THE CYTOTOXIC AND APOPTOTIC-NECROTIC EFFECTS OF WHITENING MATERIALS ON HUMAN GINGIVAL FIBROBLASTS

ABSTRACT

Background and Aim: Carbamide peroxide (CP) and low concentrations of hydrogen peroxide ($H_2O_2$) have been used as bleaching agents for discolored vital teeth. Long-term exposure to these whitening materials (WMs) may cause irritation and ulceration of the gingiva and other oral soft tissues. The aim of this study was to investigate cytotoxicity and mechanisms of the cytotoxic effects of these WMs on human gingival fibroblasts (HGFs).

Materials and Methods: HGFs were exposed to whitening materials with various dosages ranging from 0.001 to 10 mmol/L and then they were processed for analyzing MTT and DNA fragmentation. In addition, possible mechanism of the whitening-material-mediated cytotoxicity was investigated through flow cytometric analysis.

Results: The exposure of human gingival fibroblasts (HGFs) to $H_2O_2$ and CP in vitro inhibited cell growth in a dose-dependent manner ($p<0.001$ and $p<0.001$ at 24 hours (h), respectively). Cytotoxicity was found to occur due to $H_2O_2$-induced chelation of calcium as shown by control experiments with the calcium chelator ($p<0.001$). Addition of a competitive inhibitor of nitric oxide synthase modulated $H_2O_2$-induced cytotoxicity ($p<0.001$).

Conclusion: The results of the present study indicate that these WMs show toxic effects on HGFs, in vitro. Apoptosis was the main form of cell death due to these WMs and their effects were evaluated via several mechanisms. The direct bleaching procedure that cures these materials in situ should be used cautiously.

Key words: Apoptosis, Cytotoxicity, Gingival Fibroblasts, Necrosis, Whitening Materials.
INTRODUCTION

The most widely used whitening materials (WMs) are 2-10% solution of hydrogen peroxide ($\text{H}_2\text{O}_2$) in water or 10-15% carbamide peroxide (CP), which readily generates hydrogen peroxide. A 10% CP solution decomposes to 3.62% $\text{H}_2\text{O}_2$ and 6.38% urea. These WMs are applied in a tray worn through the night or for limited periods during the day. The reported results, concerning the whitening ability of $\text{H}_2\text{O}_2$ and CP have generally been positive; however, dentists have reported gingival irritation in some of their patients using these agents. One clinical study reported gingival irritation and burning of oral soft tissues after using two different CP whitening materials. Other studies, in contrast, have reported no adverse effects of WMs on oral soft tissues. Moreover, several studies reported the toxic effects of $\text{H}_2\text{O}_2$ and CP to gingival connective tissue. One study reported that a $\text{H}_2\text{O}_2$-generated WM was toxic to human gingival fibroblasts (HGFs) in vitro, causing morphological changes, cell death, and inhibition of proliferation and production of collagen and fibronectin. However, morphological changes and cell death mechanisms of $\text{H}_2\text{O}_2$-generated WMs on HGFs are not completely clear.

The purpose of this study was to determine cytotoxicity and to further investigate the cytotoxic effect mechanisms of $\text{H}_2\text{O}_2$ and a 10% CP solution (decomposing to 3.62% $\text{H}_2\text{O}_2$ and 6.38% urea) on HGFs. Our hypothesis was that the mechanisms of cell death by apoptosis and/or necrosis, induced by $\text{H}_2\text{O}_2$, could be an important indicator of cytotoxicity.

