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Efficacy of hypochlorous acid as an alternative oral antimicrobial agent on human gingival fibroblasts, *Aggregatibacter actinomycetemcomitans*, and *Candida albicans* biofilms *in vitro*

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ABSTRACT

This study compared the cytotoxicity and antimicrobial activity of hypochlorous acid (HOCl) at 50 ppm and 200 ppm and 0.2% chlorhexidine (CHX) at various time intervals, *in vitro*. Cell viability and cytotoxicity of human gingival fibroblasts (HGF) were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and the lactate dehydrogenase assay. Antimicrobial effects on *Aggregatibacter actinomycetemcomitans* and *Candida albicans* were determined using the time-kill method. All solutions exhibited a significant impact on HGFs in a dose- and time-dependent manner. 50 ppm HOCl demonstrated the highest cell viability, followed by 200 ppm HOCl. Both HOCl solutions were less cytotoxic to HGFs than 0.2% CHX. 50 ppm and 200 ppm HOCl demonstrated stronger efficiencies than CHX against *A. actinomycetemcomitans* and *C. albicans*. The data suggest that HOCl solutions have potential as an alternative antiseptic to CHX due to their lower cytotoxicity and superior antimicrobial activity, but optimal dosage of HOCl requires further investigations.

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Hypochlorous acid; chlorhexidine; fibroblasts; *Aggregatibacter actinomycetemcomitans*; *Candida albicans*

Introduction

Periodontal diseases are multifactorial inflammatory infections caused by the accumulation of oral biofilm, which is a complex microbial community that infects and destroys the periodontium (Armitage 1999). Microbial dental plaque and oral cavity pathogens cause periodontal disease, with *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis* as primary agents. Other etiological agents include such organisms as *Prevotella intermedia*, *Camphylobacter rectus*, *Fusobacterium nucleatum*, *Eikenella corrodens*, and certain fungi, especially *Candida albicans* (Haffajee and Socransky 1994; Socransky et al. 1998). *A. actinomycetemcomitans* is a Gram-negative bacteria that is part of the natural oral microbiota of humans and is associated with periodontitis, bone damage, and other non-oral infections. It is an important pathogen in aggressive, severe, and recurrent forms of periodontitis and is associated with rapidly progressive periodontitis (Gholizadeh et al. 2017). *C. albicans* is the leading cause of fungal infections in the oral cavity and can form biofilms on various surfaces, including dental implants and dentures (Lohse et al. 2018). It has colonized various body locations without causing symptoms. Changes in host immunity, stress, and other variables can lead to *C. albicans* overgrowth, resulting in opportunistic infections (Barbeau et al. 2003). Both microorganisms can coaggregate with bacteria in the dental biofilm and invade epithelial cells and connective tissue, which causes the progression of oral diseases.

Treatment for periodontal disease involves supra- and subgingival instrumentation, as well as addressing the factors that cause its involvement. However, professional mechanical removal may not always be sufficient to destroy biofilm formation completely. If necessary, additional treatments and antimicrobial agents should be used (Sanz et al. 2020). Antimicrobial agents are used to treat supragingival plaque, gingivitis, and prior to oral and periodontal surgery. They can also be used as protective and therapeutic agents in the presence of xerostomia or *Candida* infections due to prostheses, hyperplasia, mucositis, and in patients at

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risk of bacteremia or oral infections (Coelho et al. 2020).

Chlorhexidine (CHX) is an efficient oral antimicrobial agent that has been widely used in dentistry for the prevention of dental plaque formation in the oral cavity, as an adjunct to mechanical periodontal therapy in gingivitis and periodontitis, in the prevention and treatment of infections that may develop after surgical operations, and in the elimination of other oral infections (Fardal and Turnbull 1986). It has broad antimicrobial activity against both Gram-positive and Gram-negative microorganisms, but less so against fungal pathogens and some lipophilic viruses (Russell 1990). Due to its significant impact on dental plaque, it is regarded as the gold standard in dentistry (Neely 2012). Although the effectiveness of CHX as an antiseptic agent has been well established, it causes yellow-brown staining of teeth, calculus formation, and taste alteration with prolonged use (Batra et al. 2022). The use of CHX has been reported to be associated with inflammatory responses, tissue necrosis, and regeneration inhibition (Coelho et al. 2020). Its impact on tissues, especially during healing, has been called into question. Since it has side effects on teeth and oral mucosa, studies have been conducted to search for more suitable agents.

