Disinfection effect of hexadecyl pyridinium chloride on SARS-CoV-2 *in vitro*

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Research Article

Keywords: hexadecyl pyridinium chloride, SARS-COV-2, disinfection effect, cytopathic effect, oral disinfectant

Posted Date: April 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-458096/v3

Abstract

The novel coronavirus (COVID-19 or 2019-nCoV) is a respiratory virus that can exist in the mouth and saliva of patients and spreads through aerosol dispersion. Therefore, stomatological hospitals and departments have become high-infection-risk environments. Accordingly, the search for oral disinfectants that can effectively inactivate the virus has become a highly active area of research. Hexadecyl pyridinium chloride, povidone-iodine, and other common oral disinfectants are the natural primary choices for stomatological hospitals. Therefore, this study investigated the inhibitory effect of hexadecyl pyridinium chloride on SARS-CoV-2 *in vitro*. Vero cells infected with SARS-CoV-2 were used to determine disinfection effect; the CCK-8 method was used to determine cytotoxicity; and viral load was determined by real-time PCR. The results showed that hexadecyl pyridinium chloride has no obvious cytotoxic effect on Vero cells in the concentration range 0.0125–0.05 mg/mL. The *in vitro* experiments showed that hexadecyl pyridinium chloride significantly inhibits the virus at concentrations of 0.1 mg/mL or above at 2 min of action. Thus, the results provide experimental support for the use of hexadecyl pyridinium chloride in stomatological hospitals.

Introduction

The outbreak of coronavirus disease 2019 (COVID-19) began in Wuhan, China in December 2019 [1, 2], and the total number of confirmed COVID-19 cases has exceeded 40 million in 211 countries around the world [3, 4]. The most severe epidemic areas are mainly located in Asia and the Americas [5]. The impact on children has so far been relatively small compared to that on adults. The novel coronavirus (severe acute respiratory syndrome coronavirus; SARS-CoV-2) spread rapidly, sweeping across China in just a few days, causing varying degrees of respiratory disease and, in severe cases, death [6]. Accordingly, the SARS-CoV-2 outbreak poses a massive threat to public safety, and the World Health Organization (WHO) has declared a public hygiene emergency.

Since the outbreak, we have a reached a deeper understanding of SARS-CoV-2. The genome of SARS-CoV-2 has a high degree of similarity with the coronavirus carried in bats [7] and the spike protein of the coronavirus is the key target of antibodies [8]. The virus can bind to target cells via angiotensin-converting enzyme Ⅱ (ACE).

Yao et al. reported the first full three-dimensional fine structure of SARS-CoV-2 [9], while Zhou et al. reported the full-length genome of 2019-nCoV. It has the characteristics of a high infection rate, a wide range of transmission modes, and a high mortality rate. Therefore, people must find effective treatment and prevention methods. But at present, no vaccine or drug that is highly effective against SARS-CoV-2 has been approved for production and application [10, 11].

The SARS-CoV-2 is a respiratory disease that can be spread by direct transmission, aerosol transmission, or contact transmission. SARS-CoV-2 has a typical incubation period of 3–7 days and rarely more than 14 days. After infection, patients often develop fever, cough, fatigue, and other symptoms.

The virus can be present in the oral cavity and saliva of patients, presenting a high risk of infection and cross-infection to the medical staff in stomatological hospitals [12]. Thus, killing the virus in the mouth is extremely important [13] if we are to minimize the chance of iatrogenic infections, which can have serious consequences if they occur. Accordingly, the virucidal effects of oral disinfectants on SARS-CoV-2 to reduce the possibility of oral cavity transmission of SARS-CoV-2 is an active area research at present [14].

Traditional disinfectors can have virucidal effects [5], and the oral disinfectant hexadecyl pyridinium chloride has been shown to be effective for oral sterilization [15] as well as having activity against the hepatitis B virus [16] and bacteria that cause periodontitis [17]. Hexadecyl pyridinium chloride is a widely used personal care product that has been listed by the FDA as Generally Regarded as Safe (GRAS). More importantly, it exhibits a certain action against SARS-CoV-2 that can destroy the structure of the virus surface [18].

