \mu i - \mu c$).[26-30] Bonferroni-adjusted, unequal-variance t-tests ($\alpha = 0.05$) were employed to assess the hypothesis. Based on a previous study,[12] the assumed standard deviations for the control group and each mouthwash group were 8, 20, and 20, respectively. The assumed mean for the control group was -4. To have at least 80% power to detect a difference in mouthwash effects (-22 and -22) between groups (control and mouthwashes), a sample size of 16 is required for each group, resulting in a total of 48 participants. Twenty two patients are needed per group to allow for a 30% dropout rate. PASS 2023 (NCSS, LLC, Kaysville, Utah, USA) was used for sample size calculations.[31]

Statistical Analysis

The subjects' baseline characteristics were given in terms of count (percentage) and median with interquartile range. Viral titers were transformed by a log10 scale. Intention-to-treat (ITT) analyses were performed based on the original randomization assignment. The impact of CPC and on-demand aqueous chlorine dioxide mouthwash versus placebo was assessed using mixed-effects linear regression models with robust standard errors. We modeled the subject and time factors as random effects. The estimates with 95% confidence intervals (CIs) are provided for the mouthwash effects compared to placebo.

A 2-tailed statistical test with a *P*-value less than .05 was considered statistically significant. All statistical analyses were carried out using Stata 18.0 (StataCorp LLC, College Station, TX, USA).

RESULTS

Participants' Data Included in the Study

A total of 49 patients were enrolled between January 14, 2024 and February 20, 2024, and randomly assigned to receive CPC mouthwash (n=16), on-demand aqueous chlorine dioxide mouthwash (n=16), or placebo (n=17) (Figure 1). All 49 patients remained in the study for analysis.

