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Original Article

Hydrogen peroxide-induced apoptosis in human gingival fibroblasts

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Abstract: In the process of bleaching vital, discolored teeth, low concentrations of hydrogen peroxide (H_2O_2) are effective alternatives to heat-activated 30% H_2O_2. However, interest has been expressed in the assessment of pathological effects of long-term exposure to bleaching agents such as irritation and ulceration of the gingival or other soft tissues. The aim of the present study was to determine the effect of hydrogen peroxide on apoptosis in human gingival fibroblasts (HGF). Cytochrome c, Bcl-2, Bax, Bid and caspase-3 protein expression were detected by Western blotting. HGF cell apoptosis induced by H_2O_2 was both dose and time dependent. The addition of H_2O_2 resulted in the release of cytochrome c to the cytosol, and an increase of Caspase-3 cleavage. Data suggest that oxidative stress-induced apoptosis in HGF is intrinsic pathway involved the release of apoptotic signal from mitochondria.

Keywords: Human gingival fibroblasts, hydrogen peroxide, apoptosis, discolored teeth

Introduction

Human gingival fibroblasts play an important role in tissue structure, function and in the host immune defense [1]. However, when the tray (mouthguard) containing hydrogen peroxide is placed on the gums, H_2O_2 can spill onto the oral soft tissues [2-4]. Although there is scarce information available on the effects of bleaching agents on the oral soft tissues, some reports indicate that H_2O_2 caused irritation, ulceration, burning and certain adverse effects on the gums [5]. In a former report, we found that hydrogen peroxide promoted PKC and ERK 1/2 activation and induced cell viability decrease [3]. Due to the aforementioned fact, we decided to assess whether hydrogen peroxide promotes apoptosis in human gingival fibroblasts.

Apoptosis is a type of programmed cell death; it plays a major role in regulating normal development, in eliminating damaged cells and in maintaining homeostasis in multicellular organisms [6]. Mitochondria participate in the regulation of apoptosis capturing pro-apoptotic proteins from the cytoplasm and executing characteristic factors to initiate apoptotic protease cascades for cell execution [7-10]. Apoptosis may be initiated though an intrinsic pathway involving the release of apoptotic signals from mitochondria [11-13]. The intrinsic and extrinsic pathways of apoptosis converge on a cascade of cysteine proteases denominated caspases which play an important role in apoptosis initiation [14, 15]. Stimulation of the death receptor pathway leads to caspase-8 activation following the recruitment of the pro-caspase to the death-inducing signaling complex [16-18]. In contrast, mitochondrial pathway requires the release of mitochondrial cytochrome c and the formation of a large multiprotein complex comprising cytochrome c and pro-caspase 9 [19-22]. Caspases 8 and 9 will then proteolytically activate downstream caspases, in particular caspases 3 and 7, which are responsible for the apoptotic destruction of the cell [23, 24]. Caspase-3 is essential in DNA fragmentation processes [25-27]. On the other hand, effector pro-apopototic members such as Bax and Bak are the direct pro-apoptotic effectors of mitochondrial outer membrane permeabilization to induce apoptosis that can translocate and insert into the outer mitochondrial membrane, thus oligomerize and form pores [28].

Otherwise, in the classical model, the antiapoptotic members of the protein family such B-cell lymphoma-2 (Bcl-2) plays key roles in inhibiting the mitochondrial death pathway [29]. By means of the interaction between these members, the mitochondrial outer membrane's permeability can be altered with the interaction among three classes of proteins: Bcl-2, Bcl-xL and Mcl-1 which protect cells against death. Bcl-2 inhibits Bax and Bak activation though a direct interaction involving the so called BH domains (Bcl-2 homology domains, which define both the structural and the functional homology patterns within the Bcl-2 family) [30], Bax activation is favored by the proapoptotic BH3-only proteins [31]. These proteins can act both as direct activators of Bax by interacting directly with Bax or as de-repressors by interacting with antiapoptotic members of the Bcl-2 family [32-34]. However, recent data suggest that Bcl-2 plays an active role in the course of Bax activation [35]. Bas is mainly reported as being cytosolic in nonapoptotic cells and the mitochondrial protein upon apoptosis induction [36]. No complete correlation has been established between its relocation to mitochondria and subsequent activation leading to permeabilization of mitochondrial outer membrane. Nevertheless, the apoptotic pathway involved in hydrogen peroxide on human gingival fibroblasts has not been fully studied; therefore, the main purpose of the present study was to explore the apoptotic pathway involved in hydrogen peroxide-induced apoptosis in human gingival fibroblasts.

