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Original Article

In vitro cytotoxicity and antibacterial activity of hypochlorous acid antimicrobial agent

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KEYWORDS
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Cytotoxicity

Abstract *Background/purpose:* Bacteria-associated oral diseases such as dental caries and periodontitis are widespread epidemics that cause oral pain and loss of function. The purpose of this study was to evaluate the in vitro cytotoxicity and antibacterial activity of different concentrations of hypochlorous acid (HOCl).
Materials and methods: Five different concentrations (100, 200, 300, 400, and 500 ppm) of HOCl were evaluated for their antimicrobial efficacy against Gram-negative (*A. actinomycetcmcomitans* and *P. gingivalis*) and Gram-positive bacteria (*S. mutans* and *S. sanguinis*) after treatment for 1 and 10 min. Sodium hypochlorite (NaOCl) and chlorhexidine (CHX) were used as positive controls. In addition, HOCl was examined for L929 cytotoxicity and RAW 264.7 growth.
Results: The bacteriostatic ratio of NaOCl was comparable to that of CHX and significantly ($P < 0.05$) higher than that of all HOCl solutions. Higher HOCl concentration had significantly ($P < 0.05$) higher antibacterial effect, and the bacteriostatic ratio of 10 min treatment was slightly higher than that of 1 min treatment. CHX and NaOCl seeded into L929 cells resulted in low cell viability with only 30—39%, much significantly ($P < 0.05$) lower than all HOCl groups (greater than 80%). All HOCl solutions met the recommendations of ISO 10993—5 and showed no cytotoxicity, although there was a concentration-dependent decrease in cell viability. All antimicrobial agents showed the same trend of response to RAW 264.7 as L929.
Conclusion: Within the limit of this study, 400 ppm HOCl disinfectant may be a potential antimicrobial candidate for mouthwash, endodontic irrigants, and periodontitis treatment.

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Introduction

Oral diseases such as dental caries and periodontitis are prevalent worldwide due to oral biofilms, leading to pain and loss of oral function and eventually teeth loss.[1–4] Prevention and removal of biofilms is important to oral health care and clinical practice in periodontics and endodontics. In the treatment of periodontitis, it may be necessary to use adjunctive plaque control methods in addition to the commonly used mechanical debridement of biofilms on tooth surfaces.[5] Therefore, there is a need for adjunctive liquids that can rapidly kill and remove bacterial colonies during periodontal scaling. In medicine and dentistry, antiseptics (irrigants or therapeutic agents or antimicrobial agents) such as chlorhexidine (CHX) and sodium hypochlorite (NaOCl) are always used to destroy microorganisms. and.

CHX, a cationic bisbiguanide antimicrobial active against Gram-negative and Gram-positive bacteria, is one of the most widely used antimicrobial substances for wound and periodontitis disinfection.[2,6,7] Its antimicrobial activity stems from the positive charge at physiological pH, which causes nonspecific binding to the negatively charged membrane phospholipids of bacteria.[6] However, CHX is known to be toxic to cells.[8] Faria et al. reported that CHX induced cell death of L929 fibroblasts possibly through endoplasmic reticulum stress in a concentration-dependent increase.[9] Despite its toxic effects in vivo and in vitro, CHX has been used in the form of mouthwash, gels, varnishes, and controlled-release devices to prevent dental caries, plaque formation, and gingivitis.[10] Research on other antimicrobial substances for the treatment and prevention of oral disease is urgently needed due to concerns about adverse side effects and potential bacterial resistance from the use of CHX. Like CHX, NaOCl is a commonly used antiseptic. It dissolves organic and inorganic tissues and also causes oxidation and hydrolysis of cellular proteins, in addition to being effective against a broad spectrum of microorganisms.[11] During endodontics, NaOCl irrigants are often used to better remove and inactivate the smear layer containing residual bacteria after root canal preparation.[10,12] Accordingly, the American Association of Endodontics (AAE) Clinical Considerations for Regenerative Surgery mentions the use of 1.5% NaOCl as an irrigant.[13] However, when NaOCl degrades collagen type I, the organic component of dentin, it negatively affects the mechanical behavior of dentin tissue and its restoration.[14,15] For this reason, the development of new antiseptics with fewer complications or side effects is highly desired.

Hypochlorites such as NaOCl are the most widely used chlorine disinfectants. NaOCl is a highly alkaline solution in which free available chlorine exists as hypochlorite ion (OCl^-) in equilibrium with hypochlorous acid (HOCl). It is well recognized that HOCl is an endogenous antimicrobial substance of the innate immune response during antigen phagocytosis.[16] HOCl can be formed by combining acidic chlorine oxide (Cl^-) with water. It has been reported to have an excellent bactericidal effect against a variety of microorganisms because of its highly oxidizing capacity[17] and have practical applicability in hospitals or the food industry.[18–20] In dentistry, HOCl is introduced as a pulp irrigant[21] and as a chemotherapeutic agent in the treatment of periodontitis.[22] In an earlier study,[23] we have verified that 180 ppm HOCl is effective in cleaning biofilm-contaminated titanium implant surfaces and is comparable to NaOCl and CHX, but must be used in four times the volume of NaOCl and CHX. On the other hand, since the onset and spread of Coronavirus disease 2019 (COVID-19), HOCl has evoked considerable interest as an antiseptic.[17,24] Chatterjee pointed out that when disinfectants such as NaOCl solution were sprayed to reduce the virus infection, NaOCl can easily react with the water vapor to form HOCl, which was further photo-dissociated into various reactive oxygen species.[24] Given the growing threat of bacterial resistance worldwide, there has been growing interest in the use of HOCl to treat biofilm-associated diseases and virus infections.[3,17]

When using antimicrobial agents in clinical practice, the therapeutic concentration of the agents should be the crucial point. Ideally, there should be an urgent need to develop an ideal antimicrobial agent with considerably high antibacterial activity and low toxicity for home care or clinical application.[21] Antimicrobial agents have concentration-dependent biological functions, such as antibacterial activity and cytotoxicity.[23,25] Although HClO is increasingly used in terms of bactericidal activity and low cost, its concentration range has not been established to be effective as an antiseptic with minimal tissue damage. A literature search revealed few articles evaluating the concentration effect of HOCl as an antimicrobial agent on bacterial growth and cytotoxicity. To this end, further in vitro studies on the concentration dependence would be needed to confirm the effectiveness and safety of HOCl as an antiseptic. The purpose of the present study was to examine the efficacy of HOCl with different concentrations in eliminating Gram-positive and Gram-negative bacterial species. Commonly used NaOCl and CHX reagents were also tested for comparison. Importantly, L929 and RAW 264.7 cells were examined to verify the cytotoxicity of HOCl. From a clinical point of view of prophylaxis and treatment, shorter and slightly longer time points can be used, therefore, treatment times of 1 min and 10 min were used in this study.

