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Direct virucidal effect of a throat lozenge with fixed combination of cetylpyridinium chloride and benzydamine hydrochloride on SARS-CoV-2 – *in vitro* study

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Abstract

Objectives. *To evaluate the in vitro virucidal effect of the combination of cetylpyridinium chloride (CPC) and benzydamine hydrochloride (BH) as a throat lozenge against the novel SARS-CoV-2.*

Material and methods. *The study evaluated the viral presence and titre in cell cultures by using SARS-CoV-2 virus incubated for 1, 5, 15 minutes, with three different concentrations of three different active substances (CPC, free BH/CPC, BH/CPC lozenge). The titre of the virus was expressed as TCID50/ml calculated with the Spearman-Kärber method.*

Outcomes. *The faster virucidal effect in high concentration was seen in the combination BH/CPC as throat lozenge when compared to CPC as free active substance. A reduction of the virus concentration was seen, at 15 minutes contact, in all three concentrations.*

Conclusions*. There is a strong virucidal effect a throat lozenge with fixed combination of cetylpyridinium chloride and benzydamine hydrochloride on the novel coronavirus.*

Keywords: SARS-CoV-2, cetylpyridinium chloride, benzydamine hydrochloride, lozenge, respiratory tract infection

Rezumat

Obiective. *Evaluarea efectului virucid* in vitro *al combinației de clorură de cetilpiridiniu (CPC) și clorhidrat de benzidamină (BH) sub forma unei pastile în doză fixă împotriva noului SARS-CoV-2.*

Material și metode. *Studiul a evaluat prezența virală și titrul în culturile celulare prin utilizarea virusului SARS-CoV-2 incubat timp de 1, 5, 15 minute, cu trei concentrații de trei substanțe active diferite (CPC substanță activă liberă, BH/CPC substanță activă liberă, BH/CPC pastile). Titrul virusului a fost exprimat ca TCID50/ml calculat prin metoda Spearman-Kärber.*

Rezultate. *Un efect virucid rapid a fost observat în combinația BH/CPC pastile în comparație cu CPC ca substanță activă liberă, în cazul utilizării unor concentrații mari de substanță. S-a observat o reducere a concentrației virusului, la 15 minute de contact, în toate cele trei concentrații.*

Concluzii*. Există un puternic efect virucid în cazul utilizării unei pastile cu combinație în doză fixă de clorură de cetilpiridiniu și clorhidrat de benzidamină pe noul coronavirus.*

Cuvinte cheie: SARS-CoV-2, clorură de cetilpiridiniu, clorhidrat de benzidamină, pastile, infecții ale tractului respirator

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INTRODUCTION

Respiratory tract infections (RTI) in general and upper respiratory tract infections (URTI) in particular are one of the most common acute conditions that cause the presentation to the doctor. Their presentation can be from a common cold to a life-threatening clinical form. From a pathophysiological point of view, it represents an infectious-inflammatory process that can involve any component of the respiratory tract, which is why the symptoms can vary from nasal obstruction, rhinorrhea, cough, sore throat, fever, to respiratory difficulties [1].

Most URTI have as their starting point the viral infection (about 95%), the most common viruses incriminated being rhinoviruses, coronaviruses, adenoviruses, paramyxoviruses (influenza, parainfluenza, syncytial respiratory virus) and coxsackieviruses [2]. Human coronaviruses are considered responsible for 10-15% of all upper respiratory tract infections in human population [3]. Commonly, they generate a self-limited disease with upper respiratory tract symptoms. The emergence of the beta coronaviruses that causes severe acute respiratory syndrome (SARS, 2002) and Middle East respiratory syndrome (MERS, 2012) showed that coronaviruses can cause severe pneumoniae and even death. The novel coronavirus described in 2019, SARS-CoV-2, responsible for the COVID-19 disease, proved to be more aggressive with more than 246 million confirmed cases and more than 5 million deaths worldwide [4].

