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# Cytotoxicity of chlorhexidine-hydrogen peroxide combination in different concentrations on cultured human periodontal ligament fibroblasts

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## ABSTRACT

**Background:** A strong antimicrobial synergism between chlorhexidine (CHX) and hydrogen peroxide ($H_2O_2$) has been reported, but there is not enough data on the cytotoxicity of this combination. The primary aim of this study was to evaluate the cytotoxicity of CHX-$H_2O_2$ combination in different concentrations and secondary aim is to assess the influence of $H_2O_2$ on cytotoxicity of CHX on cultured human periodontal ligament (PDL) fibroblasts.

**Materials and Methods:** The PDL cells were cultured from healthy human third molar teeth and were exposed to six prepared solutions (0.2% and 2% CHX separately and in combination with 1% and 3% $H_2O_2$). The MTT assay was applied to assess their effects on the viability of the PDL cells. Two-way analysis of variance approach and subgroup analysis was performed to evaluate the differences in mean cell viability values. A level of $P < 0.05$ was considered as statistically significant.

**Results:** All tested solutions were toxic to PDL cells. There was a significant interaction effect between CHX and $H_2O_2$. The 2% CHX combined with 3% $H_2O_2$ was the most and 0.2% CHX was the least cytotoxic solutions. The 2% CHX was significantly more toxic than 0.2% CHX and $H_2O_2$ combinations. The cytotoxicity of 0.2% CHX and $H_2O_2$ combinations did not significantly rise by increasing the concentration of $H_2O_2$ from 1% to 3%.

**Conclusion:** $H_2O_2$ affected the cytotoxicity of CHX in a variable concentration-dependent manner. Based on the results of this study, it can be concluded that 2% CHX alone and in combination with either 1 or 3% $H_2O_2$ are significantly more toxic than 0.2% CHX alone and in combination with 1 and 3% $H_2O_2$. Therefore, to benefit from the synergistic antimicrobial effect between CHX and $H_2O_2$, with a minimal cytotoxicity, it is recommended to use 0.2% concentration of CHX combined with 3% $H_2O_2$.

**Key Words:** Chlorhexidine, cytotoxicity, hydrogen peroxide, root canal irrigation solution

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## INTRODUCTION

The intra-radicular microbial biofilms are known to be the major etiologic factor for the periapical diseases.[1] Irrigation solutions can minimize the microorganisms and help to remove the debris, owing to their flushing action. They may also have antibacterial activity and dissolving properties for organic or inorganic tissues.[2] The fact that the antimicrobial properties of the irrigants improve in higher concentrations is clearly known.[3-5] On the other hand, many of these solutions are established to have cytotoxicity when they come in contact with vital tissues.[6,7] Furthermore, severe inflammatory responses can be induced if they extrude to the periapical area.[8] Therefore, an optimal irrigation solution should be effective in bacterial reduction and be non-toxic to

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periodontal tissues. Currently, none of the available solutions can be regarded as an ideal irrigant individually. Therefore, efforts have been made to find an efficient combination of solutions with optimal antimicrobial activity and minimal cytotoxic effects.

Chlorhexidine (CHX) has gained increasing popularity as an irrigation solution because of its substantive and acceptable antimicrobial properties.[9,10] However, its activity is diminished in the presence of organic components.[11] Besides, it has little to no ability to dissolve organic and inorganic tissues.[12] Hydrogen peroxide ($H_2O_2$) is also another antimicrobial agent, which has been used as an irrigation solution for a long period of time. It is effective against bacteria, viruses and yeasts. Although its antibacterial effectiveness is considered weak, it acts on the organic tissues and enhances the effectiveness of the other disinfectants.[13]

A strong antimicrobial synergism between CHX and $H_2O_2$ has been reported by Heling and Chandler.[14] Likewise, Steinberg *et al*.[15] confirmed this synergistic effect and found that this combination could eradicate *Enterococcus faecalis* in lower concentrations compared with the instances that they were used alone. The anti-plaque inhibitory effect of this combination is also reported by Dona *et al*.[16] Apart from these positive reports of synergistic antibacterial activities, there is no published report evaluating their cytotoxicity yet.

The aim of this study was to evaluate the cytotoxicity of CHX-$H_2O_2$ combinations and to assess the influence of $H_2O_2$ on cytotoxicity of CHX in different concentrations on cultured human periodontal ligament (PDL) fibroblasts.