Materials and methods

Solution preparation

HOCl stock solution was 1000 ppm (0.1%) from Union Biomedical Corporation (New Taipei City, Taiwan), which was diluted with bacterial broth or cell culture medium to a

final concentration of 100 (0.01%), 200 (0.02%), 300 (0.03%), 400 (0.04%) and 500 ppm (0.05%), depending on antibacterial or biocompatibility assays. Water was also used as solvent for the purpose of comparison. To simplify the code, as an example, 100 ppm HOCl solution was marked as 1HOCl. Two commonly used antiseptics, 1.5% NaOCl (Shimakyu's Pure Chemical, Osaka, Japan) and 0.2% chlorhexidine digluconate (Sigma-Aldrich, St. Louis, MO, USA) as control were prepared using stock solutions. The pH of various antimicrobial agents diluted in different liquids was measured by using a HORIBA pH meter (LAQUAtwin-pH 11; Tokyo, Japan). Analysis was performed in five dependent sets of experiments.

Bacterial culture

Gram-negative bacteria (*Aggregatibacter actinomycetemcomitans* (*A. actinomycetcmcomitans*, IDH 781) and *Porphyromonas gingivalis* (*P. gingivalis*, A7436)) and Gram-positive bacteria (*Streptococcus mutans* (*S. mutans*, ATCC 700610) and *Streptococcus sanguinis* (*S. sanguinis*, ATCC 10556)) were used. All bacteria species (10^7 CFU/mL) were grown anaerobically at 37 °C for 1 day in Wilkins-Chalgren broth (Oxoid, Hampshire, UK).[23]

Spread plate method

The conventional spread plate method is usually used to count the number of bacterial colonies.[26] After growing 100 μL of bacteria in a 96-well plate for 1 day and removing the broth, 100 μL of test reagents was added to each sample for 1 or 10 min. Plates with the culture broth were considered negative controls. Adhered bacteria were ultrasonically detached at a frequency of 40 kHz for 5 min in a 150 W ultrasonic bath (DC150H, Taiwan Delta New Instrument Co. Ltd., New Taipei City, Taiwan). PBS solution was used to dilute the bacteria sample. Then, 50 μL of the bacterial suspension was spread on a 15 mL of Trypticase soy agar (Conda, Madrid, Spain) Petri dish (9 cm in diameter) and incubated at 37 °C for 1 day of incubation. The total numbers of colony-forming unit (CFU) in each dish were counted and the bacteriostatic ratio (%) was calculated as follows:

$$\text{Bacteriostatic ratio (\%)} = (N_{control} - N_{experiment})/N_{control} \times 100\%$$

Where $N_{control}$ and $N_{experiment}$ are the number of bacterial colonies in the negative control and experimental groups, respectively.[26] Three specimens per group were examined.

AlamarBlue assay

In addition to conventional counting, the antimicrobial ability of various test agents against bacterial species was also examined using the alamarBlue assay.[27] After removing the broth from the 96-well plate, 100 μL of test reagents was added to the bacterial sample for 1 or 10 min. After treatment, a 1:10 solution of alamarBlue (Invitrogen, Grand Island, NY, USA) to broth was added to each well and incubated at 37 °C for 10 min. The absorbance of the solution was then read at 570 nm in a BioTek Epoch microplate reader (Winooski, VT, USA) with a reference wavelength of 600 nm. The bacteriostatic ratio (%) was calculated based on the absorbance.[27] Each value represented the average of three measurements.

Bacterial colony observation

A 10 × 10 mm^2 grade 2 Ti surface (Spemet Co., Taipei, Taiwan) was mechanically polished by #1500-grit SiC sandpaper. One milliliter of each bacterial suspension (10^7 CFU) was seeded on the surface of a polished Ti disk in a 24-well plate for 1 day. After removing the broth, 1 mL of test reagent was added to the disk for 1 or 10 min. Afterwards, samples were washed with PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 min, then dehydrated for 20 min at each concentration using a graded ethanol series, mounted on a stub, and then coated with gold layer.[26] The dried samples were viewed using a scanning electron microscope (SEM; JEOL JSM-7800 F, Tokyo, Japan).

L929 cytotoxicity

The in vitro cytotoxicity test with various concentrations of HOCl was carried out using the L929 mouse fibroblast cell line (BCRC RM60091, Hsinchu, Taiwan) according to ISO 10993—5.[28] Cells (4^*10^4 cells/well) were cultured in a 48-well microplate with Dulbecco's modified Eagle medium (DMEM; HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Langley, OK, USA) and 1% penicillin/streptomycin solution (Gibco) at a humidified atmosphere of 5% CO_2 at 37 °C for 1 day to allow attachment. Afterwards, the culture medium was replaced with DMEM containing test reagents for a culture time of 1 and 10 min. 10% DMEM alone was used as a control. Cytotoxicity was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assay. Microplates were read at 563 nm by using a BioTek Epoch spectrophotometer. Absorbance results from night separate measurements were recorded. Cell viability for each sample was calculated using the equation: (absorbance of the experimental group/absorbance of the DMEM control group) × 100%.[28]

RAW 264.7 cell response

RAW 264.7 macrophage cells (BCRC 60001, Hsinchu, Taiwan) were used to examine osteoclastic responses to the respective reagents using the MTT assay, as previously described.[29] Cells were cultured in 90% DMEM with 4 mM L-glutamine containing 1.5 g/L sodium carbonate and 4.5 g/M glucose, 10% FBS at 37 °C with 5% CO_2. To induce inflammation, cells were treated with 1 μg/mL lipopolysaccharides (LPS; Sigma-Aldrich) in DMEM for 12 h. After LPS stimulation, medium was exchanged with various reagents and 10^4 cells/well were seeded in a 96-well plate for 1 and 10 min. The culture medium on a tissue culture plate (TCP) without test reagent was used as a control. Results were reported based on three independent measurements of absorbance.

Statistical analysis

Significant differences between means were assessed using one-way analysis of variance (ANOVA). Scheffe's multiple comparisons were used to determine the significance of the standard deviations between sample measurements. Results were considered statistically significant when the *P*-value was less than 0.05.