Nevertheless, there is no significant research on SARS-CoV-2 disinfection using mouthwash disinfectant. Therefore, in the present study we evaluated the inhibitive effect of hexadecyl pyridinium chloride against SARS-CoV-2 in the Vero cell model. It is hoped that this study will provide invaluable information for the use of disinfectants in oral clinical treatment during the epidemic.

Materials And Methods

Virus and cells

SARS-CoV-2 (nCov-19 / Hangzhou / ZJU-01 / 2020, GISAID, ID: 415709) was isolated from a patient in the Medical College of the First Affiliated Hospital of Zhejiang University. The Vero cells (ATCC CCL-81) were cultured in modified Eagle medium (MEM) (Life Technologies, USA) supplemented with 2% fetal bovine serum (Life Technologies, USA). The cells were cultured in a 5% CO_2 incubator at 35 ℃. The virus titer was determined by 50% tissue culture infectious dose assay (TCID50). All the experiments involving infectious viruses were conducted in the approved biosafety level III laboratory (CNAs bl002, National Key Laboratory of Infectious Diseases Diagnosis and Treatment, Zhejiang University).

CCK-8 cell proliferation toxicity test

Vero cells were seeded on 96-well plates and incubated for 24 h at 35 ℃ under 5% CO_2. The cells were then treated with five-fold serial diluted 0.2 mg/mL hexadecyl pyridinium chloride (0.2, 0.1, 0.05, 0.025, 0.0125 mg/mL) for 48 h. Then, 10 μL of CCK-84 solution (MedChemExpress, USA) was added to each well, and the absorbance value 450 nm was measured after 3 h. The dose toxicity and maximum non-toxic concentration of disinfectant were then calculated.

Evaluation of SARS-COV-2 disinfection effect

Vero cells were inoculated in a 96-well plate with 1×10^5 cells/well and incubated under 5% CO_2 at 35 ℃ to the most active 75%–90% slice cells in the logarithmic growth phase. The cell culture medium was discarded and washed twice with Hank's solution. Then, 3 μL cell solutions were mixed with SARS-COV-2 and 3 μL of a twice-diluted solution (0.2, 0.1, 0.05, 0.025, or 0.0125 mg/mL) of hexadecyl pyridinium chloride. The cells were exposed to different concentrations of disinfectant for 30 s, 1 min, 2 min, or 5 min. Culture medium was then added to each test tube for 500-times dilution.

We also performed ultrafiltration with centrifuge tubes (AMICON ULTRA 50K, Millipore, US). First, 0.5 mL of virus (7.0_LogTCID$_{50}$/mL) and 0.5 mL hexadecyl pyridinium chloride of different concentrations (0.2, 0.1, 0.05, 0.025, 0.0125 mg/mL) were mixed for 2 min, then the mixture was transferred to an ultrafiltration centrifuge tube and centrifuged at 8000 rpm for 5 min, and then repeatedly suck the mixture to make them mix evenly. We also set up a control group using 0.5 mL medium, which was added into the normal cells. The cells were cultured under 5% CO_2 at 35 ℃ for 48 h and washed with Hank solution twice. All the experiments were performed three times. See in Figure. 1.

Cytopathic effect (CPE) was observed at 48 hpi. TCID50 was determined by the Reed-Muench method[19].

Viral RNA extraction and RT-PCR

The virus RNA was extracted from 200 μL Vero cell supernatant (48 h after infection) by an automatic nucleic acid extraction system (MVR01, Liferiver, Shanghai, China). A one-step RT-PCR Kit (Cat No: Z-RR-0479-02-50, Liferiver, Shanghai, China) was used to simultaneously detect the RdRp, N, and E genes of the virus with a LightCycler 480II Real-Time PCR system (Roche, Rotkreuz, Switzerland). All the experiments were performed in triplicate. The $2^{-\triangle\triangle Ct}$ method was used to determine the relative expression levels between the treatment group and the control group.