The viral particles replicate in the nasopharynx and have to penetrate the mucosa of the upper airways. For the viruses to invade the membrane, they have to pass the local physical and immunologic barriers. To resist destruction, the invading viruses have different protective mechanisms, such as toxins which impair the body's defense system or outer structural proteins (spike protein) which helps the viral particle to not being recognized. A first step in preventing infection is to reduce the viral load on the site of infection, respectively at the level of upper respiratory tract mucosa. Different substances with virucidal action have been demonstrated to be effective in local administration, such as cetylpyridinium chloride [5,6].

Cetylpyridinium chloride (CPC) is a cationic quaternary ammonium compound used in different oral drugs as an antiseptic and a virucidal effect against influenza virus. Its virucidal properties seem to result from the direct action on the viral envelope. Considering the present literature data with no sufficient information about the treatment of SARS-CoV-2, the demonstration of the efficacy of a drug with antiseptic effect and with local virucidal action would mean an important step in the treatment of COVID-19.

MATERIALS AND METHODS

The present study was performed at the Institute of Microbiology and Immunology from Ljubljana, Slovenia, and the results were first published by Steyer et al. in 2021, in the article "A Throat lozenge with Fixed Combination of Cetylpyridinium Chloride and Benzydamine Hydrochloride Has Direct Virucidal Effect on SARS-CoV-2" [7]. The objective of the study was to evaluate, *in vitro*, the virucidal effect of a fixed combination of cetylpyridinium chloride (CPC) and benzydamine hydrochloride (BH) as a throat lozenge against the novel SARS-CoV-2. The test products used in this study were provided by KRKA, d. d., Novo mestro, Slovenia. The reagents were prepared at the Institute of Microbiology and Immunology, Ljubljana, Slovenia.

For the study Vero E6 cells, seven days old, were used. The Vero E6 cells were treated with trypsin-EDTA and then suspended in the Dulbecco's Minimal Essential Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), in order to obtain a concentration of 1×105 cells/ml. Subsequently, 100 µl of the above prepared suspension was transferred to each of the 96-well cell culture plate, with an overnight incubation at 37°C, 5% CO2, to achieve the 80% cell confluence needed for the quantitative suspension tests [7]. The isolation of SARS-CoV-2 in the cultivated Vero E6 cells was made from a throat swab of a COVID-19 positive patient. After the isolation, the concentration of the virus was determined by using an endpoint dilution assay. The titre of the tested virus was expressed as TCID50/ml (50% virus infection dose/ml) calculated with the Spearman-Kärber method.

The interfering substance was prepared by dissolving 3 g of bovine albumin in 97 ml of water, followed by the mixture of 97 ml of the prepared bovine solution with 3 ml of sheep erythrocytes (BioSap SEA, BioGnost, Zagreb, Croatia) [7]. The hard water used during the tests was prepared under septic conditions according to SIST EN 14476:2013 + A2:2019 "Chemical Disinfectants

and Antiseptics-Quantitative Suspension Test for the Evaluation of Virucidal Activity in the Medical Area" (standard) [8] and it was used within 12 hours.

The test products used in this study were: cetylpyridinium chloride (CPC) as free active substance (1mg CPC), the combination of CPC and benzydamine hydrochloride (BH) as free active substances (3 mg of BH and 1 mg of CPC), the combination of CPC and BH as throat lozenge (containing 3 mg BH and 1 mg CPC) and a placebo lozenge (eucalyptus oil, levomenthol, anhydrous citric acid (E330), sucralose (E955), isomalt (E953), brilliant blue FCF (E133)).

For each tested product and placebo, three dilutions were prepared by dissolving the substances in different quantities of hard water: 4 ml (high concentration), 20 ml (medium concentration), 30 ml (low concentration). Each test product was first diluted in the hard water, then combined with the virus and interfering substance in a dilution of 1:10, and incubated at 37°C for 1, 5, and 15 minutes. After the contact time, a sample of each suspension was transferred on an ice-cold cell medium DMEM with 2% FBS to suppress the virucidal action of the active substance. The titres of infectivity were calculated after a week according to Spearman-Kärber [9].

Control experiments were also performed in accordance with the standard procedure and included untreated cells as negative controls. 0.1 ml of viral suspension were combined with 0.1 ml of interfering substance and 0.8 ml of hard water and incubated at 37°C, 15 min. A volume of 0.1 ml was sampled before and after incubation. Ten-fold serial dilutions were prepared in DMEM supplemented with 1% FBS, and 100µL of prepared dilutions were added per well in eight replicas [7].