MATERIAL AND METHODS

**Tissue Culture**

HGFs were obtained from patients with healthy gingiva, who underwent oral surgery at Hacettepe University, Faculty of Dentistry for the purpose of removing impacted wisdom teeth. Informed consent based on an appropriate protocol was obtained from the donors. The gingival tissues were cut into 1 to 2 mm³ pieces, and then washed twice with Hanks' salt solution. Thereafter, the cut biopsies were placed into 25 cm² tissue culture flasks. The specimens were incubated with culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10 mM HEPES, glucose (4.5 g/L), NaHCO$_3$ (3.7 g/L), penicillin (100 U/ml), and streptomycin (100 mg/ml) (GIBCO, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal calf serum. The tissue samples were grown at 37°C in a humidified atmosphere of 5% carbon dioxide in air. When the outgrowth of cells was observed, the medium was replaced twice weekly until cells reached confluence. Cells were detached from the monolayer by a brief treatment with trypsin-EDTA (0.25% trypsin, 0.02% EDTA), and re-cultured in 75-cm² tissue flasks until confluent monolayers were re-obtained. Cell counts before plating revealed 95% to 98% cell viability determined by the trypan blue exclusion test. In all experiments, cells grown to a monolayer at the fourth or seventh passage were used. Unless otherwise specified, all chemicals were of cell culture grade and obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

**Trypan Blue Dye Exclusion and MTT Assay**

HGFs were resuspended in medium at $1 \times 10^6$ cells/ml after verifying cell viability by the trypan blue dye exclusion assay. The MTT (3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide) assay determines the activity of mitochondrial dehydrogenase, which is used as an indicator of cell viability. For in vitro evaluation of the cytotoxicity of bleaching agents, MTT assay was performed as previously described. Cell suspension (100 µL) was distributed into each well of 96-well microtiter plates (Costar Co., Cambridge, MA, USA), and each plate was incubated for 24 hours (h). Thereafter, 100 µL of reagent solutions at the desired concentrations were distributed into each well and treated for 24h. Wells containing 200 µL of medium alone without cells were used as the negative control and those containing 200 µL of medium and cells were used as the positive control. Following the treatment for the stated incubation times, 20 µL of MTT solution (5 mg/ml) was added to each well, and the microplates were further incubated at 37°C for 4h. The unreactive supernatants in the well were then discarded, and acidified isopropanol (100 µL) (0.04 N HCl in isopropanol) was added to the cultures and mixed thoroughly to help dissolve the dark blue crystals of formazan. The absorbance values of each well were determined with a microplate ELISA reader equipped with a 570 nm filter. The negative control well was used for the baseline zero absorbance. Results are presented as percent cytotoxicity determined by the pertinent formula (percentage of viable cells = $A/B \times 100$, where $A$ is viable cells in the experimental well and $B$ is viable cells in the positive control well).

**Assessment of Apoptosis on Gingival Fibroblast Cells by Annexin V/PI Double- Staining Assay**

To confirm apoptosis induction, annexin V and propidium...
iodide (PI) double staining was performed using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer’s instructions (Cat. No. 556420, BD Biosciences Pharmingen, USA). HGFs were cultured in 24-well costar plates at a density of 1.5 x 10^4 – 2x10^4 cells/cm² in 1.5 ml of cultured medium. Cells treated with 50 µmol (50% inhibitory dose- ID_{50}) H₂O₂ for 24h were harvested and washed twice with cold phosphate buffered saline (PBS) and then cells were resuspended in 1 x binding buffer at a concentration of ~ 1 x 10^6 cells/mL. Binding buffer 100 ml with 4 ml annexin V was added to the cells and incubated for 15 min in the dark. Each sample was also incubated with 4 ml PI for 15 min in the dark. The cells stained with annexin V FITC were analyzed with a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA) using CellQuest Software, which was also used to determine the percentage of apoptotic and/or necrotic cells.

**Cell Cycle Analysis**

PI analysis was performed as described previously. Briefly, HGFs were washed once in PBS and then stained with PI using a commercial kit (Cycle Test Plus, DNA Reagents Kit, Becton Dickinson, San Jose, CA, USA) for 1.0 min at 4°C in the dark and cells were analyzed on FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Apoptotic cells were detected as a hypodiploid population. Analysis was carried out on three separate experiments.