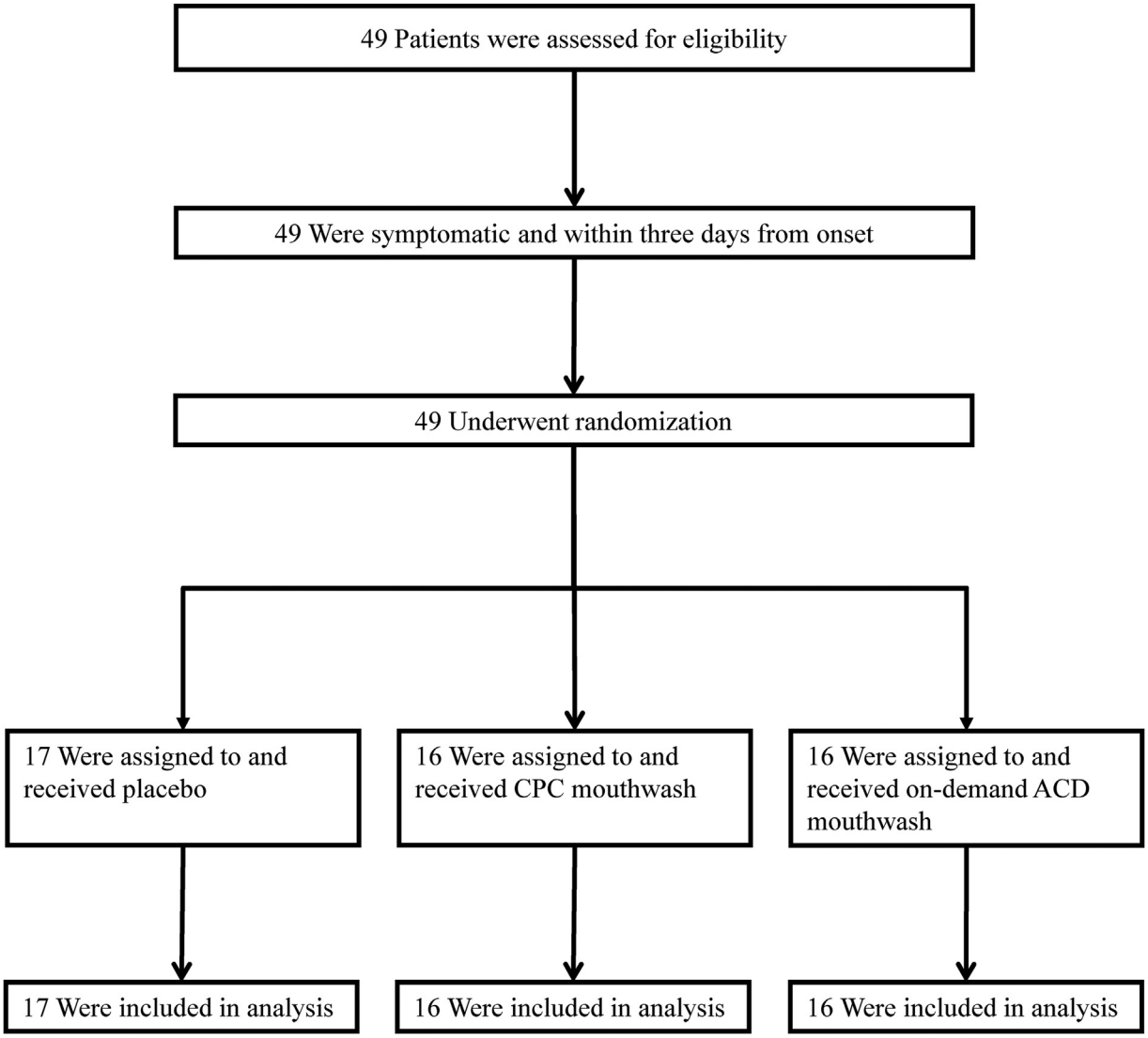
Basic Characteristics of the Patients

Table 1 shows the basic characteristics of the patients. There were no missing data in our study. 25 (51.0%) patients were male, median age was 49 years (interquartile range [IQR], 39-60), and median body mass index was 23 kg/m^2 (IQR, 20.7-24.8). 18 (36.7%) patients had a smoking history, 19 (38.8%) had underlying medical conditions, and 42 (85.7%) had a history of SARS-CoV-2 vaccination. The median time from the onset of symptoms to starting the mouthwash trial was 1 day (IQR, 1-2) and median pulse oximetry (SpO_2) was 97% (IQR, 97-98). Median salivary viral titer at baseline was 3.1 $\log_{10}$ PFU/mL (IQR, 2.3-3.8).

Difference Versus Placebo in SARS-CoV-2 Salivary Viral Titer

The relationship between a 0.05% CPC mouthwash and a 0.01% on-demand aqueous chlorine dioxide mouthwash with salivary viral titers is depicted in Table 2 and Figure 2. 0.05% CPC mouthwash was superior to placebo with regard to change from baseline in the salivary viral titer at 10 minutes (difference vs placebo, -0.97 $\log_{10}$ PFU/mL; 95% confidence interval [CI], -1.64 to -0.30; $P = .004$). In contrast, however, these effects were not shown at 30 minutes (difference vs placebo, -0.26 $\log_{10}$ PFU/mL; 95% CI, -0.92 to 0.40; $P = .435$) or 60 minutes (difference vs. placebo, -0.05 $\log_{10}$ PFU/mL; 95% CI, -0.68 to 0.58; $P = .877$). Moreover, 0.01% on-demand aqueous chlorine dioxide mouthwash proved ineffective in

Figure 1. Participants' data included in the study. ACD = aqueous chlorine dioxide; CPC = cetylpyridinium chloride.



reducing salivary viral titer compared to placebo at 10 minutes (difference vs. placebo, 0.29 $\log_{10}$ PFU/mL; 95% CI, -0.23 to 0.81; $P = .280$), 30 minutes (difference vs. placebo, 0.02 $\log_{10}$ PFU/mL; 95% CI, -0.62 to 0.66; $P = .951$), or 60 minutes (difference vs placebo, 0.22 $\log_{10}$ PFU/mL; 95% CI, -0.44 to 0.88; $P = .514$).

DISCUSSION

In this multicenter, randomized, open-label, placebo-controlled study, we assessed the impact of a 0.05% CPC and 0.01% on-demand aqueous chlorine dioxide mouthwash versus placebo on salivary SARS-CoV-2 viral titers for patients with mild COVID-19. Our study found 10-minute salivary viral titer reduction occurred with use of 0.05% CPC compared to placebo. In contrast, 0.01% on-demand aqueous chlorine dioxide mouthwash did not reduce SARS-CoV-2 viral titer in saliva. We assessed not only viral titer but even conducted a cell infection assay, which is highly worthwhile to give data not only on viral load but also on viral infectivity.[32] Our study suggests that 0.05% CPC mouthwash might reduce COVID-19 viral titer for a duration of 10 minutes.

Although use of 0.05% CPC mouthwash led to a decrease in SARS-CoV-2 salivary viral titer at 10 minutes compared with placebo, this effect was not observed at 30 or 60 minutes. Our results are consistent with previous RCTs which

Table 1. Baseline characteristics.