Immunofluorescence microscopy

Vero cells were washed with phosphate-buffered saline (PBS) 48 hpi, fixed in 80% precooled acetone (Sigma-Aldrich, USA) for 30 min, incubated in 1% bovine serum albumin PBS at room temperature, and incubated with anti-Spike RBD Rabbit PAb (1:1000; Sino Biological Inc, Beijing, China; Cat: 40592-T62) at 4 ℃ overnight. Then, the cells were washed twice with PBS, incubated at room temperature for 2 h, and treated with Alexa Fluor488®-conjugated Goat Anti-rabbit IgG secondary antibody (1:1500, Abcam, Cat No. ab150077). The stained cells were observed under a fluorescence microscope and photographed. The nuclei were stained with DAPI.

Statistical analysis

EC50 was calculated by nonlinear regression, and PRISM (GraphPad Software, San Diego, CA, USA) was used to analyze the data.

Results

Toxic effect of hexadecyl pyridinium chloride on Vero cells

CCK-8 cell proliferation toxicity test and cell pathology observation were used to determine the toxicity of the disinfectant on Vero cells. The results are shown in Figure 2. Hexadecyl pyridinium chloride has an apparent toxic effect on Vero cells at a concentration of 0.05 mg/mL or above after 30 s, 1 min, 2 min, and 5 min. Upon dilution and ultrafiltration, the toxic effect of disinfectant on cells is greatly reduced, and the disinfectant is non-toxic after ultrafiltration. The results are shown in Figure 2.

Inhibitory effect of hexadecyl pyridinium chloride on SARS-COV-2 *in vitro*

The Vero cell culture medium was treated 48 hpi with hexadecyl pyridinium chloride solution (0.2, 0.1, 0.05, 0.025, or 0.0125 mg/mL) and a 1:500 dilution of hexadecyl pyridinium chloride under ultrafiltration conditions. The supernatant of the culture was collected for RT-PCR, and the inhibition% was calculated: Inhibition%= $(1- 2^{-\triangle\triangle Ct})$ ×100%.

The results shown in Figure 2 demonstrate that the disinfection effect of hexadecyl pyridinium chloride is time- and concentration-dependent. When the action time is 30 s, the disinfection effect is poor, but when it is 2 min or above, the disinfection effect is better. There is no significant difference in inhibition rate after 2 min. When the concentration is 0.1 mg/ mL or above, there is a clear virus-elimination effect. The results show that, in the Vero cell model, hexadecyl pyridinium chloride has the strongest virus-elimination effect at a concentration of 0.1 mg/mL for 2 min.

Immunofluorescence microscopy and TCID50 testing verified the disinfection effect of hexadecyl pyridinium chloride

The supernatants (200 μL) of 48 hpi cells were collected and treated with hexadecyl pyridinium chloride at different concentrations (0.2, 01, 0.05, 0.025, and 0.0125 mg/mL) for TCID50 analysis, and the results are shown in Figure 4. The titer of the virus decreases with increasing hexadecyl pyridinium chloride concentration, showing a dose-dependent relationship. The results are shown in Figure 3.Hexadecyl pyridinium chloride at 0.1 mg/mL or above exhibits a good inhibitory effect on virus proliferation when

the action time is longer than 2 min. In summary, these results show that the disinfection effect is most apparent when the concentration of hexadecyl pyridinium chloride is 0.1 mg/mL and the action time is 2 min, and that it is significantly correlated with the inhibition of the CPE.

Discussion

COVID-19 caused by SARS-COV-2 has spread worldwide, having a huge impact on global public health. Accordingly, countries around the world are making every effort to cope with this severe challenge. On the front-line of the battle against coronavirus is stomatological hospitals, where the risk of infection is particularly high. Thus, effective antiviral oral disinfectants are urgently required to protect medical staff and stem the spread of the virus. Previous experimental studies have shown that the oral disinfectant hexadecyl pyridinium chloride exerts an inactivation effect on certain bacteria and viruses.