**Determination of Apoptosis in Stained Slides**

Cells obtained from the cell cycle analysis described above were also used to generate cytopreparations on slides that were subsequently stained with Giemsa. Apoptotic cells were defined on cytoplasmic blebbing and nuclear morphology, including condensation of chromatin, and necrotic cells were defined on fragmented nuclei. The percentage of apoptotic and/or necrotic cells was determined in a total of 500 cells per experimental group, and each experiment was repeated on three occasions.

**DNA Isolation and Fragmentation Analysis**

Cellular DNA was extracted from control and H₂O₂-applied HGFs, precipitated and dissolved as described previously with some modifications. Briefly, control and H₂O₂-applied HGFs were collected by centrifugation at 800 rpm for 5 min at room temperature. All cells were then treated with 3 ml lysis buffer (400 mM NaCl, 10 mM Tris-HCl, 2 mM EDTA, pH 8.0), 200 µl 10% SDS and 150 µl proteinase K (1.0 mg/ml), mixed gently and incubated overnight at 37°C. The next day 1 ml of ammonium acetate (saturated) was added to the mixture and incubated for 15 min at room temperature. Then the mixture was centrifuged at 4000 rpm for 30 min at room temperature. Supernatant was transferred into another tube and 2.5 volume of absolute ethanol was added. DNA was dissolved in TE buffer (pH 8.0). Extracted DNA fragments were electrophoretically separated on 2% agarose gel containing ethidium bromide (0.5 µg/ml) in TAE buffer at 90 V for 1 h. DNA samples were visualized in an UV transilluminator.

**Statistical Analysis**

The Student’s two-tailed t-test was used to determine statistical significance of detected differences and a value of p<0.05 was considered statistically significant.

**RESULTS**

H₂O₂- and CP-Induced Cytotoxicity in HGFs

The possible effects of H₂O₂ and CP on the viability of HGFs were determined using MTT assay. As shown in Figure 1a, H₂O₂ and CP treatment resulted in a dose-dependent reduction in survival of HGFs (Statistical evaluations were performed according to the comparison of control and ID_{50}, p<0.001 and p<0.001 at 24h, respectively) (Table 1). Furthermore, H₂O₂ and CP treatment also resulted in a dose-dependent increase in the number of detaching cells from the surface of the culture dish. Approximately 50% of the cellular viability (ID_{50}) was affected when 0.048 mmol/L H₂O₂ and 0.135 mmol/L CP were used.

The Apoptotic Pathway Induced by ID50 of H₂O₂

The observed cytotoxicity in HGFs was not directly due to the removal of free calcium from the culture medium. As shown in Figure 1b, ethylene-glycol-bis (β-amoethylether) N,N,N,N’-tetraaceticacid (EGTA) alone at 100 µM had no effect on HGFs. Addition of 100 µM EGTA to both ID₅₀ and 0.1 mmol/L H₂O₂ resulted in a significant decrease in cytotoxicity (Figure 1b) (Table 2; p<0.0001) for both concentrations of H₂O₂ in HGFs rather than an enhanced cytotoxicity, which one would expect if the H₂O₂ was working via chelation of calcium. A decrease in extracellular calcium concentration actually protected HGF cells from the cytotoxic effect of H₂O₂. To determine whether stimulation of HGFs with H₂O₂ induces apoptosis, and if so, whether H₂O₂ stimulated expression of nitric oxide synthase is involved in this process, HGFs were treated with both ID₅₀ and 0.1 mmol/L H₂O₂ in the presence or the absence of 400 µM of Nω-nitro L-arginine methyl ester (L-NAME), a
competitive inhibitor of nitric oxide synthase. The addition of L-NAME could prevent the H$_2$O$_2$-induced death of HGFs (Table 3; p<0.0001 for both concentrations of H$_2$O$_2$ in HGFs) (Figure 1c). Staurosporine is widely used as a protein kinase inhibitor in several cells. A significant decrease in the number of apoptotic cells was detected when protein kinase C (PKC) was inhibited by the simultaneous addition of 1nM staurosporine to cultures containing both ID$_{50}$ and 0.1 mmol/L H$_2$O$_2$, respectively (Table 4; p<0.001 and p<0.0001, respectively) (Figure 1d).