Hypochlorous acid (HOCl) is a physiological molecule that neutrophils produce during phagocytosis, and it plays an active role in eliminating pathogens (Vissers and Winterbourn 1995). HOCl is a non-antibiotic compound produced by the reverse reaction of sodium hypochlorite and hydrogen peroxide *in vitro* (Levine 2013). It has been demonstrated that it is quite effective against a wide variety of bacterial, viral, and fungal species (Armstrong et al. 2015). Even a small amount of it can quickly kill spore-forming and non-spore-forming bacteria, as well as biofilm-forming microorganisms. It has dose-dependently favorable effects on keratinocyte and fibroblasts migration and the inflammatory response (Sakarya et al. 2014). The HOCl is used as an ideal wound care solution in many fields of medicine, such as the treatment of open, chronic, and surgical wounds; the removal of microorganisms; diabetic foot ulcers; mouth, nose, throat, and lip ulcers and infections (Armstrong et al. 2015). Its use in medicine as a perfect wound care solution has led to the belief that it can also be used to treat oral infections. An animal study (Morita et al. 2011) was conducted to evaluate the systemic and gastrointestinal impacts of HOCl ingestion and investigate what would happen if some HOCl was swallowed when used as mouthwash. HOCl water was given to mice as drinking water. Visual examinations of the oral cavity, histopathologic outcomes, and assessments of the surface enamel roughness revealed no aberrant findings, indicating no systemic influence. In a clinical study (Yu-Rin Kim 2018) on dentistry, patients were gargled with 10–30 ppm HOCl and 0.005% CHX mouthwash for 1 min, and results were obtained in favor of the group using HOCl in all the tested parameters (halitosis, dental caries activity, bacterial species, and motility). According to the results, it has been suggested that the use of HOCl may contribute to the prevention of oral diseases.

Hence, the aim of the present study was to comparatively evaluate *in vitro* the cytotoxic activities of 50 ppm HOCl, 200 ppm HOCl, and 0.2% CHX solutions on human gingival fibroblasts (HGF) and their antimicrobial efficacy on *A. actinomycetemcomitans* and *C. albicans* at different time points.

Materials and methods

Test materials

In this study, 0.2% CHX and two concentrations of HOCl [50 ppm (0.005%) and 200 ppm (0.02%)] were evaluated at 30 s, 2 min, 1 h, and 24 h. To reach the final concentration of CHX, 20% chlorhexidine digluconate (Doga Pharmaceuticals, Istanbul, Turkey) was dissolved in the growth medium just before processing. The HOCl solutions, 100 and 400 ppm, were obtained from a chemical corporation (Anolit Co., Ankara, Turkey). To achieve the final concentrations, the HOCl was dissolved in the growth medium in half and half just before use.

Cytotoxic activity

Cell culture

A HGF cell line (HGF-1, ATCC, CRL-2014, Manassas, VA, USA) was used in this study. The HGF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with 1% penicillin/streptomycin, 1% L-glutamine, 0.1% amphotericin B, and 10% fetal bovine serum (FBS) (GibcoTM, Thermo Fisher Scientific, USA) at 37 °C in a humidified incubator with 5% CO_2. The cell cultures were grown in 25 cm^2 culture flasks. After reaching 70%–80% confluency, HGFs were washed with phosphate buffered saline (PBS) (Multicell, Wisent Inc., Canada), and then trypsin/EDTA (Pan-Biotech, Germany) was added. The flasks were allowed to sit at 37 °C until the cells detached, and then the fresh culture medium was added to

inactivate the trypsinization. The cell suspension was centrifuged at 1200 rpm for 5 min, and the supernatant was discarded. The cell pellet was re-suspended in growth medium and dispensed into new culture flasks. To obtain the cell counts, 10 µL trypan blue and 10 µL cell suspension were mixed, and the unstained (viable) and stained (nonviable) cells were counted separately under a light microscope. Approximately the cells were seeded in eight 6-well culture plates with a density of 1.5×10^5 cells per well in 2 mL of growth medium.

