TITLE: Cetylpyridinium chloride-containing mouthwashes reduce *in vitro* SARS-CoV-2 infectivity

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ABSTRACT (105)

Oral mouthwashes decrease the infectivity of several respiratory viruses including SARS-CoV-2. However, the precise agents with antiviral activity present in these oral rinses and their exact mechanism of action remain unknown. Here we show that Cetylpyridinium chloride (CPC), a quaternary ammonium compound present in many oral mouthwashes, reduces SARS-CoV-2 infectivity by inhibiting viral fusion with target cells. We also found that CPC and CPC-containing mouth rinses decreased a thousand times the infectivity of SARS-CoV-2 *in vitro,* while the corresponding vehicles had no effect. CPC-containing mouth rinses could represent a cost-effective measure to reduce SARS-CoV-2 infectivity in saliva, aiding to reduce viral transmission from infected individuals.

BACKGROUND

Several studies have shown the antiviral potential of mouthwashes, which decrease the infectivity of relevant airborne transmitted infectious viruses, such as influenza and distinct coronavirus, including SARS-CoV-2 (Meister et al., 2020; O'Donnell et al., 2020; Popkin et al., 2017; Staktuke, 2020). If proven effective in the oral cavity, this antiviral strategy could represent a globally accessible cheap measure easily implemented worldwide, that could reduce the infectivity of SARS-CoV-2 in saliva and cut viral transmission chain. Moreover, as mouthwashes are also produced in oral spray formats, they are also ideal strategies for vulnerable populations such as the elderly. Despite the universal applicability of this antiviral approach, and the diverse reports proving the activity of various oral products *in vitro*, we still don't know which are the individual components present in these mouthwashes that exert the antiviral effect, and what is their precise mechanism of action. Here we focused on the effect of Cetylpyridinium chloride (CPC), a quaternary ammonium compound used in many oral mouthwashes and breath sprays with broad antiseptic and anti-microbicide activity. We compared the anti-SARS-CoV-2 activity of CPC and CPC-containing mouth rinses against their vehicles, and found that CPC-containing mouth rinses inhibit SARS-CoV-2 entry into target cells while decreasing a thousand times the infectivity of SARS-CoV-2.

MATERIAL & METHODS

Cell Cultures. Vero E6 cells (ATCC CRL-1586) were cultured in Dulbecco's modified Eagle medium, (DMEM) with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen). HEK-293T overexpressing the human ACE2 were kindly provided by Integral Molecular Company and maintained in DMEM (Invitrogen) with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin, and 1 µg/mL of puromycin (all from Invitrogen).

Pseudovirus production. HIV-1 luciferase reporter pseudoviruses expressing SARS-CoV-2 Spike protein were generated using two plasmids. pNL4-3.Luc.R-.E- was obtained from the NIH AIDS repository. SARS-CoV-2.SctΔ19 was generated (Geneart) from the full protein sequence of SARS-CoV-2 spike with a deletion of the last 19 amino acids in C-terminal, human-codon optimized and inserted into pcDNA3.4-TOPO (Ou et al., 2020). Spike plasmid was transfected with X-tremeGENE HP Transfection Reagent (Merck) into HEK-293T cells, and 24 hours post-transfection, cells were transfected with pNL4-3.Luc.R-.E-. Supernatants were harvested 48 hours later, filtered with 0.45 µM (Millex Millipore) and stored at -80ºC until use. The $p24^{gag}$ content of all viruses was quantified using an ELISA (Perkin Elmer) and viruses were titrated in HEK-293T overexpressing the human ACE2.

Pseudovirus assay. HEK-293T overexpressing the human ACE2 were used to test mouth rinses and their vehicles at the indicated concentrations. A constant pseudoviral titer was used to pulse cells in the presence of the mouth rinses. 48h post-inoculation, cells were lysed with the Glo Luciferase system (Promega). Luminescence was measured with an EnSight Multimode Plate Reader (Perkin Elmer).

