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Effect of Three Chlorhexidine-Based Mouthwashes on Human Gingival Fibroblasts: An In Vitro Study

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Abstract: Mouthwashes containing chlorhexidine (CHX) are deemed to be associated with dose-dependent side effects, including burning sensation and taste alteration. To overcome these drawbacks, mouthwashes with CHX at lower concentrations with or without adjunctive agents are proposed. The aim of this in vitro study was to investigate the effects of three CHX-based mouthwashes on human gingival fibroblasts (HGFs). After 3 days of cell culture, groups were randomly treated for 30 s, 60 s or 120 s with (a) CHX 0.05% in combination with cetylpyridnium chloride (CPC) 0.05%; (b) CHX 0.1%; (c) CHX 0.2%; or (d) NaCl as control. Cell viability, cytotoxicity and apoptosis were evaluated at 2 h, 3 days and 6 days after the exposure to the different solutions. Similar cell viability values were found among the test groups at all time points. At day 0, higher cytotoxicity was measured in the group treated with CHX 0.2%, in particular after long application time (120 s), while no significant difference was found between CHX + CPC and the control group. All the investigated mouthwashes were well tolerated by HGF cells for the tested application times. The highest cytotoxic effect was observed for CHX 0.2%; therefore, clinicians should consider limiting its usage to carefully selected clinical situations.

Keywords: antiseptic; apoptosis; cetylpyridnium chloride; mouthrinse

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1. Introduction

Oral biofilm is considered the principal etiologic factor responsible for the onset, the development and the recurrence of periodontitis and peri-implantitis [1–6]. Furthermore, tissue healing can be impaired by the presence and accumulation of oral biofilm after the surgical treatment of periodontal and peri-implant diseases, when effective mechanical self-care cannot be adequately performed [7]. Thus, plaque control is deemed to be essential for both the recovery and the maintenance of healthy tissue conditions [7,8].

In adjunction to professional mechanical debridement, adequate self-administered daily home care is fundamental for the long-term success of the treatments [8–10]. At-home measures frequently include the use of antiseptic mouthwashes. In addition to adequate antibacterial activity, these products should not trigger any allergic reactions or provoke tissue damage [11,12].

Concerns may arise in cases of prolonged usage or when the antimicrobial agent comes in direct contact with the connective tissues, for instance during postoperative wound healing [11,13]. In vitro assays are frequently utilized for analyzing the cytotoxicity of antiseptic agents as well as of some filling resin frequently used dental materials, owing

to their reduced costs and their high repeatability and reproducibility [14]. In particular, human gingival fibroblasts are commonly used to mimic connective tissue exposure to mouthwashes and to investigate cell-induced stress [15–19].

Chlorhexidine (CHX), a bisbiguanide broad-spectrum antiseptic, has been widely used for chemical plaque control [20]. However, it is well documented that the prolonged use of mouthwashes with CHX at high concentration can lead to several undesired side effects, including tooth staining, taste alteration and burning sensation [20–23]. CHX-based mouthwashes on the market are generally at a concentration of 0.1%, 0.12% or 0.2% CHX digluconate, or they present a low concentration equal to or below 0.06% [20]. Research has tended towards the formulation of mouthwashes presenting lower cytotoxicity, while maintaining high antibacterial properties. To overcome the aforementioned drawbacks, mouthwashes containing low concentrations of CHX, alone or in combination with additional compounds, have been proposed [20]. Among them, cetylpyridnium chloride (CPC) seems to be particularly promising [24–28]. CPC is an amphiphilic cationic quaternary ammonium compound, whose antimicrobial activity is mainly related to its capability to bind to and destroy the bacterial cell membrane. Whereas, at low concentrations, it indirectly promotes cell autolysis through the activation of intracellular latent ribonucleases [29]. Several mouthwash formulations containing both CHX and CPC have been investigated, including solutions with CHX at low concentration, such as CHX 0.05 % + CPC 0.05 % [26,30,31] or CHX 0.03 % + CPC 0.05 % [24,25], but also at higher concentration (e.g., CHX 0.12 % + CPC 0.05 %) [32].