Cell viability assay

The cells were seeded in eight 6-well flat-bottom plates and maintained at 37 °C with 95% humidity and 5% CO_2 overnight. Different solutions (HOCl 50 ppm, HOCl 200 ppm, and 0.2% CHX) were treated, and the cells were incubated for another 30 s, 2 min, 1 h, and 24 h separately. Wells with growth medium served as the control group. After incubation, the solutions were removed. Cell viability assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Ortega-Pena et al. 2017). The wells were washed three times with PBS, and 132 µL of the MTT staining solution (5 mg/mL in PBS) was added to each well. The plates were incubated at 37 °C. After 3 h, the MTT solution was removed, and 200 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. After 10 min, 200 µL medium from each well was transferred to 96-well plates, and the optical density (OD) was recorded at 570 nm using a plate reader (Perkin Elmer, Boston, USA). When calculating cell viability (%), the viability of the control group was accepted as 100 and calculated with the following formula:

$$\left[(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank})\right] \times 100$$

Lactate dehydrogenase assay

Extracellular lactate dehydrogenase (LDH) releases were used to detect cytotoxicity as an indicator of necrosis in fibroblasts (Kamiloglu et al. 2020). The cytotoxicity assay was performed according to the directions of a commercial kit (CytoScan™, G-Biosciences, MO, USA). Cells treated with 0.1% Triton X-100 were used as the maximum LDH activity, while untreated cells with 10 µL sterile, ultrapure water were used as the spontaneous LDH activity control. The percentage release of LDH from the sample-treated cells was calculated by comparing it to the maximum release of LDH. The absorbance was measured at 490 nm using a plate reader (Perkin Elmer, Boston, USA). Cell cytotoxicity (%) was calculated by the following formula:

$$\left[\left(\text{LDH Activity}_{sample} - \text{LDH Activity}_{control}\right)/\left(\text{LDH Activity}_{maximum} - \text{LDH Activity}_{control}\right)\right] \text{ x } 100$$

Antimicrobial activity

Culture of microorganisms

In this study, the antimicrobial activity was evaluated against *A. actinomycetemcomitans* (ATCC 29522) and *C. albicans* (ATCC 10231). Cultures of *A. actinomycetemcomitans* were grown in 10 mL of brain-heart infusion broth (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA) and incubated at 37 °C with 5% CO_2 in an incubator (CB-S 260 Binder GmbH, Tuttlingen, Germany) for 24–36 h. *C. albicans* were grown in 10 mL of Sabouraud dextrose broth (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 35 °C in an incubator (UN110, Memmert GmbH + Co. KG, Schwabach, Germany) for 24–36 h. Both microorganisms were suspended in 10 mL of sterile PBS (pH = 7.4; Merck KGaA, Darmstadt, Germany), and the concentration was adjusted to 1×10^8 CFU (colony-forming units)/mL. These were then plated out on 5% sheep blood agar medium (BioMerieux, Marcy l'Etoile, France) for growth control.

The measurement of antimicrobial activity

The 'time-kill' method was used for determining the antimicrobial activity (Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure 1. International Standards Worldwide 2004). The steps of the process were applied separately to each of the previously described microorganisms (Balouiri et al. 2016).

The prepared microorganism suspensions were diluted 1/10 with sterile PBS (pH = 7.4) so that their final concentrations were adjusted to be 1×10^7 CFU/mL. Two concentrations of HOCl (50 and 200 ppm), CHX (0.2%), and sterile PBS were prepared in four sterile tubes of 500 µL each. Tubes containing antiseptic test agents were inoculated with 500 µL of microorganism suspensions. During the incubation, 50 µL of samples were removed aseptically from each tube at 30 s, 2 min, 1 h, and 24 h and carefully transferred to another tube containing 450 µL of sterile PBS. These were then vortexed to resuspend the microorganisms. Then, 100 µL of prepared microorganism suspensions were transferred and inoculated on 5%

sheep blood agar plates using the colony counting method. The plates for *A. actinomycetemcomitans* were then incubated at 37 °C with 5% CO_2 for 24 h; other groups for *C. albicans* were incubated at 35 °C with ambient atmosphere. The number of CFU per microorganism and the antimicrobial activity rates (%) were calculated for each time point.

Statistical analysis

The cytotoxicity assays were repeated twice, and three independent experiments were performed in triplicate for the antimicrobial activity assays. The data were presented as means ± standard deviations (SD). The factorial repeated measures ANOVA was used for the comparisons. The level of significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, USA).