Materials and methods

Reagents

Dulbecco's modified Eagle medium and fetal bovine serum (FBS) were purchased from Invitrogen (Carlslab, CA, USA), Western blot chemiluminescent detection system and primary and secondary antibodies to cytochrome c, Bax, Bcl-2, Bid, caspase-3, γ-tubulin, anti-goat, anti-rabbit and anti-mouse were purchased from Santa Cruz (Santa Cruz, CA, USA).

Cell culture

HGF cells were obtained from a sample of healthy tissue from patients (healthy young individuals aged 18-20 years) attending the Exodontia Clinic seeking premolar extraction. Patients signed informed consent forms (Format FBQ-LIFO-001 ISO 9001:2008). The protocol for our study in humans was approved by the Ethical Committee of the Universidad Nacional Autónoma de México. Gingival tissue was isolated at the cementoenamel junction of the extracted tooth by means of a surgical blade. The harvested tissue was rinsed several times in Dulbecco's modified Eagle medium containing antibiotics (penicillin 100 U/mL; streptomycin 125 μg/mL and amphotericin 5 μg/mL). The tissue was cut into small pieces and cultured with a medium containing 10% FBS. When cells growing from explants had reached confluence, they were detached with 0.025% (w/v) trypsin in PBS (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose supplemented with 200 mg digitonin/mL) (Sigma Chemical Co) for 10 min and subcultured in flasks. HGF were cultured in humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cell cultures used in all experiments were between passage 5 and 9 [37].

Hoeschst 3342 staining

HGF cells were treated with different concentrations of H_2O_2 for 60 min. After that period the culture medium was gently removed, and cells were stained with Hoeschst 3342 (1 μg/mL) for 10 min. Fluorescence was observed with a fluorescence microscope, and images were captured with an electronic camera.

Measurement of cytochrome c release

Release of cytochrome c from mitochondria into the cytosol was assessed by Western blotting. HGF cells were treated with hydrogen peroxide and later collected and centrifuged at 10,000 rpm for 10 min. Subsequently, cells were rinsed twice with PBS. After being re-suspended, cells were centrifuged at 15,000 rpm for 10 min at 4°C and the supernatant, corresponding to the cytosolic fraction was recovered [38]. Total protein concentration was quantified by using Bradford method. Fifty micrograms of protein extract were loaded onto sodium dodecyl sulfate (SDS) polyacrilamide gel. The separated proteins were electro-transferred to a polyvinylidene difluoride membrane (PVDF) (Amersham Pharmacia Biotech, USA). Membranes were blocked for 1 h at room temperature with non-fat milk. The blots were incubated with primary mouse monoclonal antibody