In a recent study by our group, a CHX 0.05 % + CPC 0.05 % was found to be effective against oral bacteria in vitro; however, limited data on its cytotoxicity are available [30]. Therefore, the aim of the present in vitro study was to investigate the effects of three mouthwashes containing CHX at different dilutions, alone or combined with CPC, on human gingival fibroblasts (HGFs) by examining cell viability, cytotoxicity, and apoptosis after 0, 3 and 6 days from the exposure. The null hypotheses were that, at the three time points, there would be no significant difference among groups in cell viability, cytotoxicity and apoptosis.

2. Materials and Methods

The current in vitro study was reported in accordance with the modified Consolidated Standards of Reporting Trials (CONSORT) guidelines [33].

2.1. Cell Culture

Two hundred eighty-eight wells were seeded with human gingival fibroblasts (HGFs), using 96-well binding plates (Costar® 9102, Corning, New York, US). As previously described [16], 5000 HGFs (HGFIB, passage 5, Provitro AG) per well were cultured for 3 days in 200 µL of high-glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, Missouri, US), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco, Invitrogen, Waltham, MA, USA) at 37 °C, 5% CO_2, and 95% humidity.

To simulate oral rinse, the culture medium was carefully aspirated, and cells were gently rinsed with phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) before treatment.

2.2. Treatment Procedure

Following cell culture, wells were randomly assigned to four different groups: (a) CPC 0.05% + CHX 0.05% (PERIO-AID® Active Control, Dentaid® GmbH) (regarded as CPC + CHX); (b) CHX 0.1% (Chlorhexamed® Fluid 0.1%, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG) (regarded as CHX 0.1); (c) CHX 0.2% (Chlorhexamed® Forte 0.2%, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG) (regarded as CHX 0.2); (d) control, i.e., sterile saline (regarded as NaCl). Three treatment times (i.e., 30 s, 60 s and 120 s) were tested in each group. The mouthwashes were removed, the wells were gently rinsed with PBS

and 200 µL nutrition medium was added (high-glucose DMEM). The measurements were conducted after 2 h (day 0), 3 days and 6 days following the treatment with mouthwashes. The culture medium was refreshed at day 3 in the 6-day groups. Before carrying out the experiments, the culture medium was removed, and the wells were gently washed with PBS. Figure 1 shows the flowchart of the study.

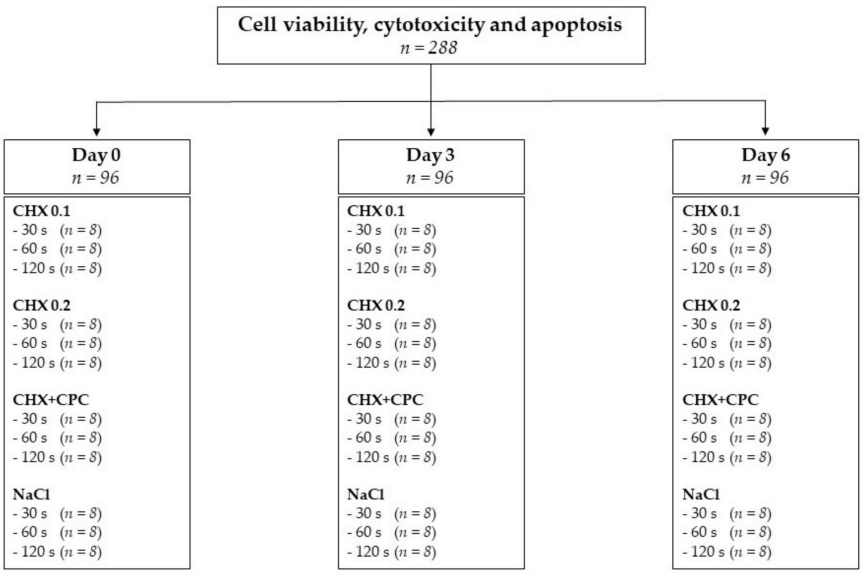

Figure 1. Flowchart of the study. The number of wells utilized for each time point and mouthwash is indicated in brackets (n =).