Virus isolation, titration and sequencing. SARS-CoV-2 was isolated from a nasopharyngeal swab collected from an 89-year-old male patient giving informed consent and treated with Betaferon and hydroxychloroquine for 2 days before sample collection as described in (Rodon, 2020). The virus was propagated for two passages and a virus stock was prepared collecting the supernatant from Vero E6. Genomic sequence was deposited at GISAID repository (http://gisaid.org) with accession ID EPI_ISL_510689.

Antiviral activity. We tested two mouthwashes from Dentaid containing CPC: Perio Aid Intensive Care (with 1.47 mM of CPC plus 1.33 mM of Chlorhexidine) and Vitis CPC Protec (with 2.063 mM of CPC). Vehicles containing the same formulation but without CPC were also tested in parallel. We also assayed 10 mM of CPC diluted in distillated water. Of note, colorants were removed from all formulations to avoid any interference with luciferase reactions. 1 mL of mouth rinses or their corresponding vehicles were mixed with 1mL of SARS-CoV-2 with $10^{5.8}$ $TCID_{50}$/mL for 2 minutes. Virus was also mixed with 1 mL of media as positive control. After 2 minutes incubation, mixes were diluted in PBS and filtered for 10 minutes at 1000g in macrosept® advance centrifugal devices of 100 K MWCO of exclusion (Pall Corporation) to wash away mouth rinses twice. Washed viruses were resuspended in 2 mL of fresh media and titrated in triplicates on Vero E6 cells for 10 serial dilutions. 3 days post infection, cells were assayed in a microscope for viral induced cytopathic effect. To detect any associated cytotoxic effect, mouth rinse formulations were also mixed with media, washed and centrifuged as previously described, and were equally cultured on Vero E6, but in the absence of virus. Cytotoxic effects of these products were measured 3 days after infection, using the CellTiter-Glo luminescent cell viability assay (Promega). Luminescence was measured in a Fluoroskan Ascent FL luminometer (ThermoFisher Scientific).

IC_{50} calculation. Response curves of mouth rinses against pseudoviral entry were adjusted to a non-linear fit regression model, calculated with a four-parameter logistic curve with variable slope. Cells exposed to the pseudovirus in the absence of products were used as positive controls of infection, and were set as 100% of viral fusion to normalize data and calculate the percentage of viral entry inhibition. Cells not exposed to the mouthwashes nor the pseudovirus were used as positive controls of viability, and were set as 100% to normalize data and calculate the percentage of cytopathic effect. All analyses and figures were generated with the GraphPad Prism v8.0b Software.

RESULTS

We first tested the capacity of CPC-containing mouth rinses to inhibit SARS-CoV-2 entry into target cells. We employed a luciferase-based assay using pseudotyped lentivirus expressing the spike protein of SARS-CoV-2, which allows to detect viral fusion on target HEK 293 T cells that expressed the ACE2 receptor. A constant concentration of a reporter pseudovirus containing the SARS-CoV-2 Spike protein was mixed with increasing concentrations of the indicated CPC-containing mouth rinses or their vehicles and added to target cells. To control for any mouthwash-induced cytotoxicity, target cells were also cultured with increasing concentrations of the indicated products in the absence of pseudoviruses. By these means, we calculated the concentration at which the mouth rinses blocked viral entry and achieved a 50% maximal inhibitory capacity (IC_{50}). CPC-containing mouth rinses were able to inhibit viral fusion in a dose dependent manner (**Figure 1**, left panels **A** and **C**, red lines) at concentrations where no cytotoxic effects of the mouth rinses were observed (**Figure 1**, left panels **A** and **C**, grey lines). Of note, no obvious inhibitory activity was detected on vehicles (**Figure 1**, right panels **B** and **D**), clearly pointing to CPC as the antiviral compound contained in the oral formulations. To further confirm the antiviral activity of CPC, we also tested this compound resuspended in water, and found that it also inhibited SARS-CoV-2 pseudoviral fusion and entry into target cells (**Figure 1**, bottom panel **E**). These results indicate that CPC and CPC-containing mouth rinses are able to block SARS-CoV-2 viral entry into target cells due to the activity of CPC.