In this study, it was found that, in the test concentration range 0.0125–0.05 mg/mL, hexadecyl pyridinium chloride has no apparent cytotoxic effect. Furthermore, the results revealed significant inhibition of the virus with 2 min of action at a concentration of 0.1 mg/ mL or above. The disinfection effect is the most important in the clinical use of disinfectants. To further verify that 2 min is the optimal disinfection time, we carried out ultrafiltration centrifugation experiments.

In short, due to the oxidation strength of chlorinated compounds, hexadecyl pyridinium chloride has a disinfection effect on bacteria and viruses and thus application prospects as a disinfectant in stomatological hospitals.

The Vero cell model may be a relatively simple means for the study of the disinfection effect of hexadecyl pyridinium chloride on SARS-CoV-2. However, we intend to study the disinfection effect of hexadecyl pyridinium chloride in other oral mucosal cell models and animal models that provide more complex virus environments. We will also further explore the disinfection mechanism of hexadecyl pyridinium chloride. Furthermore, we may also consider the use of different ways to apply the disinfectant. For example, previous studies have shown that the combined use of multiple disinfectants can increase the effectiveness of disinfection, so we also propose further research from the aspect of the combined use of disinfectants.

Declarations

Authors' contributions

Keda Chen, Feike Ma contributed to conception, design, data acquisition, and analysis, drafted and critically revised the manuscript; Ying Wang, Xinyi Zhuang, Xuning Zhang, contributed to design and data acquisition, drafted and critically revised the manuscript; Haiyan Mao, Yanjun Zhang, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Competing interests

All the authors declare that they have no conflict of interest.

Availability of data and materials

Data are derived from public domain resources.

Consent for publication

All of the authors have agreed to the submission of this manuscript and to be responsible for its contents.

Ethics approval and consent to participate

Not applicable.

Funding

Grant support for this paper was provided by:

[1] The Key Technologies R&D Program of the National Ministry of Science (2018ZX10734-401).

[2] General project of the Traditional Chinese Medicine Administration of Zhejiang Province(2016ZA134).