Results

Cytotoxic activity

Cell viability assay

The MTT test was used in order to evaluate whether 50 ppm HOCl, 200 ppm HOCl, or 0.2% CHX solutions would affect the cell viability of HGFs (Table 1 and Figure 1). All of the solutions reduced cell viability at all time points compared to the control group ($p < 0.0001$). For the CHX, the viability of cells decreased over time ($p < 0.01$), except that it was statistically similar at 2 min and 1 h ($p > 0.05$). For the 50 ppm HOCl solution, the viability of cells decreased statistically over time ($p < 0.0001$). Also, the cells' viability decreased in the 200 ppm HOCl solution throughout time ($p < 0.0001$), while there was no difference between 1 h and 24 h ($p > 0.05$). CHX served as the most viability-reducing agent at all time points ($p < 0.01$) except for 1 h, when it was statistically similar to 50 ppm HOCl ($p > 0.05$). With the exception of 24 h, the viability of 50 ppm HOCl was significantly higher than 200 ppm HOCl ($p < 0.0001$). At that time point, 50 ppm HOCl showed less viability than 200 ppm ($p < 0.0001$).

Lactate dehydrogenase assay

The LDH assay was applied to investigate the cytotoxicity of the solutions on HGFs (Table 1 and Figure 2).

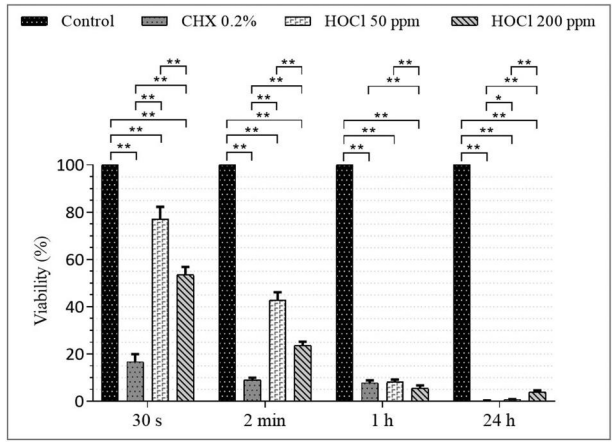


Figure 1. Effect of two different concentrations of HOCl, 50 ppm and 200 ppm, and 0.2% CHX solutions treatment on HGF cell viability in the MTT assay. The evaluation was conducted at 30 s, 2 min, 1 h, and 24 h time points. The *p*-values were determined through factorial repeated measures ANOVA ($p < 0.05$). *Statistically significant *p* value ($p < 0.01$). **Statistically significant *p* value ($p < 0.0001$).

Table 1. The effect of tested agents on HGF viability as evaluated by the MTT assay and cytotoxicity as measured by the LDH assay.

		HGF viability (MTT assay)			Cytotoxicity towards HGF (LDH assay)		
Agent	Period	Mean ± S.D.	Standard error	*P* value*	Mean ± S.D.	Standard error	*P* value*
Control		100	.00		100	.00	
CHX (0.2%)	30 s	16.72 ± 3.26	1.152	<0.0001	51.17 ± 11.3	3.982	<0.0001
	2 min	9.14 ± 0.85[a]	0.300	<0.0001	53.30 ± 16.9	5.977	<0.0001
	1 h	7.80 ± 1.06[a]	0.375	<0.0001	55.77 ± 13.6	4.792	<0.0001
	24 h	0.25 ± 0.15[a,b,c]	0.055	<0.0001	84.30 ± 6.69[a,b,c]	2.364	0.0129
HOCl 50 ppm	30 s	77.02 ± 5.24	1.851	<0.0001	14.16 ± 4.39	1.551	<0.0001
	2 min	42.82 ± 3.3[a]	1.171	<0.0001	23.69 ± 9.12	3.226	<0.0001
	1 h	8.25 ± 0.90[a,b]	0.319	<0.0001	29.19 ± 10.9a	3.852	<0.0001
	24 h	0.82 ± 0.10[a,b,c]	0.036	<0.0001	85.14 ± 11.4[a,b,c]	4.020	0.0198
HOCl 200 ppm	30 s	53.47 ± 3.35	1.186	<0.0001	17.35 ± 8.18	2.891	<0.0001
	2 min	23.66 ± 1.56[a]	0.551	<0.0001	25.69 ± 2.51	0.888	<0.0001
	1 h	5.50 ± 1.20[a,b]	0.425	<0.0001	33.42 ± 6.12	2.163	<0.0001
	24 h	4.06 ± 0.55[a,b]	0.195	<0.0001	46.55 ± 13.6[a,b]	4.820	<0.0001