2.3. In Vitro Tests

Cell viability, cytotoxicity and apoptosis were measured using a single luminescence assay (ApoTox-Glo™ Triplex Assay, Promega, Fitchburg, Wisconsin, US) in a luminometer/fluorometer (Victor 2030, PerkinElmer, Waltham, Massachusetts, US). The measurements are reported in counts per second (CPS). This experiment is characterized by two consecutive phases. First, cell viability and cytotoxicity were simultaneously assessed by fluorometry, measuring two protease activities. The live-cell protease activity was measured using glycyl-phenylalanyl-amino fluorocoumarin. This is a cell-permeant peptide, which enters intact living cells, where it is converted into amino fluorocoumarin (AFC). The resulting fluorescent signal is proportional to the amount of living cells. Cytotoxicity was determined using a fluorogenic cell-impermeant peptide (i.e., bis-alanyl-alanyl-phenylalanyl- rhodamine 110). It is converted by dead-cell protease in rhodamine 110 (R100), which is released only by cells that have lost their membrane integrity. AFC and R110 were detected simultaneously, due to the different mission spectra (green and red, respectively). In the second part of the assay, analysis was performed to determine whether the investigated solutions could cause apoptosis. This was measured using a luminogenic caspase-3/7 substrate. Luminescence was proportional to the degree of caspase activity present.

2.4. Statistical Analysis

Data analysis was performed utilizing the free software R [34]. A sample of convenience was used. Boxplots were created for descriptive purposes for each selected variable. To determine the presence of any significant difference in cell viability, cytotoxicity and apoptosis among the three treatment groups per time point, a Kruskal–Wallis test with post hoc multiple comparison test with the Bonferroni method for p-value adjustment was used, and adjusted p-values were reported. A p-value < 0.05 was considered statistically significant.

3. Results

The results of cell viability, cytotoxicity and apoptosis of HGFs are presented in Figures 2–4. No sign of bacterial or fungal contamination was observed along the entire experimental period.

3.1. Cell Viability

An overview of cell viability results is presented in Figure 2. The highest values were predictably found in the NaCl group for all time points and exposure times to the mouthwashes. Against our expectations and not in line with the graph (Figure 2), no significant difference was shown between NaCl and CHX + CPC groups at day 0 (120 s), at day 3 (30 s, 60 s, and 120 s), and at day 6 (60 s and 120 s). Moreover, no significant difference was identified between NaCl and CHX 0.2 at day 6 (30 s) (Table 1). The significance level reported in Table 1 is after Bonferroni correction. The uncorrected p-values were <0.05 in all these cases but one (CPC + CHX vs. NaCl 60 s at day 6: $p = 0.07$).

After 3 days of culture, the CHX + CPC group exhibited significantly higher cell viability compared to the CHX 0.1 group for both 60 s and 120 s application times; similarly, on day 6 this was observed after a treatment time of 60 s (Table 1).

Table 1. Cell viability. A multiple Kruskal–Wallis test was performed to compare the groups at 0, 3 and 6 days. In case of significance, a post hoc test was performed, and the adjusted p-values from the post hoc test are reported here.

Grouping Variable	Comparator 1	Comparator 2	p-Value (day 0)	p-Value (day 3)	p-Value (day 6)
CHX 0.1	30 s	60 s	-	-	0.442
	30 s	120 s	-	-	0.143
	60 s	120 s	-	-	0.002 **
CHX 0.2	30 s	60 s	-	-	0.002 **
	30 s	120 s	-	-	0.143
	60 s	120 s	-	-	0.442
CHX + CPC	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
NaCl	30 s	60 s	1.000	-	1.000
	30 s	120 s	0.049 *	-	0.009 **
	60 s	120 s	0.022 *	-	0.033 *
30 s	CHX 0.1	CHX 0.2	1.000	1.000	0.420
	CHX 0.1	CHX + CPC	1.000	0.151	1.000
	CHX 0.1	NaCl	0.016 *	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.000 ***	0.001 **	0.106
	CHX + CPC	NaCl	0.007 **	0.226	0.001 **
60 s	CHX 0.1	CHX 0.2	1.000	0.373	1.000
	CHX 0.1	CHX + CPC	1.000	0.020 *	0.046 *
	CHX 0.1	NaCl	0.022 *	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	1.000	1.000	0.198
	CHX 0.2	NaCl	0.000 ***	0.010*	0.000 ***
	CHX + CPC	NaCl	0.005 **	0.226	0.420
120 s	CHX 0.1	CHX 0.2	1.000	0.092	1.000
	CHX 0.1	CHX + CPC	1.000	0.043 *	0.445
	CHX 0.1	NaCl	0.008 **	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	0.420	1.000	1.000
	CHX 0.2	NaCl	0.000 ***	0.043 *	0.003 **
	CHX + CPC	NaCl	0.054	0.092	0.079