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Figures

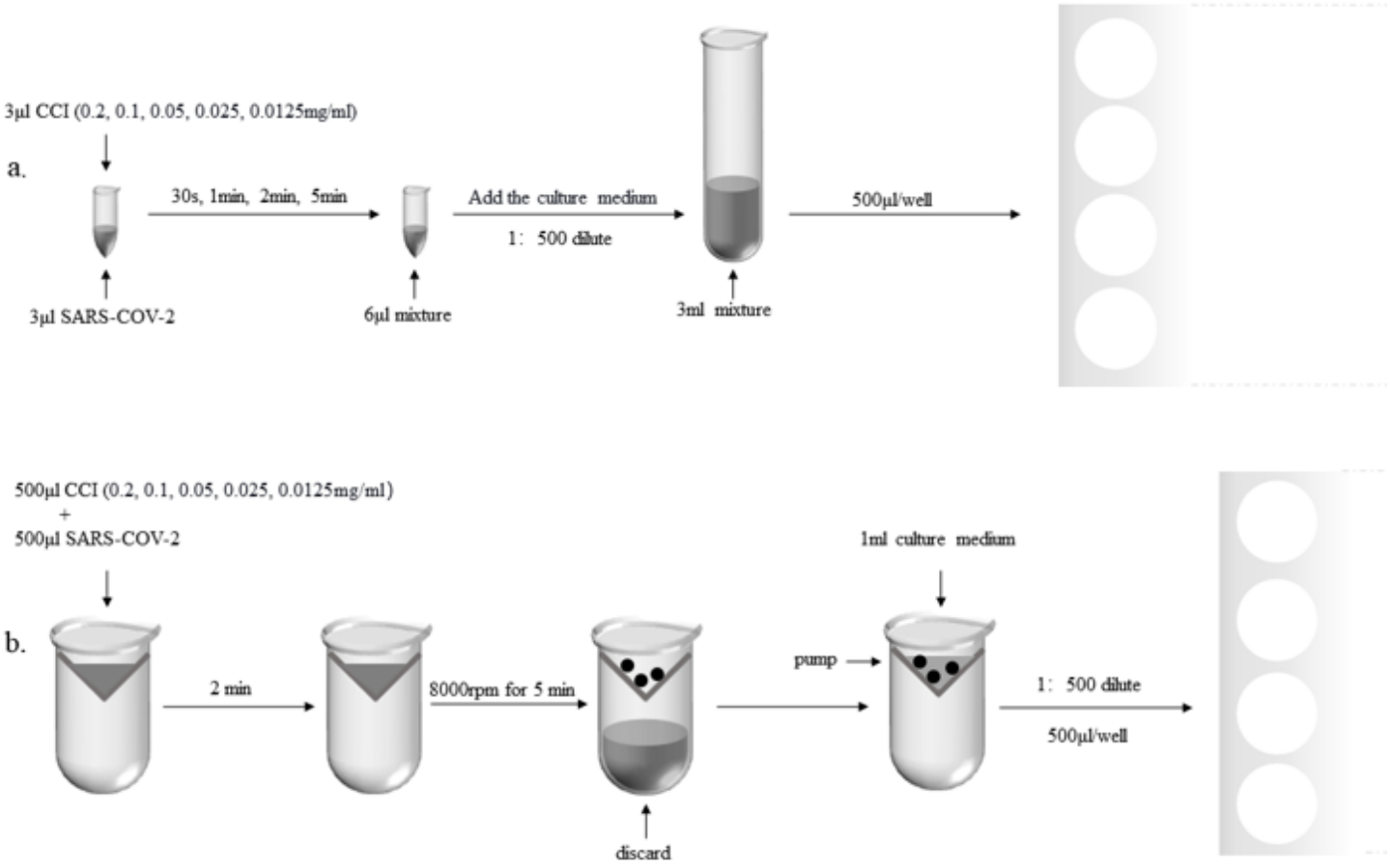

Figure 1

Experimental steps for determining the disinfection effect of hexadecyl pyridinium chloride on SARS-COV-2. (**a**) SAS-COV-2 was exposed to different concentrations (0.2, 0.1, 0.05, 0.025, 0.0125 mg/mL) of hexadecyl pyridinium chloride for different durations (30 s, 1 min, 2 min, 5 min) then diluted 1:500 to test the disinfection effect. (**b**) Then, the hexadecyl pyridinium chloride (0.2, 0.1, 0.05, 0.025, or 0.0125 mg/mL) and 500 μL SARS-COV-2 is added. After 2 min, centrifugation is performed. Finally, 1 mL culture medium is added and pumped up and down, and 1:500 dilution is carried out to detect the virus residue.