Factorial repeated measures ANOVA, $p < 0.05$, S.D.: standard deviation.
*Compared to control ($p < 0.05$).
[a]Compared to 30 s ($p < 0.05$).
[b]Compared to 2 min ($p < 0.05$).
[c]Compared to 1 h ($p < 0.05$).

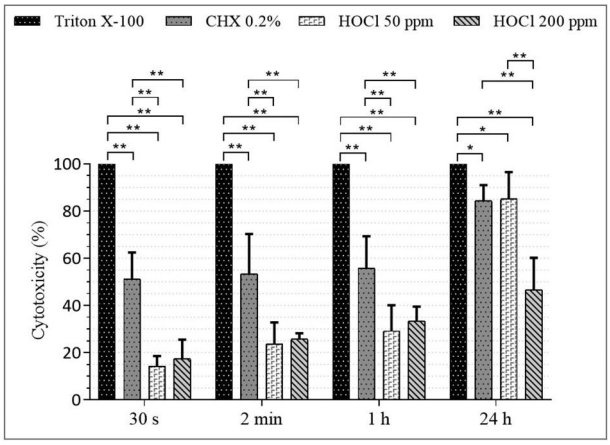


Figure 2. Effect of two different concentrations of HOCl, 50 and 200 ppm, and 0.2% CHX solutions treatment on HGF cytotoxicity measured by the LDH assay. Cells were treated with triton X-100 were considered the control group. The evaluation was conducted at 30 s, 2 min, 1 h, and 24 h time points. The *p*-values were determined through factorial repeated measures ANOVA ($p < 0.05$). *Statistically significant *p* value ($p < 0.05$). **Statistically significant *p* value ($p < 0.0001$).

The cytotoxicity values for all the agents were statistically lower than those of the control (Triton X-100) group ($p < 0.05$). There were no significant differences in the cytotoxicity of CHX at 30 s, 2 min, and 1 h ($p > 0.05$), but it significantly increased at 24 h ($p < 0.05$). For the 50 ppm HOCl, the cytotoxic activity tended to increase at 30 s, 2 min, and 1 h ($p > 0.05$), but there was only a significant difference between 30 s and 1 h ($p < 0.01$). It was also statistically higher at 24 h versus other time points ($p < 0.001$). It was found that the cytotoxic activity of 200 ppm HOCl was similar at 30 s, 2 min, and 1 h ($p > 0.05$). The cytotoxic activity, which increased at 24 h ($p < 0.05$), was not statistically significant compared to 1 h ($p > 0.05$). Both of the HOCl solutions were less cytotoxic on HGFs than CHX ($p < 0.0001$); only at 24 h, CHX was similar to 50 ppm HOCl ($p > 0.05$). No statistically significant difference was observed between HOCl solutions except that 50 ppm HOCl was more cytotoxic to the cells at 24 h ($p < 0.0001$).

Antimicrobial activity

The antimicrobial activities of the solutions on *A. actinomycetemcomitans* and *C. albicans* are shown in Figure 3. All of the treatments significantly inhibited the viability of microorganisms when compared to the negative control group at all time points ($p < 0.0001$).

For *A. actinomycetemcomitans,* the antimicrobial activity of CHX decreased from 99.38 ± 0.1% to 98.36 ± 0.1% at 30 s and 2 min ($p < 0.0001$). Afterwards, it increased to 98.72 ± 0.1% at 1 h ($p < 0.0001$) and 100% at 24 h ($p < 0.0001$). The antimicrobial activity of 50 ppm HOCl was 99.79 ± 0.09% at 30 s and 100% at 2 min, 1 h, and 24 h, resulting in significant differences observed just between 30 s and the other time points ($p < 0.05$). It was 100% at all time points for 200 ppm HOCl ($p > 0.05$). Both HOCl solutions demonstrated higher antimicrobial activity against *A. actinomycetemcomitans* than CHX at 30 s, 2 min, and 1 h ($p < 0.01$). And a statistical difference was observed between HOCl solutions at only 30 s ($p < 0.0001$).