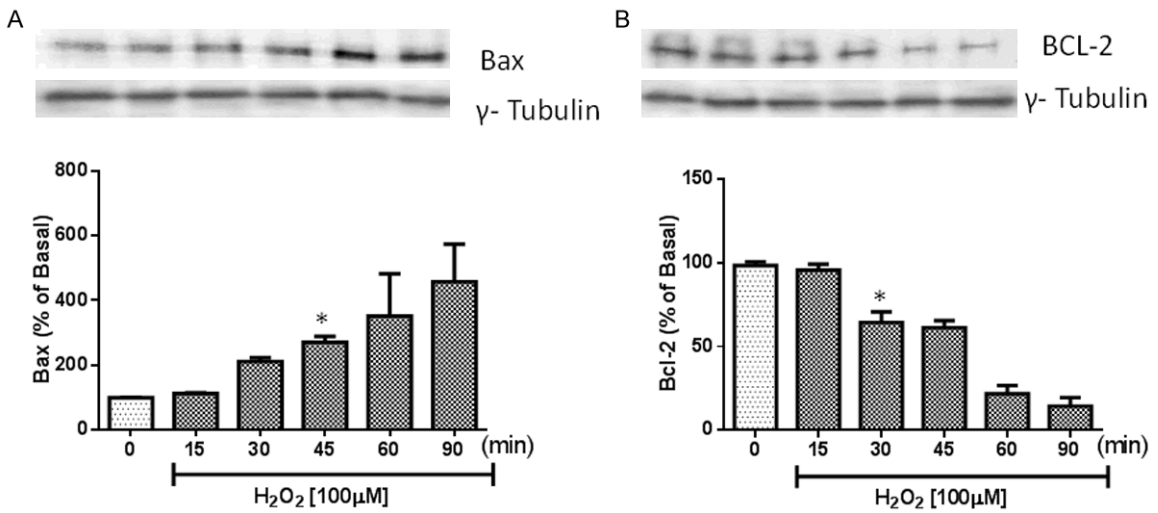

Figure 1. Effect of hydrogen peroxide on Bax and Bcl-2 expression. Cell lysates were prepared from the human gingival fibroblasts treated with hydrogen peroxide. Representative western blot image for detecting, (A) Bax and (B) Bcl-2 protein expression derived from HGF treated with 100 μM H_2O_2 for different time. Values in bar graphs are the mean ± SEM of at least three independent experiment $^*P<0.05$.

against the denatured form of cytochrome c (1:500), for 12 h. Membranes were then washed 3 times with PBS for 10 min. The bands were revealed with chemiluminescent reaction and bands visualized by autoradiography. The graph of each experiment corresponded to media ± SD. Blots equal loading was demonstrated by stripping the blots and re-probing with anti-tubulin.

Western-blot analysis

Human gingival fibroblasts (HGF) (1×10^6 cells/well) were cultured in 6-well plates. Cells were serum-starved for 16 h and treated with hydrogen peroxide in DMEM with 2% fetal bovine serum. After treatment, the media was aspirated, cells were washed twice with PBS and 50 ml cold lysis buffer was added. Lysis buffer was composed of: 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet p-40, 0.5 M PMSF, 10 mg/ml leupeptin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate. All aforementioned materials were supplied by Sigma Chemical Co. (St. Louise, MO, USA). Treated cells were removed from the plate by scraping and the lysate was transferred to a microcentrifuge tube and pulse sonicated (1 s × 30) on ice. Western blot for protein presence was performed on whole cell extracts. Protein concentrations were measured using Ponceau solution (Sigma Chemical Co., St Louis MO, USA). Protein (50 mg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.05% bromophenol blue, and 1.25 M Tris-HCl, pH 6.8; all the materials supplied by Sigma Chemical Co). Equal amounts of protein resolved by 10% SDS-PAGE. Protein was transferred to PVDF (Bio-Rad, Hercules, California, USA) overnight at 30 V. The membrane was blocked with 150 mM NaCl, 100 mM Tris-HCl and 5% bovine serum overnight at 30 V. The membrane was blocked with 150 mM NaCl, 100 mM Tris-HCl and 5% bovine serum albumin for 1 h, washed and then incubated with Bax rabbit polyclonal IgG; Bcl-2 rabbit polyclonal IgG, Caspase-3 goat polyclonal IgG, caspase-9 rabbit polyclonal IgG; g-tubulin goat polyclonal IgG all in 1:1000 dilution. Immunoreactive bands were developed using a chemiluminiscent reaction and bands visualized by autoradiography. Experiments were performed separately three times and analyzed with DigiDoc-It system (Ultra-violet products, Cambridge, UK). The graph of each experiment corresponded to media ± S.D. Blot equal loading was demonstrated by stripping the blots and re-probing wit Abs for tubulin.

Statistical analysis

Statistical analysis of densitometric data was performed by determining the integrated optical density of each sample and using Student's