**Table 1.** Statistical comparison of H$_2$O$_2$ and CP on fibroblasts.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>H$_2$O$_2$ Mean±SD</th>
<th>CP Mean±SD</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>0.001</td>
<td>-1.92±6.15</td>
<td>2.10±4.21</td>
<td>NS</td>
</tr>
<tr>
<td>0.01</td>
<td>3.11±5.24</td>
<td>-1.33±6.77</td>
<td>NS</td>
</tr>
<tr>
<td>0.025</td>
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<td>4.41±7.94</td>
<td>0.010</td>
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<tr>
<td>0.05</td>
<td>53.42±10.99</td>
<td>16.17±7.98</td>
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</tr>
<tr>
<td>0.1</td>
<td>82.25±10.18</td>
<td>36.83±8.97</td>
<td>&lt;0.001</td>
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<tr>
<td>0.2</td>
<td>89.91±9.63</td>
<td>78.08±10.27</td>
<td>NS</td>
</tr>
<tr>
<td>0.5</td>
<td>92.33±7.29</td>
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<td>NS</td>
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<td>1</td>
<td>95.25±8.89</td>
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</tr>
<tr>
<td>10</td>
<td>94.66±6.86</td>
<td>97.67±5.21</td>
<td>NS</td>
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**Table 2.** Statistical comparison of H$_2$O$_2$ and 100µM EGTA on fibroblasts.

<table>
<thead>
<tr>
<th>H$_2$O$<em>2$ (ID$</em>{50}$)</th>
<th>400LNAME</th>
<th>H$_2$O$_2$ (0.1 mM) + 400LNAME</th>
<th>400LNAME</th>
<th>H$_2$O$_2$ (0.1 mM) + 400LNAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>H$_2$O$<em>2$ (ID$</em>{50}$)</td>
<td>50.0±7.2</td>
<td>11.1±1.7</td>
<td>12.5±2.1</td>
<td>8.7±5.9</td>
</tr>
<tr>
<td>400LNAME</td>
<td>90.0±6.4</td>
<td>9.8±5.4</td>
<td>9.8±5.4</td>
<td>6.3±3.4</td>
</tr>
<tr>
<td>H$_2$O$_2$ (0.1 mM)</td>
<td>87.2±5.9</td>
<td>9.8±5.4</td>
<td>8.7±5.9</td>
<td>6.3±3.4</td>
</tr>
<tr>
<td>400LNAME</td>
<td>90.0±6.4</td>
<td>9.8±5.4</td>
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<tr>
<td>400LNAME</td>
<td>90.0±6.4</td>
<td>9.8±5.4</td>
<td>9.8±5.4</td>
<td>6.3±3.4</td>
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</tbody>
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* Comparison of H$_2$O$_2$ (ID$_{50}$) + 1000EGTA
** Comparison of H$_2$O$_2$ (0.1 mM) + 1000EGTA

Figure 1. (a) Cytotoxic effects of H$_2$O$_2$ and CP on fibroblasts. HGFs were cultured with increasing concentrations of H$_2$O$_2$ and CP for 24 hours and assayed by MTT assay for determination of percentage cytotoxicity. The data depict the mean ± SD of 3 separate experiments. (b) Effect of calcium chelation from the culture medium by EGTA. EGTA at 100µM had no effect on HGFs. Addition of 100µM EGTA to ID$_{50}$ (white column) and 0.1 mM (gray column) concentrations of H$_2$O$_2$ resulted in a significant decrease in cytotoxicity at 24 hours (p<0.001 and p<0.0001, respectively). (c) Effect of the nitric oxide synthase inhibitor L-NAME. L-NAME at 400µM had no effect on HGFs. Addition of 400µM L-NAME to cultures containing ID$_{50}$ (white column) and 0.1 mM (gray column) concentrations of H$_2$O$_2$ resulted in a significant decrease in cytotoxicity at 24 hours (p<0.001 and p<0.0001, respectively). (d) Effect of protein kinase C inhibition by staurosporine. Staurosporine at 1nM had no effect on HGFs. Addition of 1nM staurosporine to cultures containing ID$_{50}$ (white column) and 0.1 mM (gray column) concentrations of H$_2$O$_2$ resulted in a significant decrease in cytotoxicity at 24 hours (p<0.001 and p<0.0001, respectively).
Table 3. Statistical comparison of H2O2 and 400µM L-NAME on fibroblasts.

