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ORIGINAL ARTICLE

Oral malodorous compound activates mitochondrial pathway inducing apoptosis in human gingival fibroblasts

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Abstract Hydrogen sulfide (H_2S) is a main cause of physiologic halitosis. H_2S induces apoptosis in human gingival cells, which may play an important role in periodontal pathology. Recently, it has been reported that H_2S induced apoptosis and DNA damage in human gingival fibroblasts (HGFs) by increasing the levels of reactive oxygen species. However, the mechanisms of H_2S-induced apoptosis have not been clarified in HGFs. The objective of this study was to determine the apoptotic pathway activated by H_2S in HGFs. The HGFs were exposed to 50 ng/mL H_2S, resulting in 18 ng/mL in the culture medium, which is lower than the concentration in periodontal pockets. The number of apoptotic cells after 24 and 48 h incubation was significantly higher than that in the control cultures ($p<0.05$). Mitochondrial membrane depolarization and the release of cytochrome *c*, and caspase-3, and caspase-9 were also significantly increased after both 24- and 48-h incubation ($p<0.05$), whereas caspase-8, a key enzyme in the receptor ligand-mediated pathway causing apoptosis, was not activated. The present study shows that H_2S triggered the mitochondrial pathway causing apoptosis in HGFs but did not activate the receptor ligand-mediated pathway.

Keywords Hydrogen sulfide · Apoptosis · Periodontitis · Halitosis · Fibroblast

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Introduction

Halitosis, a general term for unpleasant breath odor originating either in the oral cavity or from a systemic source [1], often affects people not only physically but also socially and/or psychologically [2–4]. Oral malodor is primarily caused by volatile sulfur compounds (VSCs) such as hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) [1]. Many studies have demonstrated the relationship between offensive breath odor and periodontal disease, including a significant correlation between the presence of VSCs and the severity of the periodontal condition [5–12].

VSCs are also known to be periodontally toxic [13]. By increasing the permeability of gingival crevicular epithelia, VSCs allow greater penetration of both lipopolysaccharide and prostaglandin into the tissue [13]. Furthermore, VSCs suppress wound healing, particularly the formation of basal membrane and type IV collagen synthesis [13, 14], and stimulate interleukin-1 production, resulting in an increase in prostaglandin E and matrix metalloproteinase 1 [15]. Thus, VSCs may be the agent responsible for the initial damage to the epithelial barrier in the progression of periodontal disease. As the primary cause of physiologic halitosis in the absence of periodontal conditions, H_2S is regarded as a periodontal risk factor for periodontally healthy subjects [3, 5, 10–12].

Apoptosis, or programmed cell death, is observed in experimental periodontitis [16] and could play an important role in periodontal pathology, since one cause of periodontitis is the imbalance between destruction and wound healing of gingival tissues. Recently, it was reported that apoptosis in human gingival fibroblasts (HGFs) and epithelial cells (HGECs) was caused by the presence of H_2S in concentrations similar to or lower than those found

in gingival crevicular fluid [17, 18]. Yaegaki et al. found an increment of reactive oxygen species (ROS) as well as significant DNA damage during the apoptotic process in HGFs [18], although the signaling apoptotic pathway involved in the process was not clarified. Since VSCs and in particular H_2S strongly inhibit cytochrome *c* oxidase, which is a key enzyme for oxidative phosphorylation in the respiratory chain [19], H_2S may cause mitochondrial depolarization and cytochrome *c* release followed by a caspase cascade, resulting in apoptosis. H_2S also induces apoptosis by activating the mitochondrial pathway as well as the receptor ligand-mediated pathway in pancreatic acinar cells [20].

Controlling apoptosis in gingival tissue in the oral cavity might promote gingival health [21]. Therefore, the primary focus of this study is to elucidate the apoptotic mechanism and to identify which of the two molecular pathways, extrinsic or intrinsic, is responsible for activating the apoptotic process in HGFs.

Materials and methods

Cell cultures with H_2S incubation system

HGFs from a reserved cell line in the Department of Oral Health, Nippon Dental University were utilized for this study. This study were reviewed and approved by the Research Ethics Board of Nippon Dental University and by the Research Ethics Committee of Tokyo Medical and Dental University. HGFs were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. The cells were plated in the sixth through tenth passages at a density of 2×10^5 cells/mL in 25-cm^2 flasks and allowed to attach overnight. All cells were grown at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in air. After 24 h, the cells were placed in fresh medium prior to their incubation in the H_2S incubation system, as described below.