	Placebo (n=17)		CPC mouthwash (n=16)		On-demand ACD mouthwash (n=16)	
Male, n (%)	9	(52.9)	8	(50.0)	8	(50.0)
Median (IQR) age (years)	43	(34-60)	51	(43.5-61)	50	(41-55.5)
Median (IQR) body mass index (kg/m^2)	23.2	(20.7-24.8)	22.4	(20.3-23.4)	23.9	(20.8-26.9)
Smoking history, n (%)						
Non-smokers	12	(70.6)	9	(56.3)	10	(62.5)
Former smokers	4	(23.5)	5	(31.3)	4	(25.0)
Current smokers	1	(5.9)	2	(12.5)	2	(12.5)
Comorbidity (yes), n (%)	8	(47.1)	7	(43.8)	4	(25.0)
COVID-19 vaccination status, n (%)						
None	4	(23.5)	2	(12.5)	1	(6.3)
One dose	0	(0.0)	0	(0.0)	0	(0.0)
Two doses	1	(5.9)	1	(6.3)	1	(6.3)
Three doses	6	(35.3)	5	(31.3)	6	(37.5)
Four doses	3	(17.6)	4	(25.0)	3	(18.8)
Five doses	1	(5.9)	2	(12.5)	3	(18.8)
Six or more doses	2	(11.8)	2	(12.5)	2	(12.5)
Median (IQR) days from onset to diagnosis	1	(1-2)	2	(1-2)	1	(1-2)
Median (IQR) pulse oximetry (SpO_2) (%)	97	(97-98)	97	(96-98)	97	(97-97)
Median (IQR) salivary viral load (log_{10} PFU/mL)	3.1	(2.3-3.8)	3.0	(2.2-3.8)	3.1	(2.6-3.7)

Abbreviations: ACD, aqueous chlorine dioxide; CPC, cetylpyridinium chloride; IQR, interquartile range.

have indicated that CPC inactivates SARS-CoV-2.[10-13] Previous in vitro studies have also shown that 0.04-0.075% CPC mouthwashes showed virucidal activity against SARS-CoV-2 strains.[33-36] These results may be because CPC suppresses viral fusion by disrupting the viral envelope and hindering virus entry into target cells.[5] CPC disrupts lipid bilayers, and may exert cytotoxic effects; therefore, CPC can be applied in a formulation that can exert its effect for SARS- CoV-2.[6] Although our previous RCT showed that 0.05% CPC mouthwash do not lead to a reduction in salivary SARS-CoV-2 viral load,[9] there was an important limitation in that we only evaluated the presence of viral particles and not their viability or infectious capacity. Thus, to evaluate the effect of 0.05% CPC mouthwashes in patients with COVID-19, it was necessary to conduct viral culture by infecting cell cultures with samples before/after the mouthwash. Our clinical trial suggests that 0.05% CPC mouthwashes are effective in reducing SARS-CoV-2 viral titer in the short term, but that they lose their effectiveness over time.

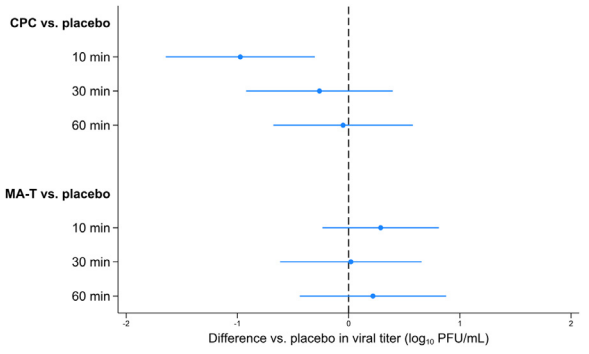
We also found that 0.01% on-demand aqueous chlorine dioxide mouthwash did not reduce SARS-CoV-2 salivary viral titers. To our knowledge, this study is the first RCT examining the relationship between using on-demand aqueous chlorine dioxide mouthwash and a reduction in salivary SARS-CoV-2 viral titer. Our study contradicts the laboratory study that indicate a suppressive impact of on-demand aqueous chlorine dioxide on SARS-CoV-2.[14] On-demand aqueous chlorine dioxide is a chemical agent that controls

Table 2. Difference vs placebo in SARS-CoV-2 salivary viral titer.

	Difference vs placebo	95% CI	*P* value
CPC mouthwash vs placebo			
Baseline	Reference		
10 min	-0.97	(-1.64, -0.30)	.004
30 min	-0.26	(-0.92, 0.40)	.435
60 min	-0.05	(-0.68, 0.58)	.877
On-demand ACD mouthwash vs placebo			
Baseline	Reference		
10 min	0.29	(-0.23, 0.81)	.280
30 min	0.02	(-0.62, 0.66)	.951
60 min	0.22	(-0.44, 0.88)	.514

Abbreviations: ACD, aqueous chlorine dioxide; CI, confidence interval; CPC, cetylpyridinium chloride.