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

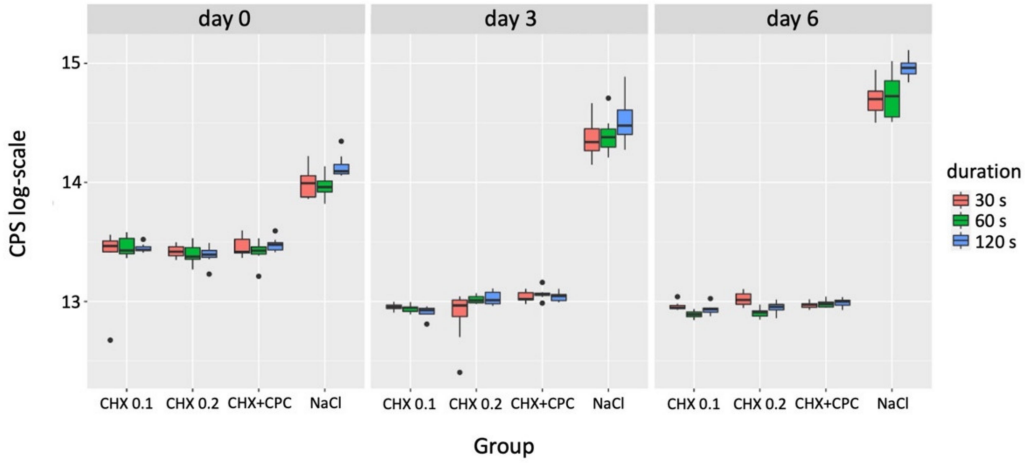

Figure 2. Overview of cell viability of HGFs after exposure to test and control mouthwashes (for 30 s, 60 s and 120 s) measured in CPS at day 0, 3 and 6.

3.2. Cytotoxicity

On day 0, the CHX 0.2 groups exhibited the highest cytotoxicity values, especially with the longest application time. Whereas, after both 3 and 6 days of culture, the highest values were registered in the NaCl group, as it clearly emerges from the boxplot (Figure 3). However, as for the cell viability assay, no significant difference was identified between NaCl and CHX + CPC groups at day 3 (30 s, 60 s and 120 s) and day 6 (60 s and 120 s). The significance level reported in Table 2 is after Bonferroni correction. The uncorrected *p*-values were <0.05 in all these cases but one (CPC + CHX vs NaCl 30 s at day 3: $p = 0.058$).

Interestingly, at day 0, after 120 s treatment time, CHX 0.2 showed significantly higher cytotoxicity not only compared to the control (NaCl) but also to the CHX + CPC group. Regarding the application time, CHX 0.2 was significantly more cytotoxic once applied for 120 s than for the short treatment time (30 s) at both day 0 and 3.

Interestingly, no significant differences could be identified on day 0 between the CHX + CPC and NaCl group for all the application times (30 s, 60 s and 120 s) (Table 2). Moreover, at day 3, significant higher values were observed in CHX + CPC than in the CHX 0.1 group after both 60 s and 120 s of treatment.

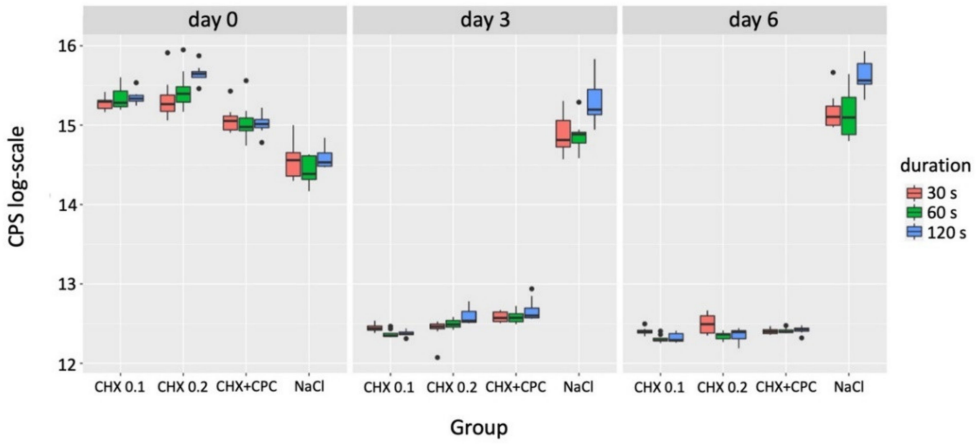

Figure 3. Overview of cytotoxicity of HGFs after exposure to test and control mouthwashes (for 30 s, 60 s and 120 s) measured in CPS at day 0, 3 and 6.