The virucidal activity was evaluated on the negative controls. A suspension of 0.8 ml of test product, 0.1 ml of interfering substance and 0.1 ml of maintenance medium was prepared. 0.1 ml of this mixture were transferred to 0.8 ml of ice-cold maintenance medium and stored at 4°C. 0.1 ml of viral suspension was added to the ice-cold suspension and placed in incubator, on ice, 15 minutes. After incubation, serial dilutions were prepared and transferred onto cell culture in eight replicas.

The next step was to identify the impact of the highest non-cytotoxic concentration of the active substance on the cell susceptibility to the virus. For this purpose, 10-fold dilutions of the tested substances in maintenance medium were prepared, the culture medium was removed and 100µL of the prepared active substance dilution were added into cell monolayers in eight replicates. Everything was incubated for 1 hour, at 37°C and 5% CO2. Next, the supernatants were removed, the cells were washed with maintenance medium and inoculated with prepared 10-fold serial dilutions of SARS-CoV-2 [7].

The transmission electron microscope was used to evaluate the effect of a high concentration of CPC as free active substance and of BH/CPC as throat lozenge on SARS-CoV-2 morphology. An 800µL test-product suspension for the studied cells or maintenance medium for negative control cells was prepared, and 100µL of the interfering substance and 100µL of the virus suspension were added. After 15 minutes, at 37°C, samples were prepared for electron microscopy. Electron-microscopy grids (400-mesh copper grids, coated with Formvar and carbon-supported) were prepared with ultracentrifugation in the Airfuge system, negatively contrasted with 2% phosphotungstic acid and examined from 30,000- to 100,000-fold magnification [7].

For the statistical analysis of the virucidal effect of the substances on SARS-CoV-2, an independent t-test in the SPSS Statistics V26 software tool was used.

RESULTS AND DISCUSSIONS

In case of controls, the virus test suspension was incubated for 15 minutes in parallel with each virucidal test. There was no significant reduction in the infective virus concentration between the test start titre and the test end titre when analysing the virucidal activity of CPC as free active substance and one BH/CPC lozenge at all evaluated concentrations (Table 1). Analysing the effect of low concentration for the combination BH/CPC as free active substance and placebo lozenge, one can see a decrease in the concentration of infectious viruses (Table 1).

Regarding the effect of the ice-cold media, one can identify an inhibitory effect of the high concentration of BH/CPC lozenge, but the titre difference was not statistically significant (Table 2).

Considering the influence of the controls upon cells susceptibility to virus infection, there was no major

TABLE 1. Stability test of the virus during the incubation period in the test procedure [7]

Test Virus	Test Product and Concentration	Titre at the Test Start (T = 0) log_{10}(c) ($TCID_{50}$/ml) ± 95% CI	Titre at the Test End (T = 15) log_{10}(c) ($TCID_{50}$/ml) ± 95% CI	Titre Difference
SARS-CoV-2 strain 751/20	Virucidal activity 0.25 mg/ml CPC and one BH/CPC lozenge/4 ml	8.94 ± 0.89	8.57 ± 0.62	<1
	Virucidal activity 0.05 mg/ml CPC and one BH/CPC lozenge /20 ml	8.50 ± 0.68	8.31 ± 0.69	<1
	Virucidal activity 0.033 mg/ml CPC and one BH/CPC lozenge/30 ml	9.76 ± 0.79	9.00 ± 0.80	<1
	Virucidal activity 0.75 mg/ml + 0.25 mg/ml BH/CPC and one placebo lozenge/4 ml	8.99 ± 0.67	9.12 ± 0.71	<1
	Virucidal activity 0.15 mg/ml + 0.05 mg/ml BH/CPC and one placebo lozenge/20 ml	9.68 ± 0.67	9.78 ± 0.77	<1
	Virucidal activity 0.099 mg/ml + 0.033 mg/ml BH/CPC and one placebo lozenge/30 ml	9.95 ± 0.67	8.93 ± 0.65	1.02