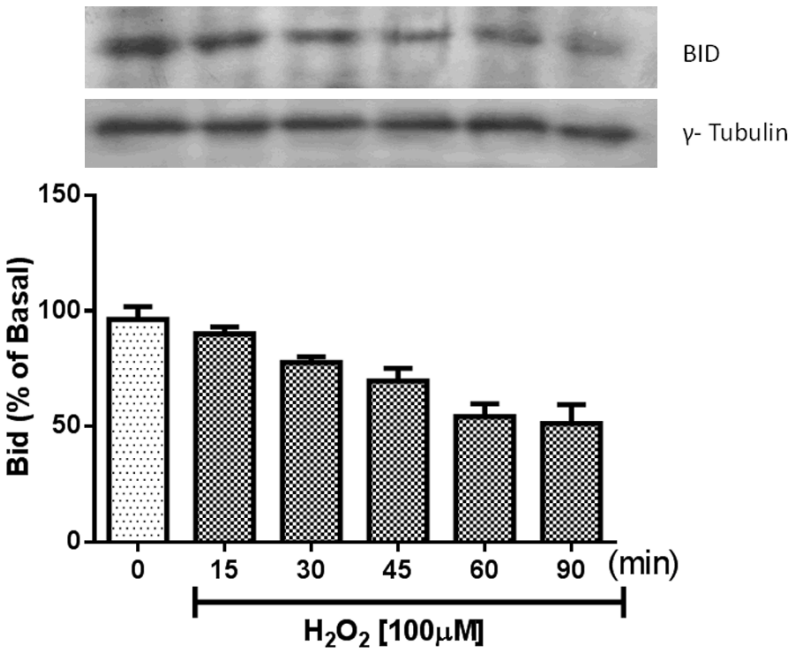

Figure 2. Effect of hydrogen peroxide on BID expression in human gingival fibroblasts. BID expression regulated by hydrogen peroxide (100 μM) at the indicated time. Results were expressed as percentage of basal (cells without treatment). Values are mean ± S.D. of three different experiments *P<0.05.

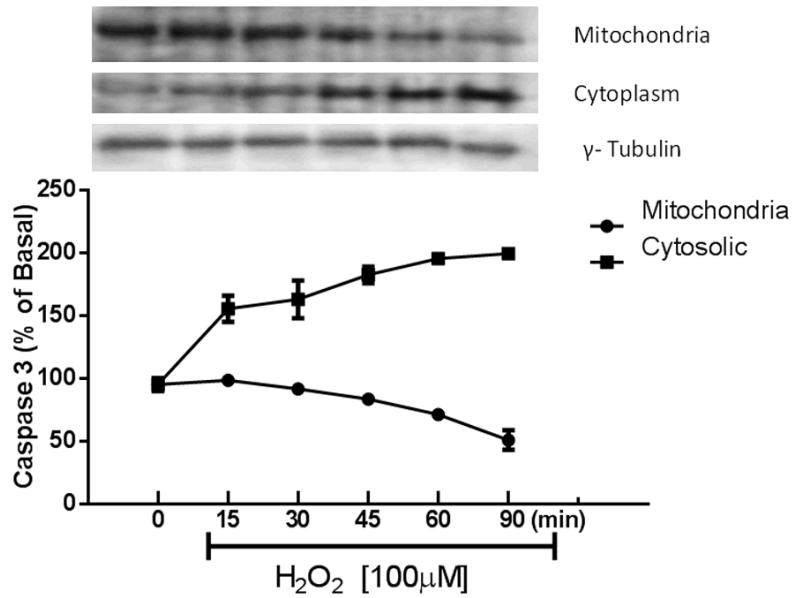

Figure 3. Effect of hydrogen peroxide on cytochrome c release to cytosol in human gingival fibroblasts. Membrane and cytosolic fractions were obtained from mitochondrial and subjected to Western blotting with a monoclonal antibody to cytochrome c. The blots were reprobed with anti-g tubulin to assure the equal amount of protein loaded in each lane. The figure is representative of three separate experiments. *P<0.05.

t-test. *P* values less than 0.05 were considered significant in all cases.

Results

Effect of hydrogen peroxide on Bax in human gingival fibroblasts

Since Bcl-2 family proteins such as BLc-2 and Bax are critical regulators on the apopototic pathway, we undertook to examine the effect of hydrogen peroxide (100 mM) on the expression of pro-apoptotic Bax protein. As shown in **Figure 1A**, the protein level of pro-apoptotic protein Bax declined in a time dependent fashion. Simultaneously, the protein level of anti-apoptotic protein Bcl-2 increased in the cells treated with hydrogen peroxide (**Figure 1B**). We next assessed the effect of hydrogen peroxide on BID expression, a pro-apoptotic protein member of Bcl-2 family protein.

Effect of hydrogen peroxide on BID expression

As shown in **Figure 2**, cells exposed to hydrogen peroxide induced Bid cleavage as demonstrated by Western blot with loss of Bid. The change was initiated within 45 min after treatment and gradually increased with incubation time. Bid induces apoptosis by mediating the release of cytochrome c from mitochondria to cytosol. We consequently evaluated the effect of hydrogen peroxide on cytochrome c release.