For *C. albicans*, the antimicrobial activity of CHX decreased from 96.20 ± 0.9% to 87.60 ± 0.7% at 30 s and 2 min ($p < 0.0001$). Afterwards, the antimicrobial activity increased to 97.60 ± 0.4% at 1 h ($p < 0.0001$) and 100% at 24 h ($p < 0.0001$). The antimicrobial activity of 50 ppm HOCl was 99.70 ± 0.05% at 30 s and statistically increased to 100% at 2 min, 1 h, and 24 h ($p < 0.05$). It was 100% at all time points for 200 ppm HOCl ($p > 0.05$). Both HOCl solutions demonstrated stronger antimicrobial efficiencies against *C. albicans* than CHX at all time points ($p < 0.0001$), with the exception of 24 h. No statistically significant difference was observed between the HOCl solutions ($p > 0.05$).

Discussion

HOCl rinses are proposed for use in dental health as a newer agent due to their low toxicity and high antimicrobial efficiency, ability to destroy biofilms, anti-inflammatory impact, and history in clinical medicine (Armstrong et al. 2015). However, little is known about its toxicity and efficacy in the oral cavity and periodontal area. This study was undertaken to compare the antimicrobial effects of 0.2% CHX, 50 ppm HOCl, and 200 ppm HOCl against *A. actinomycetemcomitans* and *C. albicans* and to investigate their impact on the viability of and cytotoxicity towards HGFs.

In spite of its well-described antimicrobial properties and widespread use in the treatment of wounds, many authors have described that CHX has a very strong cytotoxic effect on the gingival fibroblasts *in vitro* (Giannelli et al. 2008; Faria et al. 2009; Sukumaran et al. 2020; Babgi et al. 2021; Utami et al. 2022). Researchers stated that the exposure of fibroblasts to >0.01% CHX significantly reduced cell viability in time-dependent manners (Schmidt et al. 2016; Sukumaran et al. 2020; Nawrot-Hadzik et al.

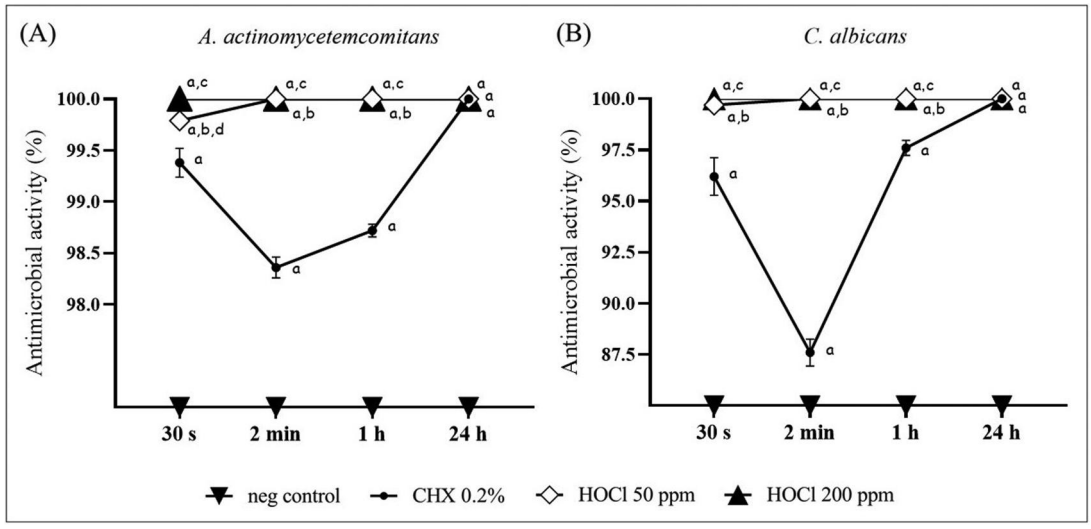


Figure 3. Antimicrobial activity percentages of 0.2% CHX and HOCl (50 ppm and 200 ppm) solutions on *A. actinomycetemcomitans* (**A**) and *C. albicans* (**B**) after 30 s, 2 min, 1 h, and 24 h treatment. The *p*-values were determined through factorial repeated measures ANOVA ($p < 0.05$). (a) versus control group ($p < 0.0001$), (b) 50 ppm HOCl versus CHX ($p < 0.0001$), (c) 200 ppm HOCl versus CHX ($p < 0.0001$), and (d) 50 ppm HOCl versus 200 ppm HOCl ($p < 0.0001$).