Table 2. Cytotoxicity. A multiple Kruskal–Wallis test was performed to compare the groups at 0, 3 and 6 days. In case of significance, a post hoc test was performed, and the adjusted *p*-values from the post hoc test are reported here.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (day 0)	*p*-Value (day 3)	*p*-Value (day 6)
CHX 0.1	30 s	60 s	-	0.014 *	0.030 *
	30 s	120 s	-	0.121	0.049 *
	60 s	120 s	-	1.000	1.000
CHX 0.2	30 s	60 s	1.000	0.609	0.040 *
	30 s	120 s	0.024 *	0.006 **	0.231
	60 s	120 s	0.269	0.214	1.000
CHX + CPC	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
NaCl	30 s	60 s	-	1.000	1.000
	30 s	120 s	-	0.024 *	0.027 *
	60 s	120 s	-	0.024 *	0.016 *
30 s	CHX 0.1	CHX 0.2	1.000	1.000	1.000
	CHX 0.1	CHX + CPC	0.292	0.099	1.000
	CHX 0.1	NaCl	0.000 ***	0.000 ***	0.001 **
	CHX 0.2	CHX + CPC	0.373	0.185	1.000
	CHX 0.2	NaCl	0.001 **	0.000 ***	0.033 *
	CHX + CPC	NaCl	0.257	0.351	0.002 **
60 s	CHX 0.1	CHX 0.2	1.000	0.420	1.000
	CHX 0.1	CHX + CPC	0.420	0.017 *	0.073
	CHX 0.1	NaCl	0.001 **	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	0.173	1.000	0.814
	CHX 0.2	NaCl	0.000 ***	0.008 **	0.002 **
	CHX + CPC	NaCl	0.226	0.257	0.226
120 s	CHX 0.1	CHX 0.2	0.591	0.185	1.000
	CHX 0.1	CHX + CPC	0.472	0.019 *	0.226
	CHX 0.1	NaCl	0.004 **	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	0.004 **	1.000	1.000
	CHX 0.2	NaCl	0.000 ***	0.019 *	0.002 **
	CHX + CPC	NaCl	0.591	0.185	0.141

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Apoptosis

Overall, the highest values were registered in the control group (NaCl) at all time points (Figure 4). In the control group, significant differences were detected between 120 s and the other application times in all cases but one (60 s vs. 120 s, day 3). Whereas, within each test group, the exposure time to the mouthwashes had no significant effect on HGF apoptosis at all time points (Table 3).

As above, contrary to our expectations and not in line with the graphical illustration (boxplot, Figure 4), no significant difference was detected between NaCl and CHX 0.1 groups at day 6 (30 s and 60 s), as well as between the NaCl and CHX 0.2 groups at day 0 (120 s). The significance level reported in Table 3 is after Bonferroni correction. The uncorrected *p*-values in all these cases were <0.05.