<table>
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<th>Condition</th>
<th>% Cytotoxicity</th>
<th>Mean</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O2 (ID50)</td>
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<td>50</td>
<td>7.2</td>
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<tr>
<td>400LNAME</td>
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<td>11.1</td>
<td>1.7</td>
<td>NS*</td>
</tr>
<tr>
<td>H2O2 (ID50) + 400LNAME</td>
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<td>2.1</td>
<td></td>
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<tr>
<td>H2O2 (0.1 mM)</td>
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<td>87.2</td>
<td>5.9</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>400LNAME</td>
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<td>9.8</td>
<td>5.4</td>
<td>NS**</td>
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<tr>
<td>H2O2 (0.1 mM) + 400LNAME</td>
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<td>13.6</td>
<td>6.3</td>
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</table>

* Comparison of H2O2 (ID50) + 400LNAME
** Comparison of H2O2 (0.1 mM) + 400LNAME

Table 4. Statistical comparison of H2O2 and 1nM staurosporine on fibroblasts.

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Cytotoxicity</th>
<th>Mean</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
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<td>5.1</td>
<td>&lt;0.001*</td>
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<td>1STAU</td>
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<td>4.9</td>
<td>2.7</td>
<td>NS*</td>
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<td>H2O2 (0.1 mM)</td>
<td></td>
<td>87.2</td>
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<td>6.3</td>
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</table>

* Comparison of H2O2 (ID50) + 1STAU
** Comparison of H2O2 (0.1 mM) + 1STAU

**CYTOTOXIC EFFECTS OF WHITENING MATERIALS**

ID50 of H2O2 induced cell death is due to apoptosis

Apoptosis was studied by morphological examination and flow cytometry. As shown in Figure 2, apoptosis and/or necrosis evident at morphological examination of slides were treated with May Grünwald-Giemsa stain. Figure 2A showed that nontreated control cells, and Figure 2B showed that many of the cells treated with ID50 of H2O2 for 24h had brightly stained fragmented nuclei and cytoplasmic blebbing.

Flow cytometric analysis of HGFs that had been stained with PI indicated the presence of a population of cells with DNA content lower than that of cells in the G1/G0 phase of the cell cycle. As shown in Figure 3, apoptotic cells were detected as a significant subG1 shoulder, representing hypodiploid cells that had lost DNA with low molecular weight through nuclear fragmentation in cultures that had been treated with both ID50 concentration of H2O2 (Figure 3b) and 0.1 mmol/L concentration H2O2 (Figure 3c), however, they were not detected in untreated cells (Figure 3a). Annexin V-FITC/PI staining and DNA quantification provided concordant results regarding the percentages of apoptotic cells for ID50 concentration of H2O2 (Figure 4). The cells were lysed and genomic DNA was analyzed by agarose gel electrophoresis. As noted in Figure 5, following staining with ethidium bromide, nucleosomal DNA fragmentation, which is the characteristic of apoptosis, was only evident for the ID50 concentration of H2O2 treated HGFs.
Figure 3. Flow cytometric analysis of the DNA content of HGFs. Cells were cultured (a) ID$_{50}$ concentration of H$_2$O$_2$, and (b) 0.1 mmol/L concentration of H$_2$O$_2$, and (c) nontreated control. Results are from a representative experiment, and the population of hypodiploid cells or cells in the G$_0$/G$_1$, phase, S phase, and G$_2$/M phase of the cell cycle are indicated. In addition to the usual distribution of cells in the G$_0$/G$_1$, S, and G$_2$/M phases of the cell cycle, a population of hypodiploid sub-G$_0$/ G$_1$,cells can be detected in the histogram of H$_2$O$_2$ treated cells, and it was increased by addition of both ID$_{50}$ concentration of H$_2$O$_2$ and 0.1 mmol/L concentration H$_2$O$_2$.