## MATERIALS AND METHODS

The PDL cells used in this study were cultured from the healthy human third molar teeth. The isolation and preparation of the primary cell cultures were accomplished with the method described by Mailhot *et al*.[17] By this means, the freshly extracted teeth were washed in a sterile saline solution to eliminate the residual blood. The PDL samples were scraped with a sterile scalpel and the scrapings were placed into a 35-mm culture dish. The explants were incubated with Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum plus penicillin 100 u/ml, streptomycin 100 mg/ml and amphotericin 2.5 μg/ml. The culture dishes were incubated at 37°C in an atmosphere of 5% $CO_2$. During a week of incubation, cells were fed every day until they reached confluence. For sub-cultivation, cells were detached by Trypsin treatment and were passage into 25 $cm^2$ tissue culture flasks. When the cultures reached confluence, they were transferred to 75 $cm^2$ flasks. Experiments with PDL fibroblasts were conducted by using cells between 3$^{th}$ and 5$^{th}$ passages.

The particular concentrations for CHX (SIGMA; St. Louis, MO, USA) and $H_2O_2$ (Merck, Darmstadt, Germany) were as follows: 0.2% and 2% CHX individually and in combination with 1% and 3% $H_2O_2$. Culture medium was served as a control. The effects of these six solutions on the mitochondrial function were measured by a colorimetric assay. This assay measures the function of active mitochondria in converting yellow water – soluble MTT dye into an insoluble purple formazan product. For this purpose, the MTT solution (SIGMA; St. Louis, MO, USA) was prepared in 5 mg/ml phosphate-buffered saline and filtered through a 0.22 μm pore size filter. PDL cells were seeded 20,000 cells per well into 96-well culture plates. After 24 h, cells were exposed to particular concentrations of each experimental solution for 15 min and 10 μL of MTT solution was added to each well. The media were discarded by overturning the plates and adding 100 μl of dimethyl sulfoxide (Merck, Darmstadt, Germany) to each well. The spectrophotometric absorbance at 540 nm was measured by an enzyme-linked immunosorbent assay reader (Awareness Stat Fax 3200, USA).

The mean optical density (OD) values of three wells containing the same extract with their standard deviations were calculated. The mean cell viability defined as the percentage of the mean OD values compared with the OD value of control (OD value of control was 0.92). Two-way analysis of variance (ANOVA) approach and subgroup analysis was used to evaluate the differences in the mean cell viability values of the experimental solutions. For the purpose of subgroup analysis, one-way ANOVA/least significant difference (LSD) tests were used. A level of $P < 0.05$ was accepted as statistically significant.

## RESULTS

All tested solutions were toxic to PDL cells. The two-way ANOVA test showed a significant interaction effect between CHX and $H_2O_2$ ($P < 0.001$) [Figure 1]. The subgroup analysis based on the one-way ANOVA/LSD tests showed that the mean of OD for 0.2% CHX was significantly higher than other

solutions [Table 1]. The combination of 2% CHX and 3% $H_2O_2$ had the most negative effect on the cell viability [Table 1]. The cytotoxicity of 0.2% CHX and $H_2O_2$ combinations did not significantly rise by increasing the concentration of $H_2O_2$ from 1% to 3% ($P$ = 0.121). The sequences of cytotoxicity for the tested irrigation solutions are summarized in Table 2.

## DISCUSSION

Currently, none of the available irrigation solutions are regarded as an ideal choice. Therefore, many publications have suggested using combination of irrigants to benefit from the combined advantages of them while minimizing their side-effects. Adding $H_2O_2$ to CHX may facilitate cleaning the pulp chamber from the tissue remnants, reduce the side effect of teeth-staining and increase the antimicrobial efficiency.[18] The mechanism of this antimicrobial synergism is not clearly understood, but it can be speculated that CHX may make the bacterial membrane more permeable to $H_2O_2$ causing more damage to the intracellular components.[15] Meanwhile, the influence of $H_2O_2$ on cytotoxicity of CHX has remained unknown and should be investigated.