Test cultures were incubated in a sealed chamber perfused with a 95% air–5% CO_2 gas mixture at 37°C. The inlet line to the test chamber was connected to a standard H_2S gas generator (PD-1B®; Gastec, Kanagawa, Japan) containing a calibrated permeation tube (Gastec) used to emit a concentration of 50 ng/mL H_2S into the air mixture for either 24 or 48 h. As a result of diffusion, the amount of H_2S in the medium was found to be only 18 ng/mL lower than that in gingival crevicular fluids from periodontal involvements [22]. Control cultures were incubated in a 95% air–5% CO_2 incubator at 37°C for 24 or 48 h. For each experiment, positive control samples using 10 μM camptothecin (Sigma–Aldrich Japan, Tokyo, Japan) were employed.

Trypan blue dye exclusion

The effect of H_2S on cell viability was assessed by Trypan blue dye exclusion. The cells were incubated as described above for 24 or 48 h and then trypsinized and resuspended in phosphate-buffered saline (PBS). Once stained with Trypan blue, the cells were counted in a hemocytometer to assess the number of dead blue cells among the total number of cells counted. The percentage of viable cells was calculated.

Detection of apoptotic cells

The extent of apoptosis of HGFs was determined a Guava EasyCyte® flow cytometer (GE Healthcare Bioscience, Tokyo, Japan). Following the period of incubation, the cells in the test and control groups were trypsinized and then resuspended in PBS at a concentration of 1×10^6 cells/mL. The cells were stained with Anexin V-PE and 7-amino-actinomycine D (7-AAD) according to the manufacturer's instruction and then were assayed by flow cytometry. Viable cells, apoptotic cells in the early and mid-stages, and necrotic or late apoptotic cells were counted separately. The viable cells were not stained with Annexin V-PE nor 7-AAD. Meanwhile, the cells in the early to mid-stages of apoptosis exhibited only Anexin V-PE-positive. The necrotic or late apoptotic cells were stained with both Annexin A-PE and 7-AAD and belonged to the same fraction. Since both necrotic and late apoptotic cells cannot be identified separately by the present method, and as the necrotic cells were already determined by Trypan blue dye exclusion, the data of the necrotic or late apoptotic cells fraction were excluded from this study. For each experiment, the results from 2,000 cells were analyzed using Guava EasyCyte Nexin® software (GE Healthcare Bioscience). The percentages of apoptotic cells among all cells were calculated.

Detection of mitochondrial membrane potential

Mitochondrial membrane potential was detected using Guava EasyCyte MitoPotential®. After incubation with or without H_2S, the cells were trypsinized and resuspended in PBS at a concentration of 5×10^5 cells/mL. Then, the cells were stained with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), which demonstrated a ratiometric dye that fluoresces green, orange, or red depending on membrane potential. In healthy, nonapoptotic cells, the JC-1 dye enters the mitochondrial matrix, accumulates as aggregates, and stains the mitochondria bright red. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 enters the cytoplasm in a monomeric form, where it

fluoresces green. After incubation of 30 min, the cells were assayed by a flow cytometer. For each experiment, 2,000 cells were analyzed using Guava EasyCyte Nexin® software, and the percentages of polarized and depolarized cells among all cells analyzed were calculated.

Measurement of cytochrome *c* released from mitochondria to cytosol

Mitochondria were separated from cytosol treated with or without H_2S for 24 and 48 h, as described below. Cells were scraped and resuspended in PBS. After centrifugation, the pellet was resuspended in a solution of 0.15 mM magnesium chloride, 10 mM potassium chloride, and 10 mM Tris-hydrochloride (pH 6.7). The resuspended cells were homogenized using the glass homogenizer. Sucrose was added to these cell breakages to make a final concentration of 0.25 M. Following centrifugation, the supernatant was used to measure release of cytochrome *c* from mitochondria to cytosol using Cytochrome *c* ELISA® (EMD Biosciences, Darmstadt, Germany). This assay employed the quantitative sandwich enzyme immunoassay technique. The plate had been precoated with a monoclonal antibody specific for cytochrome *c*. The sample cells were pipetted into the wells, and any cytochrome *c* present in the cells was bound by the immobilized antibody. After all unbound substances were washed away, an enzyme-linked monoclonal antibody specific for cytochrome *c* was added. Following a second washing to remove any unbound antibody-enzyme reagents, substrate solution was added. Color development was then terminated by means of a "stop" solution (supplied with the kit), and the final intensity of the color was measured at 450 nm. With the standard curve generated for each set of samples assayed, the cytochrome *c* concentration for each sample was calculated.