Results

The pH changes of test reagent

When testing materials for antimicrobial activity and cellular behavior, different media are used. The pH changes of HOCl, NaOCl and CHX after dilution with three different solvents, water, bacterial broth and DMEM, were measured, as shown in Fig. 1. The solution images displayed the turbidity of 0.2% CHX after dilution with broth and DMEM compared to dilution water dilution. In contrast to this finding, HOCl and NaOCl still showed clear solutions. On the quantitative analysis of pH, when the three solvents were used, the pH of NaOCl was significantly higher ($P < 0.05$) than that of all HOCl solutions and CHX solutions. Solvent type had no significant ($P > 0.05$) effect on the pH of the 0.2% CHX solution. Regarding the pH change of HOCl, when water was used as the diluting solvent, its pH value decreased as the concentration of HOCl increased, and the pH value of the 5HOCl group decreased significantly ($P < 0.05$) to 4.4. However, when HOCl was diluted with broth and DMEM solvents, there was no statistical difference ($P > 0.05$), although slightly reduced.

Antibacterial activity detected by spread plate method

Fig. 2 shows representative images of Gram-negative and Gram-positive bacteria treated by different test reagents for 10 min. CHX and NaOCl markedly reduced the four bacteria compared with the control without reagents. Higher HOCl concentration (500 ppm) presented signs of bacterial reduction efficacy than lower concentration (300 ppm). However, the number of bacteria inoculated with HOCl was higher than those inoculated with CHX and NaOCl.

Bacteriostatic ratios of different test agents against *A. actinomycetemcomitans*, *P. gingivalis*, *S. mutans* and *S. sanguinis* after treatment for 1 and 10 min using the spread plate method are shown in Fig. 3. Apparently, 1.5% NaOCl and 0.2% CHX antimicrobial agents almost completely killed the four bacteria. Not surprisingly, the efficacy of HOCl was concentration-dependent, indicating a statistical difference ($P < 0.05$). The bacteriostatic ratio of 10-min treatment time was higher than that of 1-min time. For example, 5HOCl (500 ppm) inhibited 69% and 76% of Gram-negative *A. actinomycetemcomitans* when inoculated for 1 min and 10 min, respectively. After 1 and 10 min of

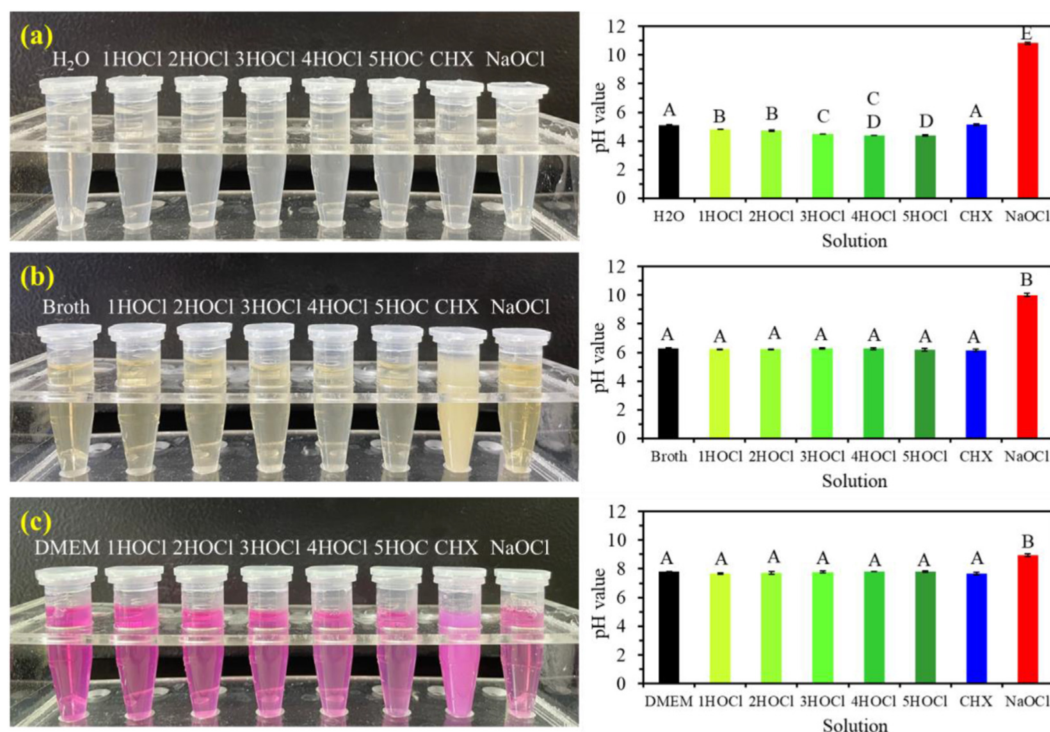

Figure 1 Pictures of various test solutions before and after dilution with (a) water, (b) bacterial broth, and (c) Dulbecco's modified Eagle medium (DMEM), and the corresponding pH values (n = 6). Different capital letters indicate significant differences at $P < 0.05$.