Effect of hydrogen peroxide on cytochrome c release

When compared with the control group, level of cytochrome c in cytosol was increased to 216 ±

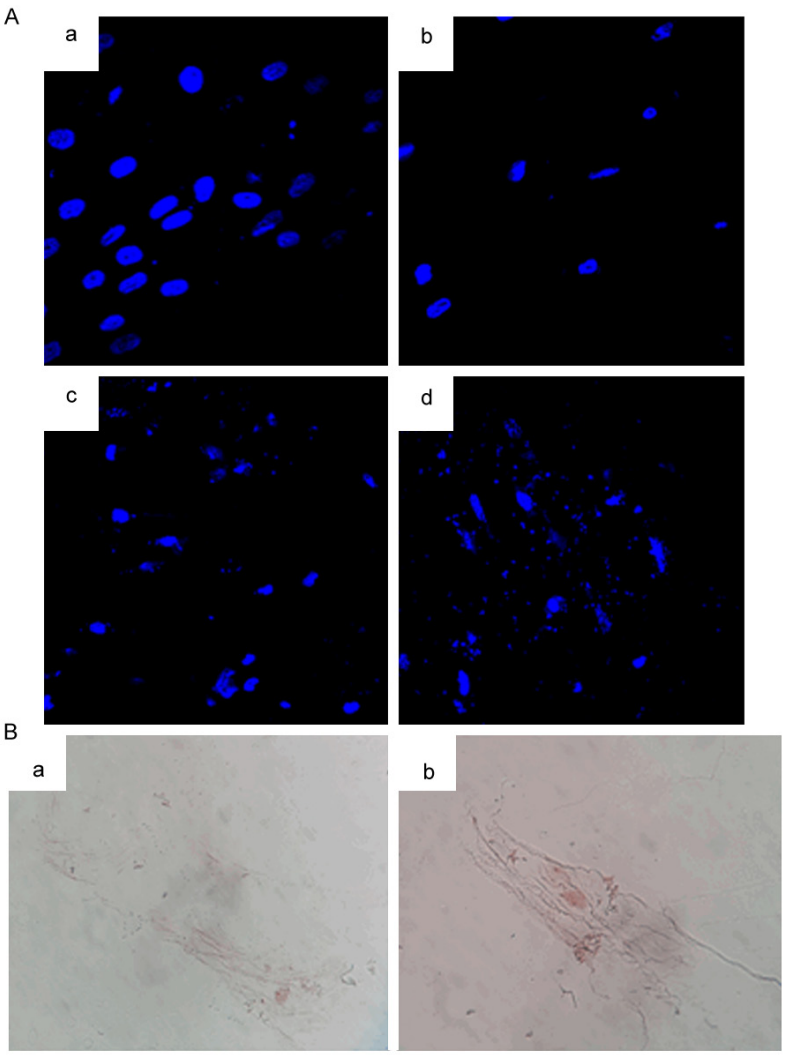

Figure 4. Hydrogen peroxide mediated apoptosis in human gingival fibroblasts detected by Hoechst 33258 and TUNEL assay. A: About 4 × 10^5 human gingival fibroblasts on the cover slips in 24 well plates were treated with different concentrations of hydrogen peroxide (100 μM); a: Basal; b: 30; c: 60; d: 90 min. The cells were washed with cold phosphate-buffered saline (pH 7.4) and then stained with Hoechst 33258 and observed under an inverted fluorescent microscope. B: About 4 × 10^5 human gingival fibroblasts were cultured on the cover slips in 24-well plates were treated with hydrogen peroxide (100 μM) for different time. After incubation cells were washed with cold phosphate-buffered saline (pH 7.4) and stained according to the instructions of TUNEL assay kit.

3.2 when the cells were treated with H_2O_2 alone, and likewise decreased in the mitochondrial membrane (**Figure 3**).