Measurement of active caspase-3, caspase-8, and caspase-9

Active caspase-3 was measured using Human Active Caspase-3 Quantikine ELISA® (R&D Systems, Minneapolis, MN), which also employed the quantitative sandwich enzyme immunoassay technique. The quantity of the active caspase-3 protein derived from the cellular lysate was measured in accordance with the manufacturer's instructions. To label active caspases in the cells, biotin-ZVKD-fluoromethylketone (fmk), a biotinylated caspase inhibitor, was used to covalently modify the large subunit of caspase-3. The inhibitor was added directly to the cell cultures treated with and without H_2S. The cells (2.2×10^5 cells/mL) were put in a microplate precoated with a monoclonal antibody specific for caspase-3. After the cultures were washed to remove any unbound substances, streptavidin conjugated to horseradish peroxidase (streptavidin-HRP), which binds to the biotin on the inhibitor, was added to the wells. After the second washing, the substrate solution was also added to the wells. The amount of active caspase-3 is directly proportional to the amount of biotin-ZVKD-fmk-modified large subunit of caspase-3. The absorbance of samples was measured at 450 nm. With the standard curve generated for each set of samples assayed, the quantity of the active caspase-3 protein was calculated.

Active caspase-8 was measured using Human Caspase-8 ELISA® (Bender MedSystems, Vienna, Austria), and active caspase-9 was measured using Human Caspase-9 ELISA® (Bender MedSystems). An antimonoclonal antibody specific for human caspase-8 or caspase-9 was first adsorbed into microwells. The cells (5×10^6 cells/mL) treated with and without H_2S for 24 and 48 h were then added to the microwells, and the enzymes present in the samples became bound to antibodies adsorbed to the microwells. The detection antibody supplied with these systems was added to bind to human caspase-8 captured by the first antibody. After all unbound detection antibody was washed away, anti-rabbit-IgG-HRP (supplied with the kit) was added and bound to the detection antibody. Following another washing to remove unbound anti-rabbit-IgG-HRP, substrate solution reactive with HRP was added to the microwells. A "stop" solution was added to terminate the reaction, and the absorbance of samples was measured at 450 nm. With the standard curve generated for each set of samples assayed, the amount of active caspase-8 or caspase-9 was calculated.

Statistical analysis

Results are presented as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance followed by Fisher's PLSD post hoc analysis. A p value of less than .05 was regarded as statistically significant.

Results

Cellular viability

To determine cellular viability, the samples were examined using the Trypan blue exclusion test. As shown in Fig. 1, there were no statistical differences between the groups at each incubation time. More than 95% of the cells were found to be viable in the H_2S-treated cells and positive controls at each time-point assessed, thereby establishing that necrosis was not induced by H_2S.

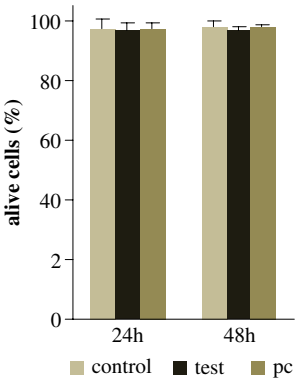

Fig. 1 Effect of H_2S on cell viability. Necrosis of HGFs was evaluated 24 and 48 h after exposure to 50 ng of H_2S with Trypan blue exclusion test. There were no significant differences between the groups. Positive control (*pc*). (N=7)

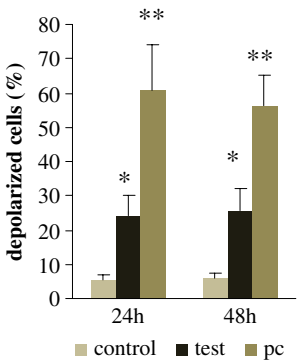

Fig. 3 Effect of H_2S on mitochondrial membrane potential. The changes in mitochondrial membrane potential were assessed by flow cytometry at 24 and 48 h. The percentage of depolarized cells was significantly increased by exposure to H_2S for 24 and 48 h and also significantly increased in positive control (*pc*). (N=5, *p< 0.05, **p<0.001)

Apoptosis assay

To determine whether H_2S induced apoptosis in HGFs, we used flow cytometry to find the proportion of apoptotic cells. Apoptotic cells at the early and mid-stages exhibited only Annexin V-PE-positive. These cells clearly demonstrated that apoptosis has in fact occurred. The ratio of apoptosis is shown in Fig. 2. A significant difference between groups was found at both 24 and 48 h in the proportion of apoptotic cells (see Fig. 2). At 24 h, the control group was 4.7±2.8%; the test and positive control groups were 11.2±3.7% and 18.8±3.6%. At 48 h, the control group was 3.9±1.7%; the test and positive control groups were 18.7±6.2% and 20.5±8.1%.