Next, we tested the capacity of CPC to reduce the infectivity of a clinical isolate of SARS-CoV-2. A 1 to 1 ratio of SARS-CoV-2 was mixed with CPC or CPC-containing mouth rinses or their vehicles for 2 minutes and washed twice with PBS to remove the formulations by ultrafiltration using a macrosept® centrifugal device. Collected viruses were titrated on Vero E6 cells to calculate the Tissue Culture Infectious Dose 50% ($TCID_{50}$) per mL after each of the treatments. While water used to dilute CPC had no effect on SARS-CoV-2 infectivity as compared to untreated virus (**Figure 2**), high doses of CPC effectively suppressed viral infection on Vero E6 (**Figure 2**). Analogously, treatment for 2 minutes with CPC-containing mouthwashes decreased 1,000 times the $TCID_{50}$/mL of SARS-CoV-2, while vehicles had no impact on SARS-CoV-2 infectivity when compared to untreated virus (**Figure 2**). To control for the presence of mouthwash remaining in the viral preparations that could induce cytotoxic effects, target cells were also cultured with the indicated products washed in ultrafiltration centrifugal devices as

previously described, but in the absence of SARS-CoV-2. By these means we confirmed that the observed SARS-CoV-2 induced cytopathic effect was effectively inhibited at concentrations where the CPC or CPC-containing mouthwashes were not toxic for the cells. Thus, CPC has antiviral activity against SARS-CoV-2 and CPC-containing mouthwashes have the capacity to reduce 1,000 times the infectivity of a viral stock when treated at a 1:1 ratio for 2 minutes.

DISCUSSION

Here we have shown that CPC has an antiviral activity against SARS-CoV-2, and that this compound exerts its activity blocking viral entry or inhibiting viral fusion on target cells. Most likely, CPC will act disrupting the integrity of the viral envelope, as previously shown for influenza virus (Popkin et al., 2017). Thus, CPC-containing mouthwashes could protect the oral mucosa from infection. Yet, since SARS-CoV-2 may most likely infect cells via the upper respiratory tract, further strategies should consider the use of CPC in nasal sprays to fully achieve the prophylactic potential of this approach. Our results also showed that in a very restrictive experiment, where we mixed equal volumes of a highly infectious SARS-CoV-2 viral stock with different CPC-containing mouthwashes for 2 minutes, these treatments reduced a 1,000 times the $TCID_{50}$ only in the presence of CPC. Moreover, as gargles are usually performed with 10 ml of mouth rinse, and saliva in mouth has an approximate volume of 1 to 2 ml that will most likely have always lower infectivity than a viral stock of SARS-CoV-2 grown in the laboratory, mouthwashes could be even more effective in the oral cavity that what we have estimated here *in vitro*. Thus, CPC-containing mouthwashes could be a cost-effective measure to reduce SARS-CoV-2 infectivity in saliva, aiding to reduce viral transmission from infected individuals. Several events where numerous people were infected at the same time, which are considered superspreading events, are related to activities where people were either talking, shouting or singing (Hamner et al., 2020; Lemieux et al., 2020). Indeed, viable viruses have been isolated from the saliva of COVID-19 infected individuals (Jeong et al., 2020), probing that exhaled saliva micro-droplets and aerosols are infectious. Future work should address if in the oral cavity of SARS-CoV-2 infected individuals, CPC-containing mouth rinses are able to decrease viral load and infectivity of viruses found in saliva. While prior studies have shown that CPC has an anti-bacterial activity that lasts for 3 to 5 hours in saliva (Elworthy et al., 1996), future studies should address the duration of the CPC antiviral activity in the oral cavity. This information will be key to effectively use this cost-effective measure to maintain a reduced infectious capacity of SARS-CoV-2 in the saliva.