CPC - cetylpyridinium chloride; BH - benzydamine hydrochloride; CI - confidence interval; $TCID_{50}$ - 50% tissue culture infectious dose

TABLE 2. Suppression efficiency of test products [7]

Test Virus	Test Product and Concentration	Titre at the Test Start (T = 0) log_{10}(c) ($TCID_{50}$/ml) ± 95% CI	Titre at the Test End (T = 15) log_{10}(c) ($TCID_{50}$/ml) ± 95% CI	Titre Difference
SARS-CoV-2 strain 751/20	0.25 mg/ml CPC as free active substance	9.76 ± 0.79	9.75 ± 0.63	<1
	1 BH/CPC lozenge/4 ml	9.76 ± 0.79	9.38 ± 0.76	<1
	0.75 mg/ml BH + 0.25 mg/ml CPC as free active substance	9.95 ± 0.67	10.25 ± 0.74	<1
	1 placebo lozenge/ 4 ml	10.46 ± 0.75	10.13 ± 0.85	<1

CPC - cetylpyridinium chloride; BH - benzydamine hydrochloride; CI - confidence interval; $TCID_{50}$ - 50% tissue culture infectious dose

TABLE 3. pH values and log reduction of the virus concentration for test product concentrations and each contact times [7]

	CPC as Free Active Substance	CPC/BH as Free Active Substance	BH/CPC Lozenge	Placebo Lozenge
High concentration (dissolved in 4 ml)				
pH value	7.61	7.80	2.85	2.99
Time (min) Δlog_{10}(c) ($TCID_{50}$/ml) ± 95% CI				
1	−0.00 ± 1.06	−1.57 ± 0.86 *	−4.32 ± 0.82 *	+0.25 ± 1.01
5	−1.88 ± 0.96 *	−4.49 ± 0.67 *	−4.44 ± 0.75 *	−2.00 ± 0.92 *
15	−4.94 ± 0.75 *	−4.49 ± 0.67 *	−4.44 ± 0.75 *	−2.88 ± 0.83 *
Medium concentration (dissolved in 20 ml)				
pH value	7.66	7.73	4.2	4.45
Time (min) Δlog_{10}(c) ($TCID_{50}$/ml) ± 95% CI				
1	−0.06 ± 0.99	−1.96 ± 0.95 *	−1.69 ± 0.96 *	−0.81 ± 0.92
5	−0.75 ± 0.98	−3.30 ± 0.96 *	−3.56 ± 0.86 *	−1.06 ± 0.98
15	−2.44 ± 0.79 *	−5.36 ± 0.77 *	−5.00 ± 0.68 *	−0.65 ± 1.05
Low concentration (dissolved in 30 ml)				
pH value	7.67	7.80	4.64	5.25
Time (min) Δlog_{10}(c) ($TCID_{50}$/ml) ± 95% CI				
1	−0.32 ± 1.08	−0.89 ± 1.03	−0.88 ± 1.11	+0.13 ± 1.06
5	−0.70 ± 1.04	−1.58 ± 0.98 *	−1.19 ± 1.03	−0.46 ± 0.98
15	−1.69 ± 1.03 *	−2.44 ± 0.94 *	−3.01 ± 1.05 *	−1.46 ± 0.97