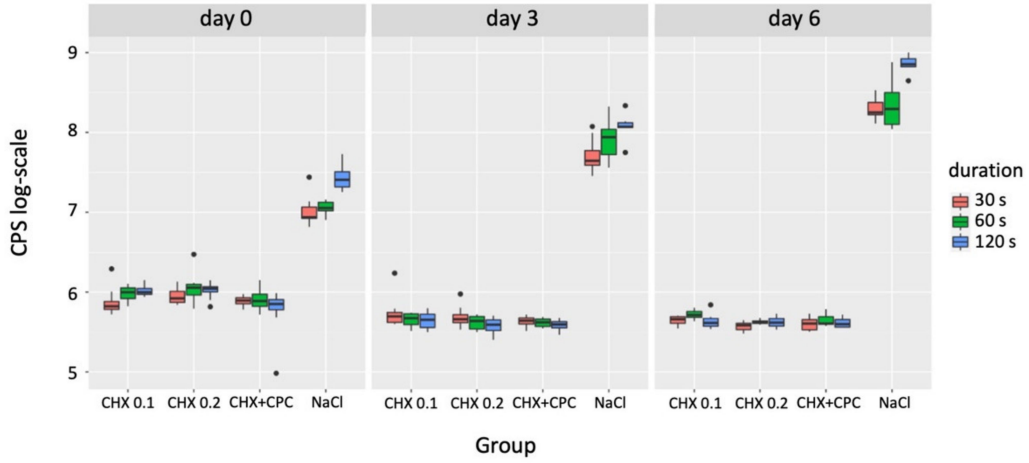

Figure 4. Overview of apoptosis of HGFs after exposure to test and control mouthwashes (for 30 s, 60 s and 120 s) measured in CPS at day 0, 3 and 6.

Table 3. Apoptosis. A multiple Kruskal–Wallis test was performed to compare the groups at 0, 3 and 6 days. In case of significance, a post hoc test was performed, and the adjusted *p*-values from the post hoc test are reported here.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (day 0)	*p*-Value (day 3)	*p*-Value (day 6)
CHX 0.1	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
CHX 0.2	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
CHX + CPC	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
NaCl	30 s	60 s	1.000	0.648	1.000
	30 s	120 s	0.002 **	0.006 **	0.004 **
	60 s	120 s	0.011 *	0.183	0.009 **
30 s	CHX 0.1	CHX 0.2	0.813	1.000	0.623
	CHX 0.1	CHX + CPC	1.000	1.000	1.000
	CHX 0.1	NaCl	0.000 ***	0.016 *	0.065
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.048 *	0.004 **	0.000 ***
	CHX + CPC	NaCl	0.004 **	0.001 **	0.003 **
60 s	CHX 0.1	CHX 0.2	1.000	1.000	0.291
	CHX 0.1	CHX + CPC	1.000	1.000	0.444
	CHX 0.1	NaCl	0.007 **	0.023 *	0.185
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.028 *	0.002 **	0.000 ***
	CHX + CPC	NaCl	0.000 ***	0.001 **	0.000 ***
120 s	CHX 0.1	CHX 0.2	1.000	1.000	1.000
	CHX 0.1	CHX + CPC	0.248	1.000	1.000
	CHX 0.1	NaCl	0.032 *	0.024 *	0.004 **
	CHX 0.2	CHX + CPC	0.167	1.000	1.000
	CHX 0.2	NaCl	0.052	0.001 **	0.005 **
	CHX + CPC	NaCl	0.000 ***	0.002 **	0.003 **

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

The purpose of the present in vitro study was to assess the possible effects of three commercially available CHX-based mouthwashes on human gingival fibroblasts (HGFs). The null hypotheses were that there would be no significant difference among the groups in cell viability, cytotoxicity and apoptosis. They were partially rejected, and the corresponding alternatives were accepted.

Cell viability, as expected, was generally higher in the saline control group (NaCl) as compared to the use of mouthwashes, with similar values among the latter at all time points. Furthermore, cell viability was found not to be influenced by mouthwash application time in the large majority of cases.

Insights into the cellular death mechanisms provoked by the different treatment procedures were also uncovered. Despite the modifications of the plasma membrane in the final stages of apoptotic cell death, the rupture and the integrity of the cell membrane are generally considered as main features of necrosis and apoptosis, respectively [35,36].

Cytotoxicity was analyzed by measuring a proteolytic biomarker dependent on cell membrane disruption. In agreement with cell viability findings, at day 0, all the mouthwashes presented higher values as compared to the control. Interestingly, at this time point, CHX 0.2 exhibited significantly higher cytotoxicity compared to the CHX + CPC group after the long application time (120 s) and was significantly more cytotoxic once applied for 120 s than for the short treatment time (30 s). Moreover, at day 0, no statistically significant differences could be observed between CHX + CPC and the control group for all the application times. By contrast, at day 3, NaCl showed the highest cytotoxicity; significant differences were also observed between CHX + CPC and CHX 0.1 groups after both 60 s and 120 s of treatment. It can be speculated that CHX-based mouthwashes induced HGF death immediately after exposure, when the phenomenon could clearly be observed. Since most of the cells were likely to be dead in the early phases after the contact with the mouthwashes, in particular at higher CHX percentages, their cytotoxicity values drastically decreased already at day 3. Whereas, in the NaCl group, a balance between living and dead cells was maintained up to day 6 of culture.