Effects of hydrogen peroxide on apoptosis of human gingival fibroblasts

Cell cycle checkpoints play an important role in cell death or survival. If conditions where cells live are favorable for successful interphase and mitosis, cells can divide and maintain growth or they might enter into a death event. Based on previous results on cell viability decrease by hydrogen peroxide in HGF, we used two methods Hoescht 33258 and TUNEL assay to assess effects of hydrogen peroxide on apoptosis in human gingival fibroblasts. After treatment with hydrogen peroxide at 100 mM and use of Hoescht staining 33258 cells were observed in search for apoptotic occurrence (**Figure 4A**). It was found that percentage of apoptotic cells increased in hydrogen peroxide treatment. Moreover, we used TUNEL assay to confirm hydrogen peroxide-mediated apoptosis in human gingival fibroblasts. As shown in **Figure 4B**, results were expectedly similar to those detected by Hoechst 33258 staining. Results confirmed that hydrogen peroxide induced apoptotic occurrence in human gingival fibroblasts. It has been well established that that the conversion of procaspase-3 to active Caspase-3 by cleavage is an important step that subsequently results in chromatin degradation and apoptosis, based on the aforementioned facts we decided to assess effects of hydrogen peroxide on Caspase-3.

Hydrogen peroxide activates caspase-3 in human gingival fibroblasts

To test whether hydrogen peroxide-mediated apoptosis was associated to Caspase-3 cleavage by Western-blotting, efficient cleavage of Caspase-3 occurred already 30 min after the addition of 100 mM hydrogen peroxide. As shown in **Figure 5**, proteolytic processing of procaspase-3 in oxidant injury results in the formation of a 17-kDa subunit of the active

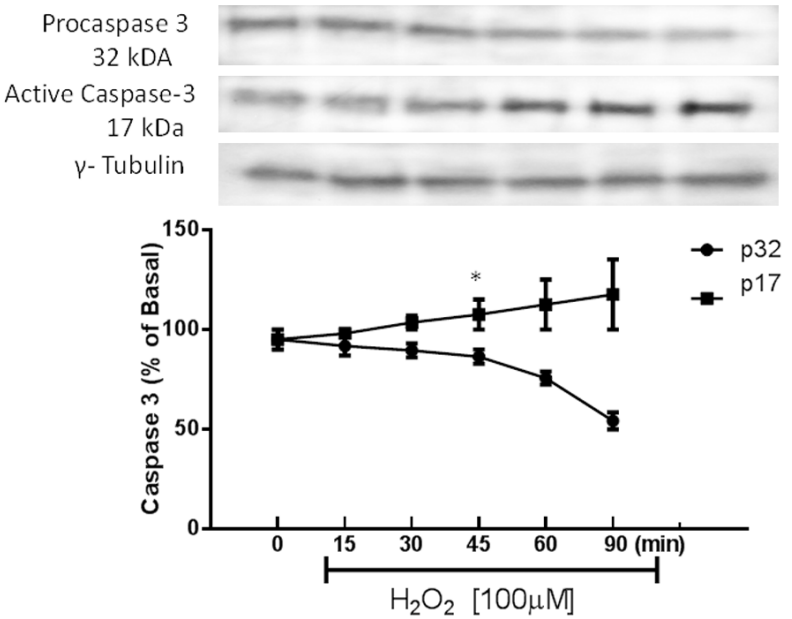

Figure 5. Effect of hydrogen peroxide on caspase-3 protein expression in human gingival fibroblasts. Cell lysates were prepared from the human gingival fibroblasts treated with hydrogen peroxide (100 μM) for different time and subjected to SDS-PAGE as described in Materials and Methods. Representative western blot images for detecting caspase-3 protein expression derived from HGF treated with H_2O_2 (100 μM) for different time. g-tubulin was used as a loading control *P<0.05 vs. the basal.