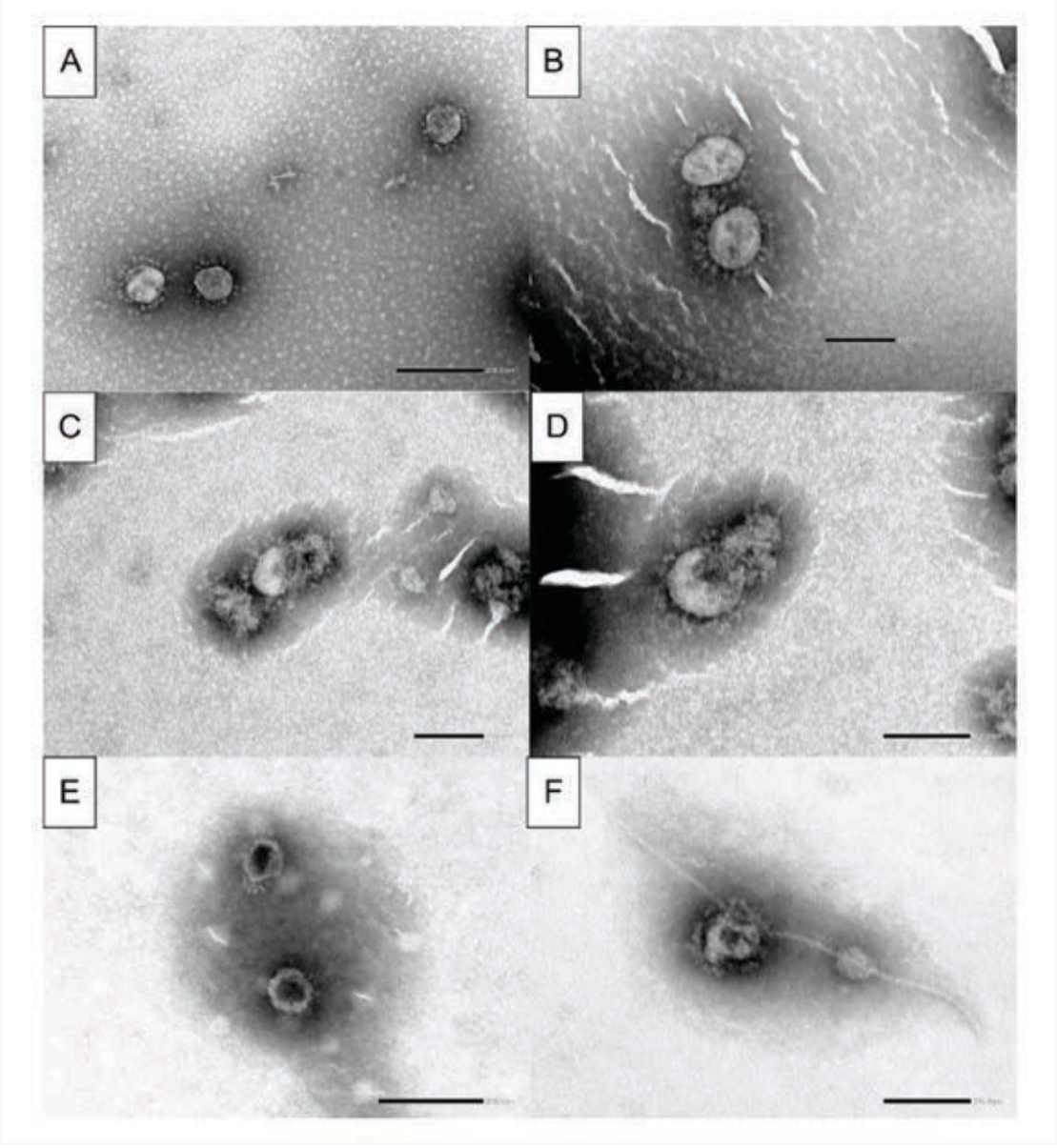

FIGURE 1. Electron micrographs of viral particles after ultracentrifugation and negative contrast. (A, B) - viral particles in maintenance media, not exposed to test product; 50,000-fold (A) and 80,000-fold (B) magnification; intact SARS-CoV-2 particles with peplomers (corona). (C, D) - 15 min exposure to high CPC concentration (0.25 mg/ml); 80,000-fold (C) and 100,000-fold (D) magnification; damaged viral particles, rarely visible peplomers, disrupted envelope and exposed internal nucleocapsid. (E, F) -15 min exposure to high concentration of a BH/CPC lozenge (1 lozenge in 4 ml); 60,000-fold (E) and 100,000-fold (F) magnification; outer-layer damage and accumulation of negative contrast agent inside the nucleocapsid (black virus centre), rare peplomers, destruction of outer layer and nucleocapsid (F) (with the permission of Steyer et al. [7])

impact. The evaluation was made separately for CPC free active substance and BH/CPC lozenge, and BH/CPC free active substance and placebo lozenge. A slightly lower virus titre was found in the exposed cells compared with the non-exposed cells.