Mitochondrial depolarization

Mitochondrial membrane potential in HGFs was assessed to investigate the contribution of the mitochondrial pathway in H_2S-induced cell apoptosis. As shown in Fig. 3, H_2S-treated cells showed depolarized mitochondrial membrane potential, whereas control cells displayed the phenomenon of polarized mitochondria. The rate of depolarized cells in the control group was 5.2±1.7% at 24 h and 6.0±1.3% at 48 h. In contrast, the rate of depolarized cells in the test group was 24.1±6.2% at 24 h and 25.5±6.8% at 48 h. Thus, a significant difference was found between test and control groups at both 24 and 48 h. These data indicated that H_2S led to mitochondrial membrane depolarizing of HGFs at 24 and 48 h.

Detection of cytochrome *c* released from mitochondria in H_2S-treated HGFs

To determine the role of the mitochondrial pathway in H_2S-induced apoptosis, the release of cytochrome *c* from mitochondria into cytosol was detected with a semiquantitative sandwich ELISA.

As shown in Fig. 4, the release of mitochondrial cytochrome *c* was dramatically increased by H_2S treatment. A significant increment was found in H_2S-treated cells at both 24 and 48 h (0.1±0.05 ng/mL in controls and 2.0± 1.5 ng/mL in test cells at 24 h; 0.3±1.5 ng/mL in controls and 2.1±0.9 ng/mL in test cells at 48 h). This means that H_2S promoted the release of mitochondrial cytochrome *c* to cytoplasm after depolarization of the mitochondrial membrane.

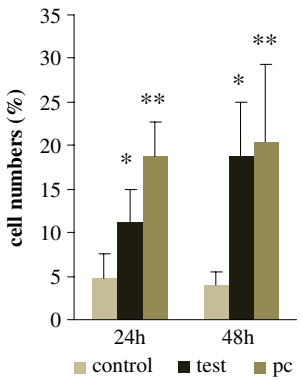

Fig. 2 Detection of apoptotic cells. Proportion of apoptotic cells induced by H_2S was evaluated 24 and 48 h after exposure to 50 ng/mL of H_2S. The percentage of apoptotic cells was significantly increased by exposure to H_2S for 24 and 48 h and also significantly increased in positive control (*pc*). (N=7, *p<0.05, **p<0.01)

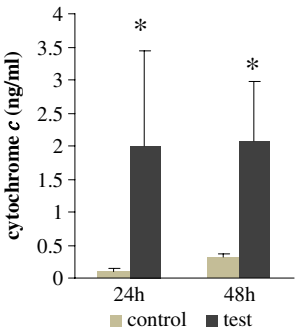

Fig. 4 Effect of H_2S on release of cytochrome *c*. Release of cytochrome *c* into cytoplasm was dramatically increased by H_2S treatment. At both 24 and 48 h, a significant difference was found between groups (N=5, p<0.05)

Assessment of caspase-3, caspase-8, and caspase-9 activities in HGFs

Figure 5 shows the quantity of active caspase-3 (Fig. 5a), caspase-9 (Fig. 5b), and caspase-8 (Fig. 5c) in HGFs. The quantity of active caspase-3 was significantly higher in H_2S-treated cells compared to control cells (0.22±0.1 ng/mL in controls and 2.9±1.3 ng/mL in test cells at 24 h; 0.3±0.1 ng/mL in controls and 4.1±3.2 ng/mL in test cells at 48 h). Similarly, active caspase-9 was significantly increased in test cells (0.2±0.1 ng/mL in controls and 2.9±1.3 ng/mL in test cells at 24 h; 0.3±0.1 ng/mL in controls and 4.1±3.2 ng/mL in test cells at 48 h). On the other hand, there were no differences between the groups with respect to the quantity of active caspase-8. These results demonstrate that H_2S activated procaspase-3 and caspase-9 only but not caspase-8.

Discussion

Gingival epithelial tissues play a key role in periodontal pathogenesis by forming a barrier against penetration of periodontal pathogens and the detrimental products of microorganisms. It has been known that VSCs both increase the permeability of the tissue in a crevicular epithelial model [23] and enhance the secretion of prostaglandin, cAMP, and procollagenase by HGFs [15]. Furthermore, VSCs increase collagen degradation and reduce collagen synthesis in HGFs [24–27], then inhibit the proliferation of gingival epithelial cells [28]. Taken together, these observations suggest that VSCs may play an important role in the etiology of periodontal disease.