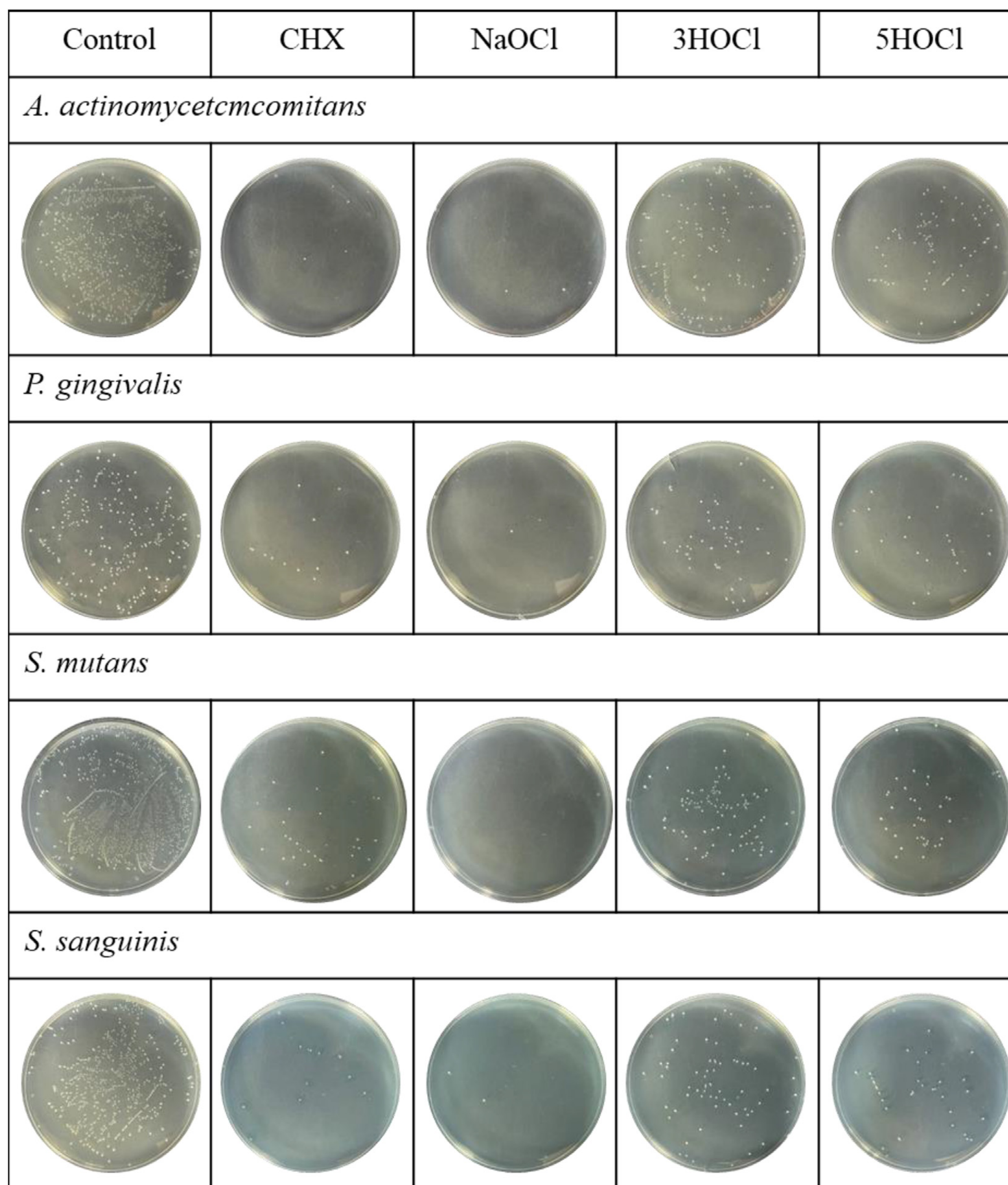

Figure 2 Representative images of spread plates for (a) *A. actinomycetemcomitans*, (b) *P. gingivalis* (c), *S. mutans* and (d) *S. sanguinis* after 10 min treatment with different test reagents.

treatment with 5HOCl, 55% and 75% of Gram-positive *S. mutans* were killed, respectively.

Antibacterial activity detected by alamarBlue assay

To further elucidate the antimicrobial ability of various antimicrobial agents, alamarBlue assay was used to assess the bacteriostatic ratios after 1-min and 10-min treatment. Fig. 4 shows that the bacteriostatic ratios of CHX against the four bacteria were comparable to NaOCl, and the trends were similar to those obtained using spread plate method. The two commonly used antimicrobial agents had the bacteriostatic ratios of 80–90% after 1-min and 10-min treatment. Undoubtedly, higher concentrations of HOCl resulted in higher bacteriostatic ratios regardless of bacterial species. For example, 1HOCl, 2HOCl, 3HOCl, 4HOCl and 5HOCl killed 54%, 60%, 65%, 69% and 75% of *P. gingivalis*, respectively, in a 1-min treatment. When *S. sanguinis* was treated for 10 min, the bacteriostatic ratios were 33%, 42%, 47%, 52% and 60%, respectively. However, the bacteriostatic ratios of the 4HOCl and 5HOCl groups against the four bacteria were not statistically different ($P < 0.05$).

Bacterial colony observation

The amounts of Gram-negative bacteria were examined by using SEM before and after 10 min of treatment. Fig. 5 shows that the surface of the control without antiseptic treatment was populated with *A. actinomycetemcomitans*