FIGURES

Figure 1. Antiviral activity of CPC-containing mouthwashes inhibiting SARS-CoV-2 entry. Percentage of viral entry inhibition on target HEK-293T cells expressing ACE2 exposed to a fixed concentration of SARS-CoV-2 in the presence of increasing concentrations of oral formulations (**A** and **C**), their vehicles (**B** and **D**) and CPC diluted in water (**E**). Non-linear fit to a variable response curve from one experiment with two replicates is shown (red lines), excluding data from drug concentrations with associated toxicity. When calculated, the particular IC_{50} value of the graph is indicated. Cytotoxic effect on HEK-293T cells expressing ACE2 cells exposed to increasing concentrations of mouthwashes or vehicles in the absence of virus is also shown (grey lines).

Figure 2. Infectivity of SARS-CoV-2 treated with CPC-containing mouthwashes. One mL of of SARS-CoV-2 with $10^{5.8}$ $TCID_{50}$ was treated with CPC (10 mM) or CPC-containing mouth rinses (2 mM) and their respective vehicles for 2 minutes at a 1:1 ratio. Untreated virus was used as positive control. Infectivity of treated viruses washed right after treatment by ultrafiltration to remove cytotoxic mouthwashes were assayed on Vero E6 cells 3 days post infection. In parallel, we confirmed that the inhibitory effect was not due to any cytotoxic effect of the mouthwashes, as tested on Vero E6 cells exposed to the media remaining from washed mouth rinses that were equally centrifuged in the absence of virus.

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AUTHOR CONTRIBUTION

Conceived and designed the experiments: R.L, VB, J.G, B.C., N.I-U.

Performed experiments: J.M-B, D.P-Z.

Analyzed and interpreted the data: J.M-B, D.P-Z, R.L, VB, J.G, B.C., N.I-U.

Wrote the paper: J.M-B, D.P-Z, N.I-U.

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COMPETING INTEREST

R.L, VB. And J.G are researchers working for Dentaid Research Center. The authors declare that no other competing interest exist.

DATA AVAILABILITY Data is available from corresponding author upon reasonable request.

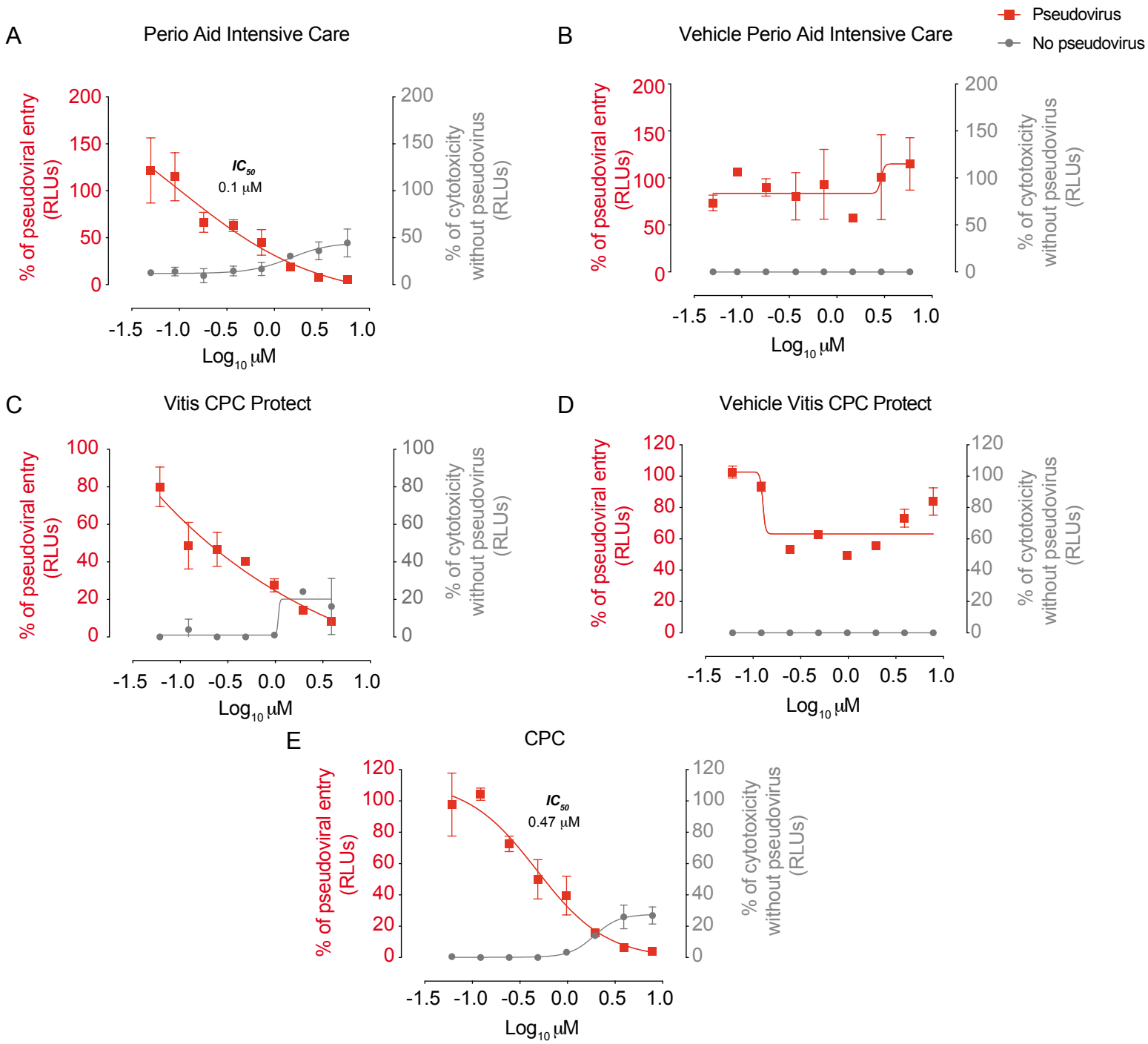
A
Perio Aid Intensive Care
IC50
0.1 µM
% of pseudoviral entry (RLUs)
% of cytotoxicity without pseudovirus (RLUs)
Log10 µM
B
Vehicle Perio Aid Intensive Care
Pseudovirus
No pseudovirus
C
Vitis CPC Protect
D
Vehicle Vitis CPC Protect
E
CPC
IC50
0.47 µM