Caspases are deemed to be responsible for the proteolytic cleavages leading to cell disassembly, which is typical of apoptosis [37]. Therefore, a luminescent assay measuring the activity of two effector caspases, which are expressed and activated in apoptotic cells, was here utilized. In accordance with a previous investigation of our team [16], the control group exhibited significantly higher values of apoptosis as compared to the test groups. The predominant cytotoxic action exerted by the mouthwashes could be an explanation for the low apoptosis values. Although optimal culture conditions were provided through constant cell coverage by culture medium, in the NaCl group, apoptosis values tended to increase over time; this might be ascribed to environmental stress, which can result from changes in cell density, nutrient depletion, or waste product accumulation [38].

For all the selected parameters, changes were mainly observed between day 0 and day 3, while the values remained almost unmodified from day 3 to day 6. As the majority of the events took place in the early phases after mouthwash application, it would be interesting to map and quantify these dynamic processes in real time and over time by means of live cell imaging [39,40].

Mouthwashes are widely used concomitant with periodontal and peri-implantitis treatments [8,41,42]. As these pathological conditions are associated with bacterial biofilm formation, antimicrobial properties are of major importance for supragingival plaque control [11,43]. Owing to its well-documented antibacterial activity, CHX is frequently employed to reduce oral bacterial load [20]. However, mouthwashes containing a high percentage of CHX have been associated with cytotoxic effects in vitro [12,16,44,45]. Therefore, usage of lower concentrations of CHX in combination with CPC have been proposed [24–28]. In a recent in vitro study by our group, a CHX 0.05% + CPC 0.05% mouthwash was revealed to be effective against oral living bacteria after in situ plaque accumulation, showing similar properties as comparted to CHX 0.1% solution [30]. Furthermore, utilizing the same study

design of the present work, the authors found the highest cytotoxicity on osteoblast-like cells at day 0 in the CHX 0.2% group, which also presented significantly higher values compared to CHX 0.05% + CPC 0.05% for all the application times [31]. Due to the limited data available on the cytotoxicity on fibroblasts of the former, the current work was conceived as a complementary study in support of our recent investigations [30,31]. In addition to the NaCl and CHX 0.1 groups, it was decided to add the CHX 0.2 group, as it still represents a commonly used solution, in particular after periodontal and peri-implantitis surgeries. In such cases, the protective epithelial barriers would no longer inhibit the direct contact between the connective tissue and the mouthwash; therefore, the cytotoxicity of the antiseptic agent should be carefully considered [11,13].

A limitation of this study is that it was based on monolayer cultures. Two-dimensional (2D) cell cultures were chosen due to the relatively easy environmental control and cell observation, which allow for the minimizing of measuring errors. Flat cultures are, indeed, considered particularly suitable for preliminary toxicity tests, but three-dimensional (3D) human oral mucosal models might be considered for further studies, due to their closer resemblance to the complex in vivo tissue microenvironment [46–49]. As the oral mucosa is characterized by multiple layers, in 3D models the direct contact of the fibroblasts with the mouthwashes can be avoided, better mimicking the tissue permeability of the agents through the epithelial outer layer [47]. This would be particularly relevant for translating the data to the chronic usage of mouthwashes, which might affect oral mucosa health in the long term [50].

Our results mark out a starting point for future clinical investigations, aiming at understanding not only the impact of different mouthwashes, but also the influence of different rinsing regimens on oral mucosa health and periodontal and peri-implant disease control.

In summary, the results obtained in the present study showed that the three tested CHX-based mouthwashes had similar effects on in vitro HGF viability, thus rejecting the null hypotheses that assumed no significant differences per time point and variable. At day 0, the CHX + CPC group presented milder cytotoxic effects as compared to the CHX 0.2 group after an application time of 120 s. In addition, at this time point, no significant differences could be identified between the CHX + CPC and the NaCl control group for all the application times, thus confirming the relatively moderate cytotoxicity of the CHX 0.05% + CPC 0.05% mouthwash.

Although extrapolating in vitro data to predict side effects in patients remains difficult, rinsing regimens should be carefully considered by the clinicians balancing the risks of cytotoxicity and the required antimicrobial effect in specific clinical circumstances.

High concentration of CHX might have detrimental effects on oral mucosa, not only in the case of prolonged usage, but also when applied directly in contact with connective tissues during wound healing.

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