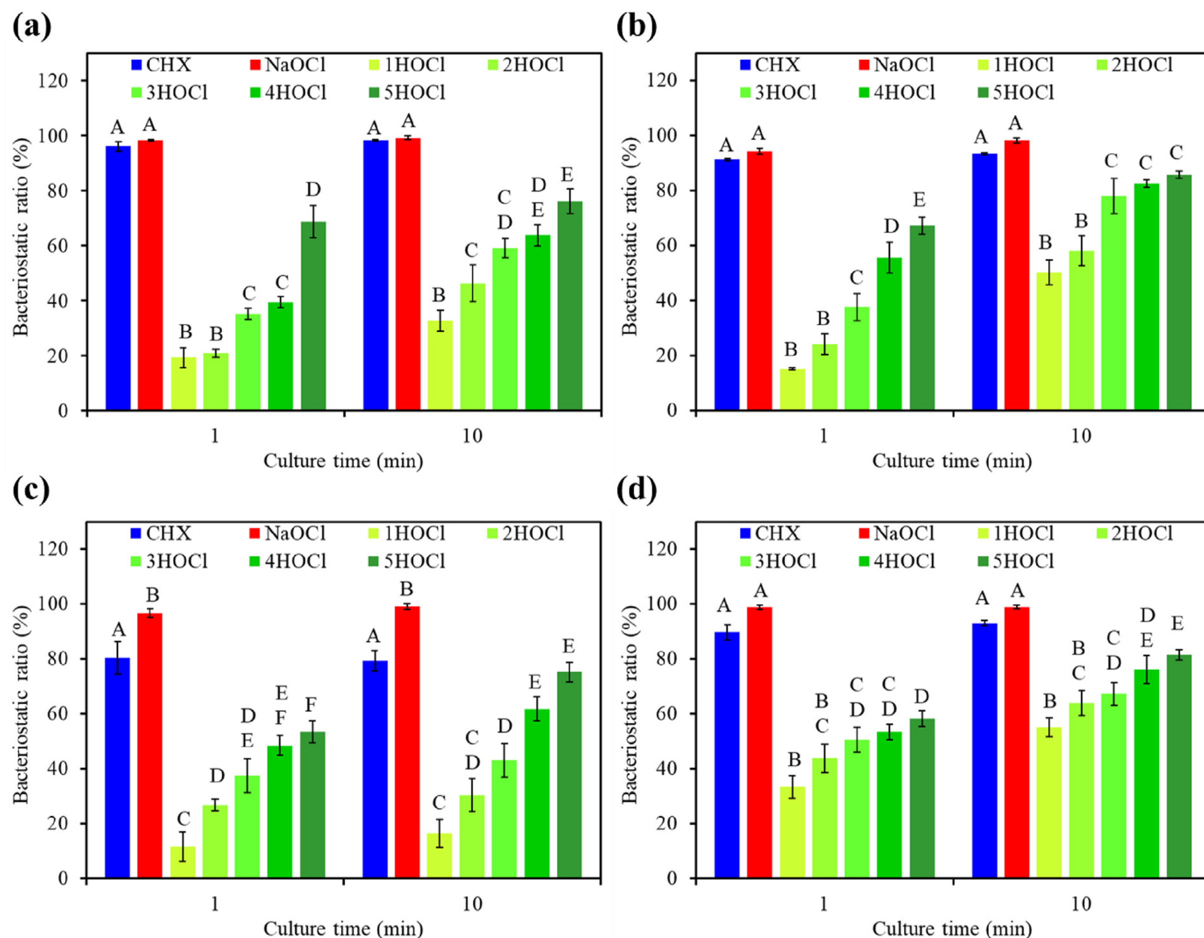

Figure 3 Bacteriostatic ratios of different test reagents against (a) *A. actinomycetemcomitans*, (b) *P. gingivalis*, (c) *S. mutans* and (d) *S. sanguinis* were analyzed using the spread plate method after treatment for 1 and 10 min. Different capital letters indicate statistically significant differences at $P < 0.05$ (n = 3).

(Fig. 5a) and *P. gingivalis* (Fig. 5b) bacteria. When inoculated with CHX and NaOCl, the numbers of both bacteria were appreciably reduced. Also, higher concentration of HOCl resulted in more potent antibacterial activity compared with the untreated control.

Fig. 6 shows the surface morphology of Gram-positive bacteria before and after treatment with various antiseptics for 10 min. Substantial removal of bacteria attached to the titanium surface was observed after treatment with NaOCl and CHX. On the other hand, HOCl also effectively reduced the number of bacteria, indicating concentration-dependent trend. The antimicrobial activity of HOCl was slightly inferior to that of NaOCl and CHX, which was in line with the bacteriostatic ratio.

L929 cytotoxicity

Fig. 7 shows the effect of different antimicrobial agents on L929 cytotoxicity using the MTT assay. A lower viability value presented a higher cytotoxic potential of the test sample. CHX and NaOCl inoculated to L929 cells resulted in a significant decrease in cellular activity, showing 30–39% viability after 1-min and 10-min treatment. When cells were seeded with HOCl, there was a concentration-dependent reduction in cell viability. At 1 min of treatment, cell viability decreased from 94% to 84% with increasing concentration, but showed no statistical difference ($P < 0.05$). Similar to the downward trend, cell viability in HOCl was between 98% and 88% at 10-min treatment, indicating no signs of cytotoxicity. Notably, after 10-min treatment, the viability in 5HOCl was higher by a factor of 2.7 and 2.3 than that of CHX and NaOCl, respectively.

Macrophage response

After treatment with NaOCl and CHX for 1 min, the absorbance of RAW 264.7 cells revealed a significant reduction ($P < 0.05$) compared with the control (TCP), as shown in Fig. 8. In contrast, HOCl exhibited lower cell growth ($P < 0.05$) than the negative control in a concentration-dependent manner, but 2–3 times higher cell growth than NaOCl and CHX. When exposed to all test reagents for 10 min, there was a similar trend in RAW 264.7 expression for all groups. More importantly, all HOCl groups showed much significantly higher ($P < 0.05$) cell growth than NaOCl and CHX. For example, when normalized to the negative control, 400 ppm HOCl (4HOCl) and 500 ppm (5HOCl) were

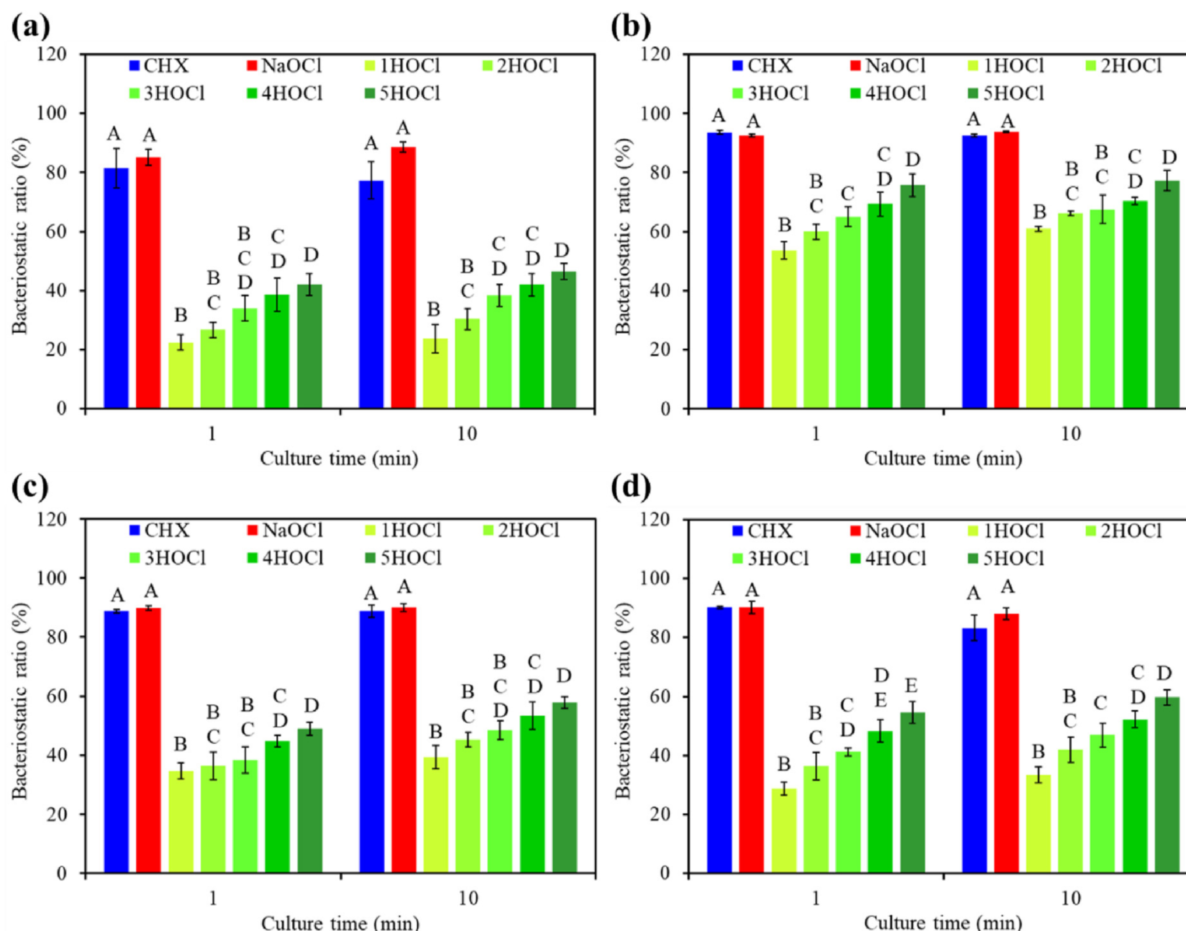

Figure 4 Bacteriostatic ratios of different test reagents against (a) *A. actinomycetemcomitans*, (b) *P. gingivalis*, (c) *S. mutans* and (d) *S. sanguinis* were analyzed using alamarBlue assay after treatment for 1 and 10 min. Different capital letters indicate statistically significant differences at $P < 0.05$ (n = 3).