Figure 2. Difference vs placebo in SARS-CoV-2 salivary viral titer. ACD = aqueous chlorine dioxide; CPC = cetylpyridinium chloride.



the generation of aqueous radicals via organic catalyst technology. When SARS-CoV-2 is present, the active aqueous chlorine dioxide radical collides with surrounding water molecules or chlorite ions and attacks the components of SARS-CoV-2, such as spike glycoprotein, membrane protein, nucleocapsid protein, and envelope protein.[14,37] However, the discrepancy between studies may be due to differences in study design (randomized controlled trial vs laboratory study), study populations, analysis methods, intervention agents, and follow-up time. Additionally, our previous RCT showed that 0.01% on-demand aqueous chlorine dioxide mouthwash did not lead to a significant reduction in SARS-CoV-2 salivary viral load.[9] Our previous and the current RCTs suggest that 0.01% on-demand aqueous chlorine dioxide mouthwash may not affect the salivary viral load and titer of SARS-CoV-2. Our results suggest the importance of further clinical trials on the effects of different concentrations of on-demand aqueous chlorine dioxide mouthwash for longer times on SARS-CoV-2 viral titer.

The oral cavity is a significant site for both the transport of SARS-CoV-2 via saliva and the initiation of its replication.[38] While antiviral drugs and vaccines have their roles in preventing the spread of COVID-19, public health officials must also consider nonpharmaceutical interventions as essential components of the COVID-19 response strategy. Mouthwash functions as an affordable daily antiseptic in addition to tooth brushing for maintaining oral hygiene.[39] Disproportionately affected by COVID-19, poor and minority populations face restricted access to oral healthcare, exacerbating preexisting oral health issues.[40] Recognizing the impact of nonpharmaceutical interventions like mouthwashes is crucial to shaping public health policies for COVID-19.[41] Our RCT showed that the use of CPC mouthwash reduces SARS-CoV-2 salivary viral titer for 10 minutes, but effects vanish within 30 minutes. Our results suggest that 0.05% CPC mouthwash may temporarily decrease SARS-CoV-2 viral titers. However, the variability of viral titers with mouthwash concentration and emerging SARS-CoV-2 variants may cause our results to change. Although mouthwash might

be effective for a short time, a systematic review suggested that using mouthwash before dental procedures might potentially decrease COVID-19 transmission to dental teams and help improve systemic issues in COVID-19 patients related to oral microbial flora.[42] Moreover, a recent review added some clinical outlook that CPC-based mouthwashes can be used as preprocedural mouthwash in the dental practice as part of the bundle of measures for infection prevention.[43] Another multicenter RCT has also indicated that using the β-cyclodextrin–citrox mouthwash (CDCM) might reduce the SARS-CoV-2 viral load in saliva.[44] Further research is required to determine the exact effectiveness of several kinds of mouthwashes, including CPC/on-demand aqueous chlorine dioxide mouthwash.

In RCTs, randomization achieves the goal of evenly distributing known and unknown factors between control and intervention groups, decreasing potential confounding.[45] Despite these strengths, our study has several methodological limitations. First, the findings cannot be generalized to patients with moderate or severe COVID-19, or to different concentrations and durations of mouthwash. Second, our study group assignment was not concealed during the trial. Thus, knowledge of assignment and adherence could have influenced patients' behavior and outcomes. Third, our results, potentially biased due to recruited COVID-19 patients, may not represent the entire population. Additionally, we were missing data on education, income, employment, nutrition, and alcohol use for individuals. Although baseline characteristics were similar across groups, a more comprehensive exploration of patient characteristics might enhance the applicability of the study findings.[46] To address these limitations, future studies should evaluate the efficacy of CPC and on-demand aqueous chlorine dioxide mouthwash against SARS-CoV-2 viral titer in diverse populations and settings.

In conclusion, our clinical study revealed a significant reduction in SARS-CoV-2 salivary viral titer for 10 minutes after using 0.05% CPC mouthwash compared to placebo. In contrast, 0.01% on-demand aqueous chlorine dioxide mouthwash had no impact on SARS-CoV-2 salivary viral titer compared to placebo. Further investigation is needed across various populations and settings.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

DAISUKE ONOZUKA: Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **KEIJI KONISHI:** Writing – review & editing, Investigation. **SATOKO TAKATERA:** Writing – review & editing, Investigation. **MASAHIDE OSAKI:** Writing – review & editing, Investigation. **SHOUGEN SUMIYOSHI:** Writing – review & editing, Investigation. **YUSUKE TAKAHASHI:** Writing – review & editing, Investigation. **SHIGETO HAMAGUCHI:** Writing – review & editing, Investigation. **YASUO IMOTO:** Writing – review & editing, Investigation. **SATOSHI KUTSUNA:** Writing – review & editing, Supervision, Funding acquisition.

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