For the cytotoxic level evaluation, dilutions between 10-1 and 10-11 were tested. A cytotoxic effect was identified in dilutions 10-1 and 10-2 and both were excluded from the following evaluations. For undiluted BH/CPC as a free active substance, the BH/CPC lozenge and the placebo lozenge, the level of cytotoxicity with respect to virus dilution was at 4.5 log10(c) ($TCID_{50}$/ml) [7]. For all other test products in medium and at a low concentration, and also for the CPC as a free active substance in a high concentration, the level of cytotoxicity was 3.5 log_{10}(c) (TCID50/ml) [7].

To test the virucidal effect, the virus was exposed to each of the three tested substances, in all three concentrations, for 1, 5 and 15 minutes.

Considering the effects of test products in high concentration (test product dissolved in 4 ml), the faster virucidal effect was seen in the combination BH/CPC as lozenge when compared to CPC as free active substance (Table 3). The 4-log reduction in virus titre was achieved after 1 minute of exposure to high concentration test product. The same happened with the free active substances but only after 15 minutes of exposure. In case of placebo lozenge, at 1 minute of exposure an increase in viral titre was seen. At 5 and 15 minutes there was a reduction of 2.00 ± 0.92 and 2.88 ± 0.83, respectively (Table 2) [7].

Evaluating the effects of test products in medium concentration (test product dissolved in 20 ml), the fastest virucidal effect was seen only after 5 and 15 minutes of contact in the BH/CPC lozenge (Table 3). We consider that this might be the concentration achieved in the actual use of a throat lozenge, taking into account the chemical characteristics of the human oral cavity, such as temperature, saliva composition, the lozenge dissolution process (time, saliva quantity).

For low concentration (test product dissolved in 30 ml), the virucidal effect was not so significant (Table 3). In case of placebo, there was an increase in the viral titre at 1 min of exposure, and a reduction at 5 and 15 minutes.

An important information is that a reduction of the virus concentration can be seen, at 15 minutes contact, in all three concentrations especially in BH/CPC combination as a throat lozenge or as free active substances. There are studies which show a high concentration of the virus in both pharyngeal mucosa and higher in the saliva, and the significant reduction of virus titre by mouth rinses [6,10,11].

Another factor which seems to influence the virucidal effect is pe pH. Different studies sustain that SARS-CoV-2 may be sensitive to pH variations, in alkaline (pH > 12) or acid (pH < 3) environment the virus being inactivated by influencing the spike protein [12,13]. In the presented study, the pH value for CPC and BH/CPC as free substances was neutral (between 7.61 and 7.80) and the virucidal effect was low. In contrast, at pH between 2.85 and 4.64 the BH/CPC lozenge determined a significant decrease of the viral titre. The present results are sustained by the one found in the literature.

An important part of this study was the electron microscopic evaluation of both controls and test samples. The negative control samples contained intact virus particles, with no visible morphological changes or damage, with a clearly expressed peplomers of the S protein (corona) and an intact viral envelope (Figure 1 A, B) [7]. The samples exposed to high concentration of test products, CPC as free active substance (Figure 1 C, D) or a BH/CPC lozenge (Figure 1 E, F), presented a destruction of the viral envelope, with unclear peplomers formation or even without peplomers and envelope, while in some samples the internal nucleocapsid was exposed and visible [7]. The damage of the outer layer of the viral envelope could explain the loss of viral infectivity. The viral envelope destruction was also noted, with a contrast accumulation in the virus internal site (black virus centre) [7].

CONCLUSIONS

The *in vitro* study performed in a controlled laboratory condition emphasises the important virucidal effect of a fixed combination of cetylpyridinium chloride and benzydamine hydrochloride as throat lozenge against the novel SARS-CoV-2. The BH/CPC combination, both as lozenge and free active substances, proved to have a greater influence upon viral inactivation compared to CPC alone.

The BH/CPC as throat lozenge can be considered an effect drug in treating the pharyngeal symptoms of COVID-19 disease.

Authors contributions

All authors contributed equally to the writing of the article.

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