71% and 64%, respectively, while both CHX and NaOCl were about 30%.

Discussion

There is great interest in the investigation of antimicrobial agents so that dental caries and periodontal disease can be significantly controlled. The most frequently used chemotherapeutic agents for endodontic and periodontal infections are CHX and NaOCl.[2,30] For example, 0.2% CHX is used as an adjunct to scaling and root planing in the treatment of periodontitis.[2] Nevertheless, in vivo studies have confirmed the lack of chemotherapeutic agents as the gold standard for complete microorganisms, in addition to side effects.[31] When applied as a mouth rinse, 0.2% CHX is swirled in the mouth for approximately 1 min,[32] at this concentration prolonged exposure may be cytotoxic. Even more worrisome, prolonged use of CHX has been associated not only with tooth pigmentation, mucosal damage, and mucosal dryness, but also with the development of CHX resistance.[3,33,34] NaOCl is often used as an irritant because it disrupts biofilms and eliminates bacteria in biofilms and dentinal canals.[10,35]

Various antimicrobial agents can reduce the action of Gram-positive and Gram-negative bacteria. Cationic CHX can electrostatically bind to the negatively charged surface of bacteria, disrupting the outer layer of the cell wall and making it permeable.[10,30] NaOCl may be harmful because it is extremely alkaline, irritating and cytotoxic at commonly used concentrations.[23,30] The antimicrobial effectiveness of highly alkaline NaOCl is based on its release of OH from solution,[35] which interferes with the integrity of the cytoplasmic membrane through irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation.[36] The concentration of undissociated HOCl in NaOCl solution may contribute to the antimicrobial ability to some extent,[30] while HOCl exerts its bactericidal activity by oxidizing the sulfhydryl groups of bacterial enzymes.[37]

Similar to one of hypochlorites, HOCl disinfectant is increasingly used in heavily contaminated hospital environments and for wound treatment and patient decolonization at low concentrations,[38,39] but concentration-dependent effect of HOCl on the cytotoxicity and antibacterial activity remain to be studied for further clinical application. This knowledge of biological properties of HOCl may allow us to formulate antiseptic agents that provide desired biological

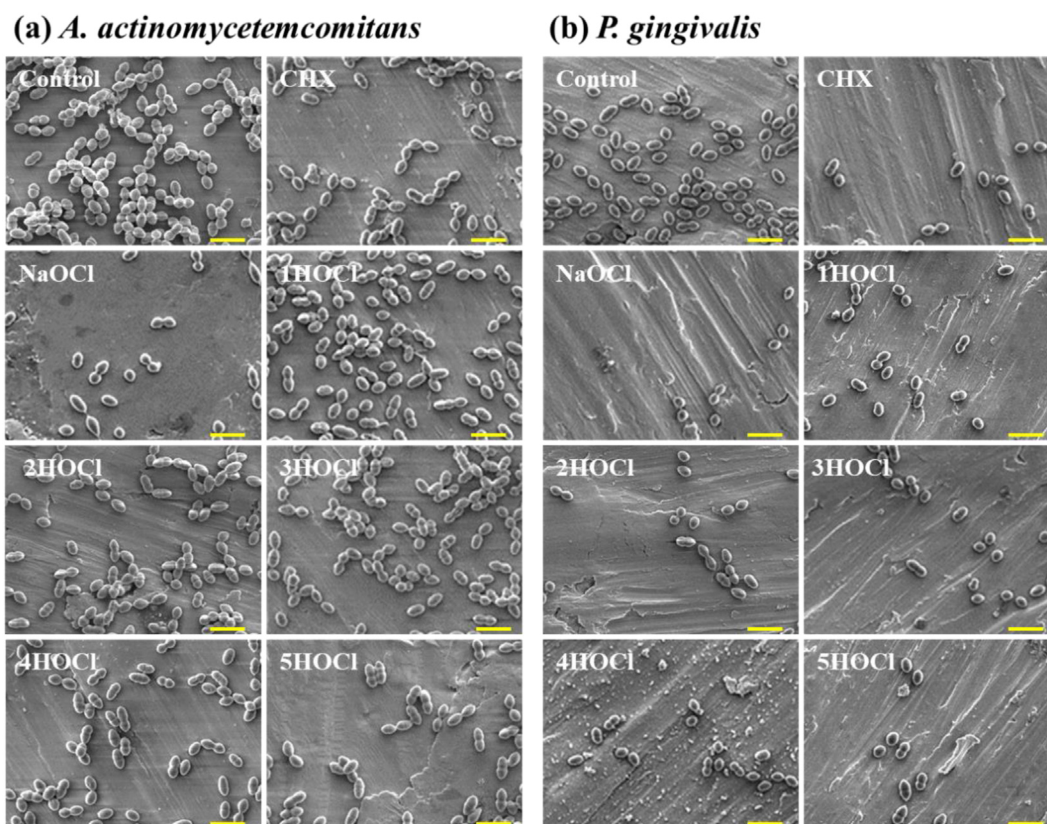

Figure 5 Scanning electron microscope images of Gram-negative (a) *A. actinomycetemcomitans* and (b) *P. gingivalis* after exposure to different antiseptics for 10 min. Scale bar, 2 μm.

response. In this study, four different bacteria were used to examine the efficacy of antimicrobial agents. Among bacterial species, Gram-negative *P. gingivalis* is associated with periodontitis and also with *peri*-implantitis,[23,40] while Gram-negative *A. actinomycetemcomitans* microaerobes are one of the pathogens causing periodontal disease.[41,42] As for Gram-positive bacteria, *S. sanguinis* is often present in the human oral cavity and is known as a pioneer bacterium of oral biofilms.[43] *S. mutans* is an important bacterial stain initiating dental caries.[44] The antimicrobial efficacy of the three antiseptics against bacterial species was determined using the conventional spread plate method and the alamarBlue assay. The former is the classical type of CFU assay, and the latter is used for real-time and repeated monitoring of bacterial viability.[27,45] The alamarBlue assay is much less labor intensive. It allows the estimation of the number of viable bacteria by the redox reaction between the indicator dye and metabolically active bacteria,[42] which is proportional to the maximum absorbance at 570 nm.