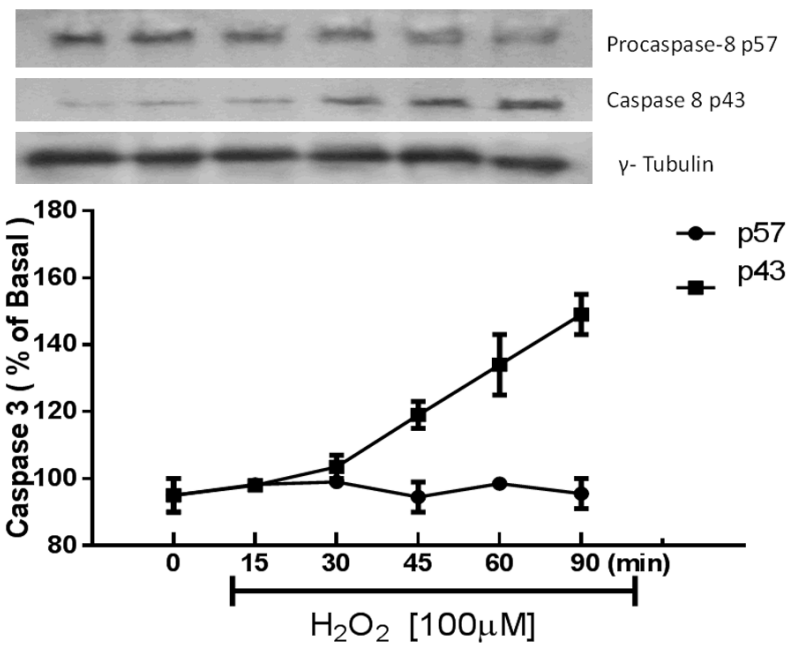

Figure 6. Western blot analysis of caspase-8 protein expression in human gingival fibroblasts. Cell lysates were prepared from the human gingival fibroblasts treated with hydrogen peroxide (100 μM) for different time and subjected to 10% SDS-PAGE as described in materials and methods. Representative western blot images for detecting caspase-8 expression. Relative expression level of caspase-8 protein from three independent experiments was quantified; g-tubulin was used as a loading control. *P<0.05 vs. basal.

caspase-3, supporting the concept that the Caspase-3 activation does play a role during this apoptotic process. Next, we followed the fate of Caspase-8 and Caspase-9.

Effect of hydrogen peroxide on caspase 8 and 9in human gingival fibroblasts

To determine the involvement of caspase-8 in H_2O_2-induced apoptosis in human gingival fibroblasts, we examined the activation of caspase-8 by detecting caspase-8 precursor and subunit expression. As shown in **Figure 6** hydrogen peroxide-mediated detectable caspase-8 subunit expression by Western blotting. We next measured caspase-9 activity using western blot technique, in human gingival fibroblasts after hydrogen peroxide treatment. Similarly, as caspase-8, caspase-9 is proteolytically processed resulting in 37 and 17-kDa subunits of the active form. As shown in **Figure 7** and consistent with the effect of hydrogen peroxide on apoptosis, Caspase-9 activity in human gingival fibroblasts treated with hydrogen peroxide was markedly elevated in a time dependent-fashion.

Discussion

Vital bleaching of teeth with hydrogen peroxide under controlled clinical conditions has widely been used to lighten teeth. Nevertheless several reports of gingival irritation and ulceration in some patients suggest that bleaching agents, under certain cir-

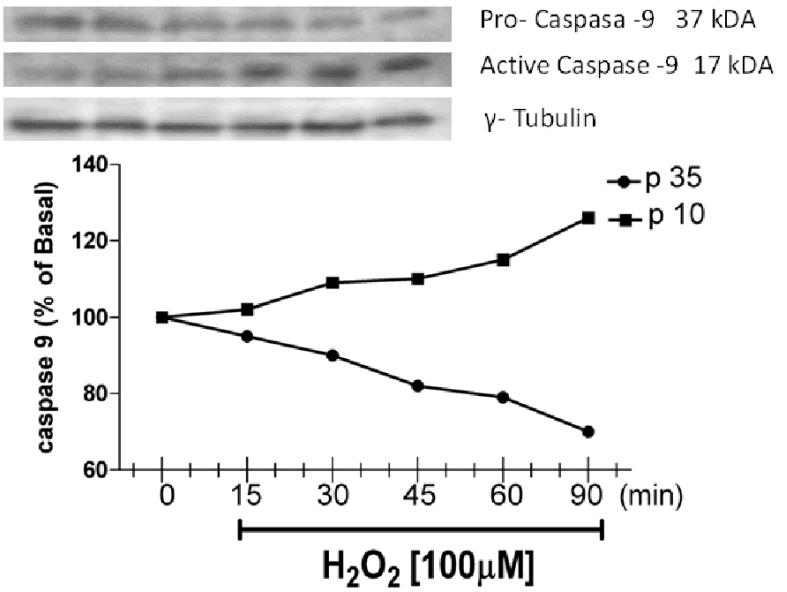

Figure 7. Effect of hydrogen peroxide on activation of caspase-9 in human gingival fibroblasts. Caspase-9 activity induced by hydrogen peroxide (100 μM) at the indicated time. Cell lysates were prepared form the human gingival fibroblasts and subjected to 10% SDS-PAGE as described in Materials and Methods. Representative western blot images for detecting caspase-8 expression. Relative expression level of caspase-8 protein from three independent experiments was quantified; g-tubulin was used as a loading control. *P<0.05 vs. basal.

cumstances, promote toxic effects on oral cells and in human gingival fibroblasts [38, 39].