Figure 4. FACS analysis of HGF after staining with Annexin. Induction of apoptosis and necrosis in HGF cultures by ID$_{50}$ concentration of hydrogen peroxide after 24 h. FACS analysis after staining with Annexin V-FITC/PI. (a) non treated cells (control); (b) cells treated with ID$_{50}$ (50µmol) hydrogen peroxide after 24 h. Three distinct cell distribution patterns are visible: normal viable cells (lower left quadrant); 18.2% apoptotic (lower left quadrant); 16.1% necrotic and/or apoptotic necrotic (upper right quadrant).

Figure 5. Agarose gel electrophoresis of DNA extracted from fibroblasts. Agarose gel electrophoresis (2 % agarose gel containing ethidium bromide (0.5 µg/ml) in TAE buffer) of genomic DNA extracted from fibroblasts and ID$_{50}$ concentration of H$_2$O$_2$ applied HGFs. Lane 1 and 4 DNA ladder from 100 bp to 100000 bp. Lane 2. Original HGFs. Lane 3. H$_2$O$_2$, applied HGFs.
DISCUSSION

Mild oxygenating agents generating of H$_2$O$_2$ are effective in bleaching discolored, vital teeth$^{1-5,19,20}$ There are concerns about possible pathological effects of long term exposure to WMs on the oral soft tissues and gingiva. The cytotoxic effects of these WMs on the HGFs were analyzed after 24h incubation period. Although the ID$_{50}$ for 24h incubation of H$_2$O$_2$ with HGFs was 0.048 mmol/L, these estimates of biological cytotoxicity may vary in the fibroblasts of various origin due to their different sensitivities. Earlier studies have also shown cytotoxicity of these WMs on HGFs. Hanks et al. reported on ID$_{50}$ for one- and six-hour incubations of with Balb/c 3T3 cells were 0.58 mmol/L and <0.44 mmol/L, respectively. A study reported that concentrations of 0.05% to 0.025% the H$_2$O$_2$ appeared to kill most of the HGFs. Microscopic examination revealed that there occurred some morphological changes from 0.025% to 0.017%. Another study reported the concentrations of H$_2$O$_2$ causing a 50% decrease in cell number were calculated as 0.00034% after 24h. The results of the study indicated that cytotoxicity of H$_2$O$_2$ and CP were influenced by the different concentrations of the peroxide, which is similar to the several studies$^{1,12,21}$ This study also showed that CP is less toxic for fibroblast than H$_2$O$_2$. CP causes fewer hazards in gingiva because of lesser H$_2$O$_2$ concentration.

The results of our study indicate that WMs cause a dose dependent cytotoxicity in vitro via direct interaction between H$_2$O$_2$ or CP and HGFs. Flow cytometric analysis of DNA content demonstrated an increase in the proportion of cells in the sub-G1 phase, possibly owing to the slowing of regression through S phase or a block between S and G2M in the cell cycle.$^{12}$ These hypodiploid cells are the characteristics of the cells undergoing apoptosis, which have lost low molecular weight DNA through nuclear fragmentation.$^{22}$

Apoptosis, in contrast to necrosis, is characterized by a specific series of intracellular events leading ultimately to DNA fragmentation. Several studies have shown that H$_2$O$_2$, cause cell apoptosis, and H$_2$O$_2$-induced apoptosis was correlated with mitochondrial dysfunction and caspase activation.$^{10,23,24}$