The efficacy of the HOCl solution against microorganisms is influenced by many factors, including pH, concentration, treatment time or volume.[23,46,47] For example, increased volume, high concentration, and long treatment time would increase bactericidal activity.[23,47] Miyaoka et al. reported concentration-dependent virucidal activities of HOCl with various concentrations of free available chlorine (100–500 ppm) against aerosolized coronavirus.[48] The weak acid HOCl can dissolve in water to form hypochlorite ion (OCl^-),[21] and the HOCl/OCl^- ratio depends on the solution pH.[49] It should be noted that as a disinfectant HOCl is much more effective than OCl^-. Therefore, when examining the concentration-dependent effect of HOCl in different culture environments, the final pH of the medium is accordingly different, as the weak acid HOCl may be affected by the type of the medium. For this reason, this study examined the pH changes of CHX, NaOCl and HOCl after dilution with water, bacteria growth broth and cell culture medium. As a result, the pH of 0.2% CHX was comparable to the solvent used. However, when diluted with Wilkins-Chalgren broth and DMEM, turbidity formed in the diluted CHX solution may be due to the complex reaction between protein (or amino acid) in the medium and CHX (N′,N′′′′′-hexane-1,6-diylbis [N-(4-chlorophenyl) (imidodicarbonimidic diamide)]), which precipitated some substances. It has also been reported that CHX can interact with albumin present in serum or saliva.[10] Therefore, we diluted CHX with water instead of broth for bacterial culture and DMEM for L929/RAW 264.7 cell culture for all in vitro bioassays. In contrast, the strong base NaOCl (pH $>$ 11) caused 1.5% NaOCl to remain basic regardless of the solvent. The pH of HOCl solutions diluted by the three solvents changed little in the pH 5–7 range, consistent with a previous study.[18] The pH value of HOCl solution was stable in broth and DMEM, while HOCl was soluble in water, resulting in a slight drop in pH.

When the weak acid HOCl was diluted with bacterial growth broth with pH $<$ 7, the broth did not affect the pH of HOCl, which were around 6.2 at concentrations of 100–500 ppm, which favored the presence of HOCl.[50,51]

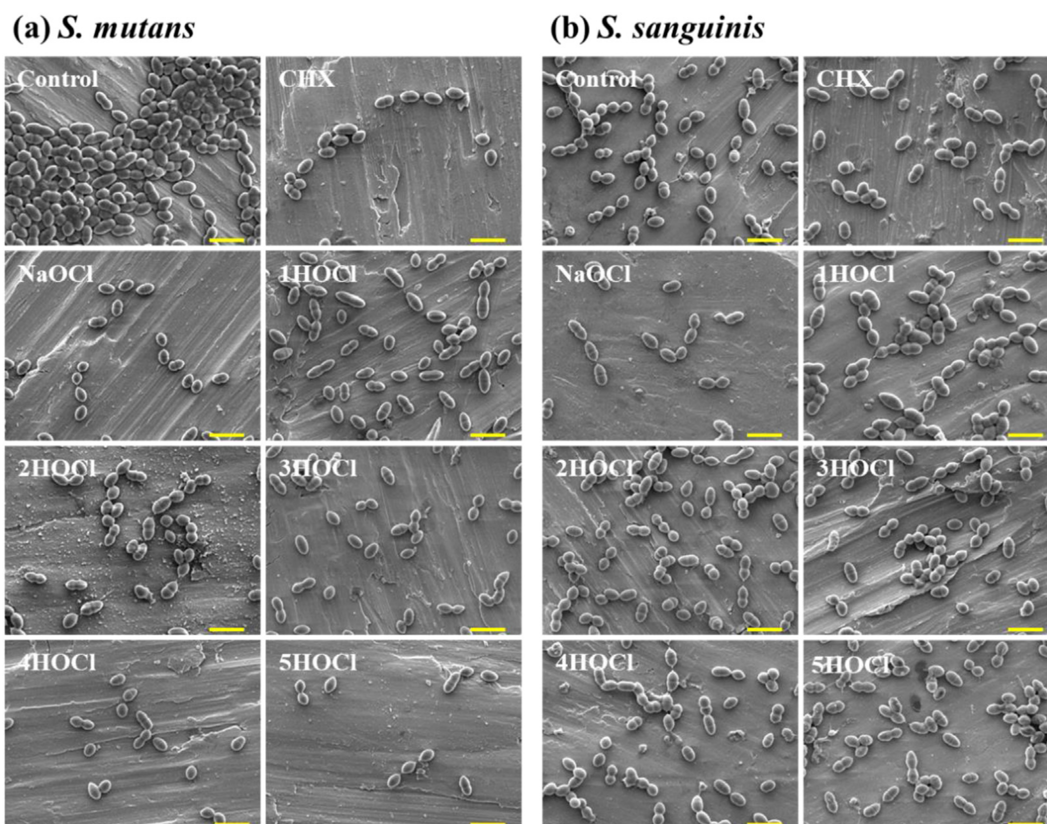

Figure 6 Scanning electron microscope images of Gram-positive (a) *S. mutans* and (b) *S. sanguinis* after exposure to different antiseptics for 10 min. Scale bar, 2 μm.

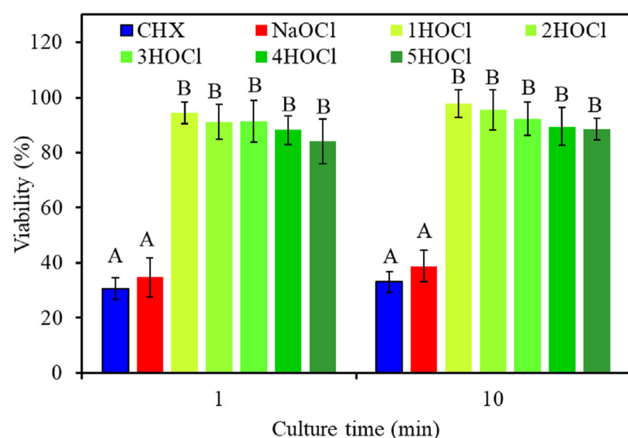

Figure 7 Viability of L929 cells cultured with different test reagents for 1 min and 10 min. Cell viability was normalized to the negative control (Dulbecco's modified Eagle medium (DMEM) only) based on absorbance (n = 9). For the same cell culture time, different capital letters indicate significant differences at $P < 0.05$.