2021). Similar to this present study, Babgi et al. detected a strong inhibition in the viability of HGFs after 2 min of exposure to CHX (Babgi et al. 2021); likewise, Utami et al. found the same result at 24 h (Utami et al. 2022). Furthermore, to detect the toxicity of CHX, the induction of necrosis (LDH release) in HGFs was investigated. However, there is a lack of studies searching for cell death after exposure; only a few studies could be found that were in relation to CHX (Giannelli et al. 2008; Faria et al. 2009; Schmidt et al. 2018). Gianelli et al. demonstrated that CHX, at a 0.12% concentration, influenced the necrosis of fibroblasts after 1 min of treatment, with rates superior to 50% (Giannelli et al. 2008), as well as Faria et al. who showed 80% necrosis at even lower concentrations after 24 h (Faria et al. 2009). These observations are in agreement with this present study, which showed a significant increase in the cytotoxicity of HGFs after 30 s and 24 h of treatment with 0.2% CHX. In light of the effects of 0.2% CHX on HGFs, its safety as an antiseptic agent for treatment should be questioned and less toxic alternative agents should be investigated in further studies.

HOCl shows only mildly cytotoxic effects on human fibroblasts (Gonzalez-Espinosa et al. 2007; Rani et al. 2014; Armstrong et al. 2015; Day et al. 2017; Kramer et al. 2018), although no research has been conducted particularly on HGFs. This study is the first to evaluate the cytotoxic effectiveness of HOCl solutions on HGFs. Sakarya et al. found that HOCl solution had dose-dependently favorable impacts on fibroblasts and keratinocyte migration (Sakarya et al. 2014). Within 24 h of treatment, the migration was inhibited at high concentrations (55–109 ppm) but increased at low concentrations (5.5–11 ppm). A recent study in a 6- to 48-h time frame revealed that HOCl (3.3, 16.5, and 33 ppm) inhibited biofilm formation without being excessively cytotoxic to fibroblasts (Ortega-Pena et al. 2017). Similar to the findings of the present study, the rate of cell viability decreased in a dose- and time-dependent manner. However, it was also shown that HOCl may result in tissue necrosis and/or apoptosis at higher concentrations (500 ppm) (Vile et al. 2000; Zhang et al. 2001). Day et al. evaluated 330 ppm HOCl and 0.05% CHX at 3 min and reported that HOCl showed similar efficiency to CHX in eliminating tested bacteria but lower cytotoxicity (Day et al. 2017). Despite the different concentrations used, the findings about toxicity are in line with the present study. However, considering the effects of HOCl on HGFs, the effects of lower concentrations of it could be studied due to their lower toxicity.

A. actinomycetemcomitans has been firmly associated with the pathogenesis of adult periodontitis resistant to conventional periodontal therapy as well as cases of early-onset periodontitis (Gholizadeh et al. 2017). Concerning the antibacterial activity of the three tested solutions for *A. actinomycetemcomitans*, the CHX with a concentration of 0.2%, known as the 'antibacterial antiseptic of choice' (the 'gold standard') (Neely 2012), caused less antimicrobial activity except for 24 h, and the HOCl solutions of 50 and 200 ppm each caused more CFU reduction and showed similar