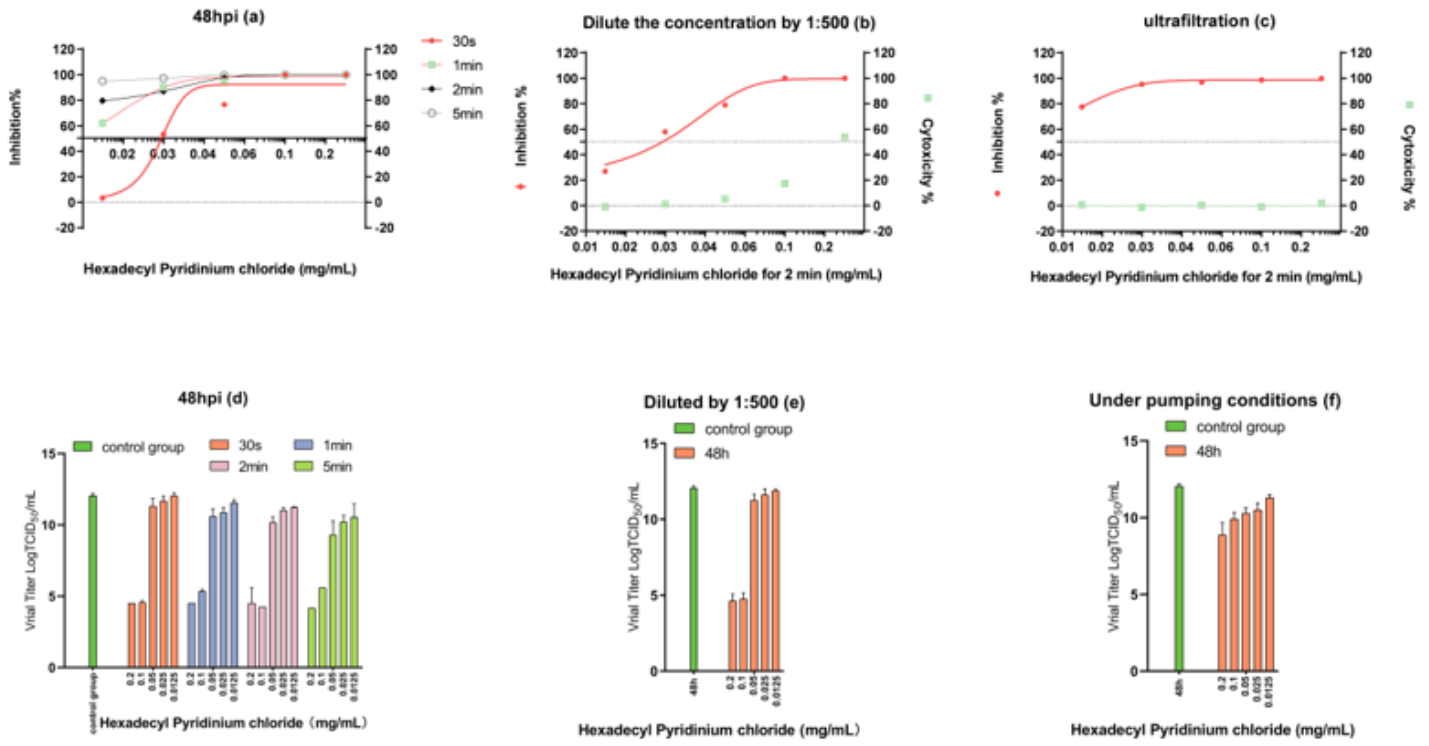

Figure 2

In vitro **disinfection and cytotoxicity of hexadecyl pyridinium chloride and viral titers for different disinfectant conditions. (a)** Inhibition of Vero cells under different concentrations (0.2, 0.1, 0.05, 0.025, or 0.0125 mg/mL) of hexadecyl pyridinium chloride for different periods of time (30 s, 1 min, 2 min, 5 min). The *x*-axis represents the concentration, and the *y*-axis represents the inhibitory rates. **(b)** Inhibition rate and cytotoxicity of the diluted hexadecyl pyridinium chloride solution on Vero cells for 2 min. The ***x***-axis represents the concentration, the left *y*-axis represents the inhibition rate, and the right *y*-axis represents cytotoxicity. (**c**) Inhibition rate and cytotoxicity of hexadecyl pyridinium chloride at different concentrations on Vero cells for 2 min under ultrafiltration. The *x*-axis represents the concentration, the left *y*-axis represents the inhibition rate, and the right *y*-axis represents cytotoxicity. **(d) (e) (f)** TCID50 was calculated by the reed-Muench method. Means ± SD were calculated from the experimental data.

Figure 3

Immunofluorescence microscopy of virus infection. SARS-CoV-2-infected Vero cells were treated with different concentrations (0.1, 0.025, 0.0125 mg/mL) of hexadecyl pyridinium chloride. After dilution (1:500), 200 μL was used for immunofluorescence analysis. When the concentration is 0.1 mg/ mL, the virus is completely inhibited. The infected cells were fixed. Then, anti-spike RBD Rabbit PAb (1:1000; Sino Biological) was used as the first antibody and Alexa Fluor488®-conjugated Goat Anti-rabbit IgG (1:1500; Abcam) was used as the second antibody. The nuclei were stained with DAPI. The scale is 100 μm.