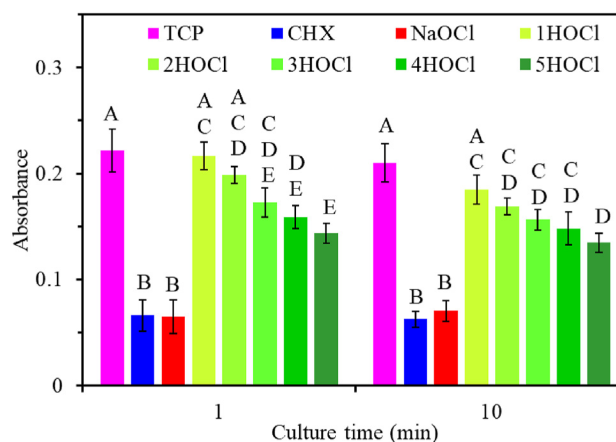

Figure 8 Growth of RAW 264.7 cells cultured with different test reagents for 1 min and 10 min. The tissue culture plate (TCP) without test reagents was used as a control. For the same cell culture time, different capital letters indicate significant differences at $P < 0.05$ (n = 3).

Rutala and Weber proposed that HOCl had the best disinfection efficacy at pH 6,[50] due to the presence of higher amounts of HOCl in solution. The bactericidal activity of chlorine disinfectants is mainly attributed to un-dissociated HOCl. At pH 6.0, HOCl accounts for 98% of the free chlorine, whereas at pH 10.0, OCl^- accounts for more than 99%.[52,53] Although the current results showed that the antimicrobial efficacy of HOCl against the four bacterial species was lower than that of NaOCl and CHX, the concentration of HOCl used (0.01–0.05%) was much lower than that of NaOCl (1.5%) and CHX (0.2%). Castillo et al. found that 0.2% CHX was more effective against *S. mutans* compared with 250 ppm and 500 ppm HOCl after 30 s of treatment.[54]

Within the limit of this study, HOCl did not completely kill the bacteria, but as reported in an earlier study,[23] increased volume may be effective way to increase the antibacterial effectiveness. Indeed, significantly higher concentrations of HOCl and longer treatment times would be required to completely kill bacteria, but the side effects of high concentrations of antibacterial agents on cells must be considered simultaneously.

Since antiseptics have a concentration-dependent eradication effect on bacterial species, attention should be paid to cytotoxicity outcomes. Low cytotoxicity should be one of the essential requirements for antiseptics. Because CHX and NaOCl are used in the management of periodontal and endodontic diseases, they should remain low toxic and non-immune function. Therefore, the cytotoxicity of all antimicrobial agents should be unveiled to confirm their biosafety. To explore this, this study analyzed the cytotoxicity of L929 fibroblasts and RAW 264.7 macrophages cultured with these antiseptics.

Not surprisingly, CHX and NaOCl had significantly lower cell viability than all HOCl solutions despite their superior antimicrobial ability. For example, CHX and NaOCl resulted in approximately 30% viability of L929 cells and 4HOCl approximately about 88% when incubated for 1 min. CHX inhibited L929 cell proliferation, which is consistent with a previous study.[6] Injection of CHX into the hind paws of mice by Faria et al. induced severe toxicity.[55] NaOCl has been shown to be toxic to periapical fibroblasts at concentrations above 0.01%.[56] Conversely, the L929 cytotoxicity results for all HOCl solutions in the 100–500 ppm range met the ISO 10993–5 recommendations, with cell viability above 70% considered non-cytotoxic.[57] Tazawa noted that, unlike NaOCl, HOCl was nontoxic to tissues but still had broad bactericidal effects.[58] Hsieh et al. demonstrated minimal in vivo toxicity of 125 ppm and 250 ppm HOCl using zebrafish embryo assays.[59] Wang et al. confirmed that HOCl was non-irritating and non-sensitizing, and it was less cytotoxic to mammalian cells than NaOCl in the effective antimicrobial concentration range.[53] In addition to the lower concentration of HOCl, pH 7.8 DMEM can be used to explain the low cytotoxicity of the HOCl solution when compared with CHX and NaOCl. Kim et al. found a HOCl/OCl^- ratio of one at pH 7.5.[51] Therefore, it can be speculated that HOCl in DMEM was less damaging to cells compared with to bacterial growth broth at pH 6.2.

Macrophages play a key role in wound healing and innate immunity, and produce pro-inflammatory mediators against invading pathogens.[60,61] Since the RAW 264.7 cell line has a stable and mature adherent macrophage phenotype, it is widely used to test the immune activity of reagents.[29] In this study, CHX and NaOCl revealed significant negative impacts on macrophage viability, whereas HOCl showed a slight concentration-dependent reduction in cell growth. In a study by the Chang group, CHX cytotoxicity in RAW264.7 cells also displayed a dose- and time-dependent manner,[60] confirming our findings. CHX induces an inflammatory reaction and tissue necrosis, and retards granulation tissue formation and wound healing.[9] An inflammatory response occurs when 2% CHX or 5.25% NaOCl is injected into the subcutaneous tissue of guinea pigs and rats.[10]

Regardless of the fibroblast (L929) or macrophages (RAW 264.7), all HOCl groups did not result in cytotoxicity over the range of HOCl concentrations used in the study except for 500 ppm HOCl solution in RAW 264.7. More importantly, relative to the findings of antimicrobial activity, HOCl produced larger cell growth in L929 and RAW 264.7 compared with CHX and NaOCl. HOCl may be a potential disinfectant because of its good antimicrobial properties and non-toxicity.

Reducing microbial populations and maintaining periodontal health is one of the concerns of home care and clinical practice. In this in vitro study, CHX and NaOCl had high antibacterial ability and large cytotoxicity. Although HOCl was inferior to NaOCl and CHX in antibacterial ability, it is far superior to them in terms of biocompatibility. HOCl exhibited a concentration-pendent antimicrobial activity and biocompatibility, could significantly reduce the populations of oral-associated bacteria without adverse cytotoxicity. Taken together, 400 ppm HOCl can be considered as a potential antiseptic in terms of antimicrobial activity and non-cytotoxicity. Therefore, the non-antibiotic antimicrobial HOCl solution could be used as a potential mouthwash to reduce gingivitis and plaque, or as a root canal irrigant and in the treatment of *peri*-implantitis. Further studies such as the maximum duration of use and in vivo response should be performed before clinical applications.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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