Figure 1

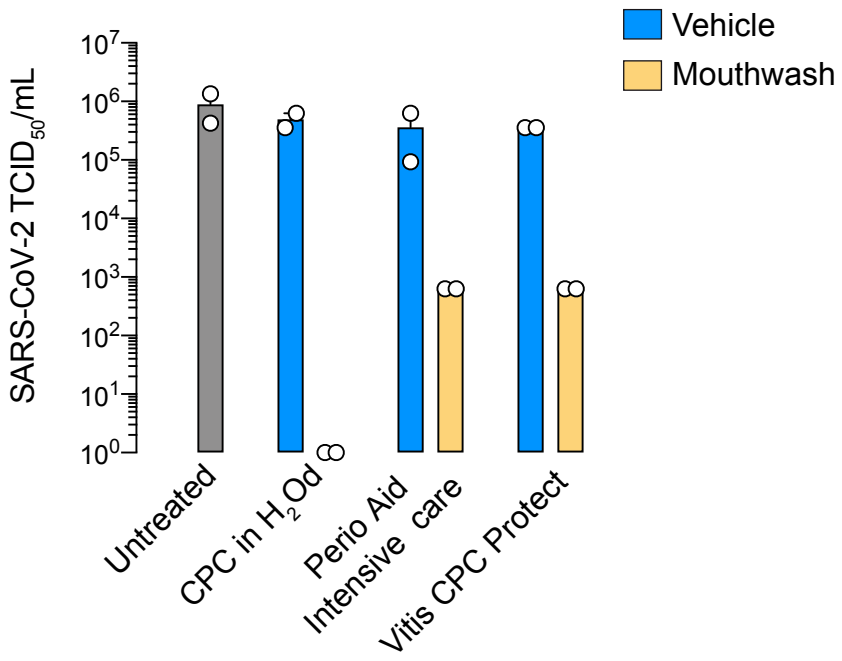
Vehicle
Mouthwash
SARS-CoV-2 TCID50/mL
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Untreated
CPC in H2Od
Perio Aid
Intensive care
Vitis CPC Protect

Figure 2