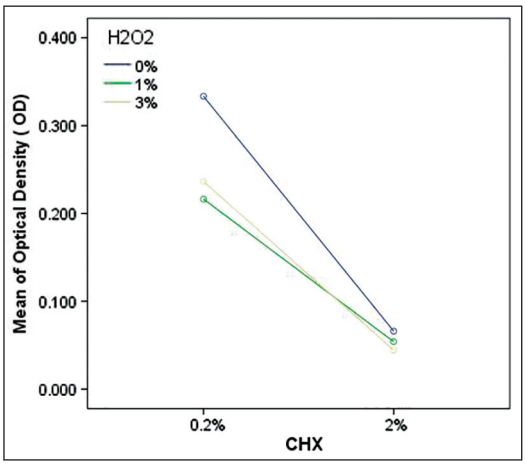


**Figure 1:** The interaction effect between chlorhexidine and hydrogen peroxide on the viability of the periodontal ligament cells

**Table 1: Mean and standard deviation of optical density and percentage of cell viability for the experimental groups**

| $H_2O_2$ % | CHX | |
|---|---|---|
| | 0.2% (%) | 2% (%) |
| 0 | 0.33±0.01 (36.2) | 0.07±0.00 (7.17) |
| 1 | 0.22±0.02 (23.48) | 0.06±0.01 (5.87) |
| 3 | 0.24±0.02 (25.65) | 0.04±0.01 (4.78) |

CHX: Chlorhexidine

The MTT cell proliferation assay is usually used to measure the cell proliferation rate, but it can also evaluate the reduction in cell viability. This assay was used in this study, since it has been considered as an accurate and sensitive index for evaluating the cytotoxicity of the irrigants. Furthermore, it does not need a washing step that could cause an unknown variation in the samples.[19] The reason for selecting an exposure time of 15 min was to simulate the clinical situation.

Our results showed that combining $H_2O_2$ with CHX could increase the cytotoxicity of CHX but this effect had a variable concentration-dependent manner.

We found a dissimilar toxic behavior for 0.2% and 2% CHX when mixed with $H_2O_2$. The cytotoxicity of 0.2% CHX was significantly increased when it was combined with both 1% and 3% $H_2O_2$, while the toxicity of 2% CHX was significantly elevated only by mixing with 3% $H_2O_2$. This difference in behavioral toxicity can be rational since the toxicity of 2% CHX was significantly much more than 0.2% CHX even when it was used alone.

CHX in 0.2% concentration had significantly lower cytotoxicity than 2% CHX and other combined solutions. Faria *et al*.[20] demonstrated that CHX at concentrations of 0.25, 0.5 and 1% could cause foci of necrosis in the paws of mice proportional to the concentration. They also documented their findings by testing CHX on cultured L929 fibroblasts. These conclusions were in line with the results of our study which indicated that the toxicity of CHX is dose-dependent.

Heling and Chandler[14] revealed that using 0.2% CHX in combination with $H_2O_2$ had more antibacterial effectiveness than 0.2% CHX alone. They also demonstrated that mixing 1.8% CHX and 3% $H_2O_2$ have similar antimicrobial efficiency to those achieved by using 0.2% CHX followed by 3% $H_2O_2$.

**Table 2: Order of toxicity for the test solutions based on the significant pairwise comparisons**

| Order of toxicity | I | II | III | IV |
|---|---|---|---|---|
| Solution (s) | 2% CHX+3% $H_2O_2$ | 2% CHX and 2% CHX+1% $H_2O_2$ | 0.2% CHX+1% $H_2O_2$ and 0.2% CHX+3% $H_2O_2$ | 0.2% CHX |

CHX: Chlorhexidine

On the other hand, our results revealed that the combination of 2% CHX with 3% $H_2O_2$ was the most toxic solution tested. Besides, we also found that the cytotoxicity of 0.2% CHX and $H_2O_2$ combination did not significantly elevate by increasing the concentration of $H_2O_2$ from 1% to 3%. All in all, to benefit from the synergistic effect of combining CHX and $H_2O_2$ with minimal cytotoxicity, it is suggested to employ 0.2% instead of 2% CHX in combination with 3% $H_2O_2$. The clinical significance of these results, however, should be evaluated further in clinical investigations. Furthermore, it is not thoroughly known whether the organic or inorganic materials of the root canal system can affect the behavior and the concentrations of irrigation solutions in contact with vital periodontal tissues during root canal treatment.

## CONCLUSION

Under the condition of this study, it can be concluded that 2% CHX alone and in combination with either 1 or 3% $H_2O_2$ are significantly more toxic than 0.2% CHX alone and in combination with 1 and 3% $H_2O_2$. To benefit from the synergistic effect of combining CHX and $H_2O_2$ with minimal cytotoxicity, it is recommended to use 0.2% concentration of CHX combined with 3% $H_2O_2$.

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