In the present study, we found that treatment of HGFs with hydrogen peroxide elicited cytotoxic effects. In a previous work [3] we have shown that hydrogen peroxide stimulated phosphorylation of PKCa and ERK 1/2, this process might have led to cellular proliferation decrease. However effects of hydrogen peroxide of HGF on apoptosis have not been previously reported. In our work, we found that hydrogen used at concentrations of 10 to 200 mM promoted apoptosis. Meanwhile, we observed characteristic apoptotic events such as morphological changes, including chromatin condensation, nuclear and DNA fragmentation, considered a hallmark of cells undergoing apoptosis, were detected in hydrogen peroxide HGFs. As an ensemble, these results indicated that hydrogen peroxide induced apoptosis in HGFs. In continuance we characterized effects of hydrogen peroxide on Caspase activation.

It has been well established that H_2O_2 modulates a large number of cellular responses, which varied with cell type and conditions of H_2O_2 treatment [40-42]. Although H_2O_2 had been reported to induce apoptosis in human gingival fibroblasts and plays an important role in periodontal tissue and alveolar bone destruction, molecular mechanism of the induction remains unclear. Many investigations have focused on the effect of H_2O_2 induced pyknotic/necrotic cell death rather than apoptosis [43]. Moreover, other studies suggested that H_2O_2 induced tumor necrosis factor (TNF) expression and an increase in membrane permeability in human gingival fibroblasts [43]. Additionally it was reported that both vitamin K and docosahexanoic acid diminished these responses [44]. These results indicated that hydrogen peroxide induced apoptosis in human gingival fibroblasts, nevertheless molecular mechanism have not been clearly researched. This prompted us to undertake research of hydrogen peroxide caused apoptosis in human gingival fibroblasts. In our work, we found that hydrogen peroxide promoted characteristic apoptotic events such a morphological changes, including chromatin condensation and nuclear fragmentation which is a hallmark of cells undergoing apoptosis as previously reported. Then we focused on researching apoptotic mechanisms in human gingival fibroblasts It has been well documented that there are at least two pathways involved in apoptosis: extrinsic (through death receptors) and intrinsic (mitochondria) which are regulated by Caspase 8 and Caspase 9 respectively. In our research, we found that Caspase-9, the apical Caspase in mitochondrial mediated apoptosis and Caspase-3, were activated during the process of apoptosis induced by hydrogen peroxide in human gingival fibroblasts. Similar results have been reported for other cells in which at 10 μM to 5 mM concentration, H_2O_2 promoted apoptosis in Jurkat cells, cardiomyocytes, skin fibroblasts and tendon cells fibroblasts. We found that 100

μM H_2O_2 promoted apoptosis in HGF. It has been reported that Bcl-2, anti-apoptotic protein and Bax pro-apoptotic resided in the mitochondrial outer membrane and were involved in mitochondrial-dependent apoptosis pathways by regulating mitochondria membrane permeability. Proapototic protein Bax induces cell apoptosis via mitochondrial membrane permeabilization that leads to the release of cytochrome c. In the present study, H_2O_2 treatment resulted in increased expression of Bax and decreased expression of Bcl-2 which played a role via mitochondrial pathways in H_2O_2-induced apoptosis in HGF, this process was followed by caspase-9 and caspase-3 activation. Results revealed that H_2O_2 exhibited potential to trigger apoptosis in mitochondria pathways as previously reported.

We therefore beg to conclude that these findings provided insight into the effects of hydrogen peroxide on apoptosis in a caspase-dependent pathway thus releasing mitochondrial cytochrome c. Nevertheless, and for the first time our experiments demonstrated that H_2O_2-induced apoptosis in HGF was mediated through mitochondrial- and caspase-dependent pathway.

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Disclosure of conflict of interest

None.

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