higher antimicrobial activity values. According to assessments of the effects of 0.2% CHX on the oral microbiome, there is agreement that it has a significant impact on *A. actinomycetemcomitans,* such as in 30 s (Decker et al. 2017), 1 min (Voos et al. 2014), 5 min (Etemadi et al. 2020), and 24 h (Vahabi et al. 2019) *in vitro* studies. These results are comparable with the findings of this study. Another *in vitro* study (Von Maltzahn et al. 2020) had shown that 0.2% CHX treatment resulted in a minor or no effect on *A. actinomycetemcomitans* at 24 h. However, this research was carried out using *A. actinomycetemcomitans* as a late biofilm maturation form grown on titanium specimens, which is a totally different technique. A recent review of the literature revealed that HOCl has wide-spectrum antimicrobial activity against bacteria, viruses, and fungi (Armstrong et al. 2015). In the literature, only one study (Castillo et al. 2015) examined the relationship between HOCl and *A. actinomycetemcomitans*. Castillo et al. exposed *A. actinomycetemcomitans* to 250 and 500 ppm HOCl and 0.2% CHX for 30 s and used the fluorescence method to determine bacterial viability (Castillo et al. 2015). Similar to the findings in this study, the viability percentages of *A. actinomycetemcomitans* were significantly affected by HOCl when compared with CHX. At the same time, although different concentrations were studied, they found that the higher concentration of 500 ppm HOCl had a greater effect than the lower concentration of 250 ppm, likewise in this study.

C. albicans is a major pathogen that causes oral candidiasis and *Candida*-associated denture stomatitis, which are common oral fungal infections that primarily affect people with compromised immune systems (Barbeau et al. 2003). Furthermore, *C. albicans* has been detected in peri-implant lesions in addition to putative periodontal pathogens (Lohse et al. 2018). According to the literature, CHX is effective at inactivating *C. albicans*, but the concentration and contact time of CHX in killing the fungus differ across studies; in some studies evaluated in 1 min (Burgers et al. 2012), 5 min (Oliveira et al. 2014; Mohammadi and Asgary 2015), and 24 h (Abbaszadegan et al. 2015), it was shown that CHX is highly effective against *C. albicans,* while in other studies, it completely eliminated it in 30 s (Vianna et al. 2004), 2 min (Medvedec Mikic et al. 2018), and 1 h (Aghili et al. 2015). On the other hand, a 24-h *in vitro* study (Tirali et al. 2013) showed that 0.2% CHX is ineffective for *C. albicans*. The study design, which was an agar plate assay, may have resulted in this varied outcome. In this method, the microbial inhibition zone depends on the solubility of the test substance and its diffusion capacity from the agar; therefore, these tests may not represent their full effective potential, even though they are fairly common (Mayrhofer et al. 2008). As mentioned earlier, HOCl is also quite effective against fungal species (Armstrong et al. 2015). Kim et al. suggested that HOCl has a 96% bactericidal effect on *C. albicans* at a concentration of less than 3.5 ppm (Kim et al. 2008). According to Harriot et al. all biofilms were destroyed after 1 h with 250 and 330 ppm HOCl solutions, showing excellent fungicidal action (Harriott et al. 2019). According to a recent study (Anagnostopoulos et al. 2018) comparing 100 ppm HOCl with 4% CHX, HOCl had an immediate effect against *C. albicans*, whereas a full bactericidal effect was observed at 1 min for CHX. Despite the different concentrations studied, similar results were reported for HOCl in this study. HOCl solutions reduced C. albicans similarly between them, but at a higher rate than CHX. This present study is the first in the literature to examine the relationship between 50 or 200 ppm HOCl and *C. albicans*. Since the efficacy of 50 ppm HOCl against *A. actinomycetemcomitans* is slightly higher than that of 200 ppm HOCl and their efficacy against *C. albicans* is similar, we recommend that future research focus on examining the antimicrobial properties of lower HOCl concentrations.

One of the limitations of our study is that it was performed only *in vitro*; it was not possible to assess the impact of numerous variable factors in the clinical setting, thereby restricting the significance of the findings. The results of the MTT and LDH tests, which were used to evaluate cell viability and cytotoxicity, were not supported by scanning electron microscopy. Another limitation of the study could be the focus on specific pathogens as the primary research targets. However, it is possible and even a common approach to test the antimicrobial efficacy of antiseptics on a limited number of microorganisms.

Conclusion

Within the limitations of this study, HOCl can be recommended as an alternative to CHX due to its lower cytotoxic potential and higher antimicrobial effects. When the risk-benefit scenarios of HOCl concentrations are evaluated among themselves, it is seen that 50 ppm and perhaps lower HOCl concentrations should be preferred. Further studies are needed to clarify the appropriate dosage of HOCl that can be used as an effective, biocompatible, and non-toxic

antiseptic agent in the microenvironment of the oral cavity and in the control of pathogen-induced oral infections.

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