It has been shown that H$_2$O$_2$ may also induce the increase in cytosolic calcium.$^{23}$ It was thus possible that the observed cytotoxicity in the present study was indirectly due to the removal of free calcium from the culture. To test this hypothesis, we compared the effect of H$_2$O$_2$ on HGFs with EGTA more efficient calcium chelator in the MTT assay. Only EGTA at 100 µM had no effect on the cells. Addition of EGTA to H$_2$O$_2$ containing culture showed a significant decrease in cytotoxicity rather than an enhanced cytotoxic effect. The data indicate that a decrease in the extracellular calcium concentration can actually protect to HGFs from these WMs. This is the first report, to our knowledge, showing enhanced cytosolic calcium with H$_2$O$_2$ in the HGFs. H$_2$O$_2$ can increase the level of intracellular calcium, down regulate GSH levels, slightly induce lipid peroxidation, and lead to change in the ratio of reduced ion components to oxidized ion components of cells. Analysis of flow cytometry indicates that H$_2$O$_2$ decreases the level of Bcl-2. The data indicate that H$_2$O$_2$ induced apoptosis requires new mRNA and protein syntheses; H$_2$O$_2$ can activate calcium/magnesium dependent endonuclease leading to internucleosomal DNA fragmentation and activation of poly (ADP-ribose) polymerase interfering with the energy metabolism of the cell. The H$_2$O$_2$ mediates downregulation of GSH, which may be more important for apoptosis than H$_2$O$_2$ induction of lipid peroxidation; the H$_2$O$_2$ induced changes in redox status of the cell may be among the original events, which lead up to other biochemical changes.$^{25}$ Therefore, an increase in the cytosolic calcium concentration obviously plays a decisive role in the cytotoxicity of H$_2$O$_2$.

Our observation of a decreased cytotoxic effect by ID$_{50}$ of H$_2$O$_2$ when this agent was combined with 1 nM staurosporine (a potent inhibitor of PKC) suggests that PKC mediated intracellular phosphorylation is involved in mediation of H$_2$O$_2$ induced apoptosis.

Our observation of a decreased cytotoxic effect by ID$_{50}$ of H$_2$O$_2$ when this agent was combined with 1 nM staurosporine (a potent inhibitor of PKC) suggests that PKC mediated intracellular phosphorylation is involved in the mediation of H$_2$O$_2$ induced apoptosis. It has also been shown that PKC-mediated pathways are the major mechanisms underlying spontaneous/physiological apoptosis in osteoclast. We conclude that the PKC signaling apoptosis may be a major signal transduction pathway for apoptosis in HGFs. High levels of nitric oxide can cause apoptosis in mouse macrophages$^{27}$, and may cause DNA damage directly by nitrosylation and deamination of purine and pyrimidine bases.$^{28}$ We investigated whether H$_2$O$_2$ induced apoptosis in HGFs could be mediated by modulation of nitric oxide synthesis. Thus, addition of 400 µM competitive inhibitor of nitric oxide synthase L-NAME to cell cultures containing ID$_{50}$ of H$_2$O$_2$ prevented H$_2$O$_2$ induced apoptosis. Therefore,
the mechanism by which \( \text{H}_2\text{O}_2 \) causes apoptosis on HGFs appears to involve increased production of nitric oxide.

**CONCLUSION**

The results of the present study show that WMs have toxic effects on HGFs in vitro by inhibiting several cellular functions. In clinical practice, WMs can be in direct contact with the gingival epithelium and there are reports in the literature showing clinical ulceration caused by these WMs. However, the gingival tissues appear to be less vulnerable than those expected from in vitro findings. Although the clinical significance of our findings remains to be elucidated, the information presented here may contribute to a better understanding of the mechanism involved in the interactions between HGFs and WMs. In addition, these WMs induced cell death by apoptosis in HGFs. The increase in apoptosis indicates that WMs activate signaling pathways involved in cell death regulation with several mechanisms, and also the levels of apoptosis reflect the cytotoxicity degree of WMs.

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