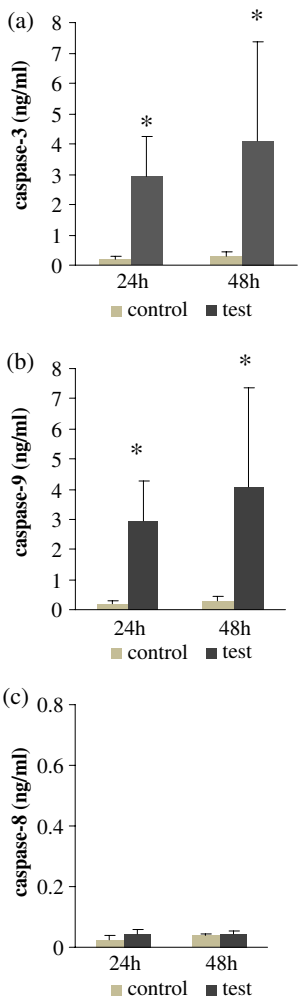

Fig. 5 Activation of caspase-3. caspase-9, and caspase-8. **a** Caspase-3 was dramatically increased by treatment of H_2S. *Asterisk* indicates a statistically significant difference between the test and control groups (N=5, p<0.05). **b** Caspase-9 in HGFs treated by H_2S was significantly elevated compared to control cells. *Asterisk* indicates a statistically significant difference between the test and control groups (N=5, p<0.05). **c** Caspase-8 protein in H_2S-induced apoptotic HGFs was not significantly elevated (N=5, p>0.05)

One of the effects of VSCs on periodontal tissues is apoptosis, as reported previously [17, 18]. In the present study, we aimed to determine the effect of H_2S on apoptosis and its apoptotic pathway in HGFs. As described above, the number of necrotic cells was not determined by a flow cytometer, Trypan blue exclusion test was carried out to verify whether necrosis was induced by H_2S at this concentration in HGFs. Our results indicated that H_2S did not induce necrosis in HGFs. Thereafter, we identified the cells in early and mid-stages of apoptosis using Annexin V staining. Annexin V detects phosphatidylserine which translocates to the surface of the plasma membrane undergoing apoptotic cell death. On the other hand, 7-AAD never stain alive cells as well as early apoptotic cells but labels necrotic and those in the late apoptosis. Three fractions of cells are distinguished with a flow cytometer using these two stains; alive cells show Annexin V-negative/7-AAD-negative, early apoptotic cells show Annexin V-positive/7AAD-negative, and necrotic or late apoptotic cells show Annexin V-positive/7AAD-positive. However, necrotic cells cannot be identified separately from late apoptotic cells in this fraction as mentioned above. Therefore, we combined our flow cytometric data with the results of Trypan blue exclusion test. It was clearly shown that H_2S induced apoptosis but did not induce necrosis in HGFs.

The change in mitochondrial membrane potential and the release of cytochrome *c* from mitochondria into cytosol were assessed in this study. H_2S decreased the mitochondrial membrane potential and increased the release of cytochrome *c* from mitochondria. Because these changes trigger a mitochondrial-dependent apoptosis, this study elucidated that the pathway for mitochondrial-dependent apoptosis was activated by H_2S.

Caspase-3 has been implicated in the effector caspase because both receptor ligand-mediated and mitochondrial pathways converge at the level of caspase-3 activation to amplify initiation signals into commitment to apoptosis [29, 30]. Detection of increased caspase-3 activity proves that apoptotic mechanisms are underway. We also showed that caspase-3 activity was dramatically increased by H_2S.

Caspase-8 and caspase-9 are known as the two major upstream activators of caspase-3. Caspase-8, an initiator

activated in the receptor ligand-mediated apoptotic pathway [29], promotes the release of cytochrome *c* indirectly through cleavage of Bid, a pro-apoptotic Bcl-2 family member [21, 29]. On the other hand, caspase-9 is activated by cytochrome *c* involved in the mitochondrial pathway [29]. Our results showed that there were statistically significant differences between control and test groups in the quantity of caspase-9, whereas the differences between the groups in caspase-8 were not statistically significant. The implication is that H_2S activated caspase-3 and induced apoptosis probably by activating the mitochondrial pathway, but that the extrinsic pathway was not involved in H_2S-induced apoptosis. Yaegaki et al. [18] reported that H_2S strongly inhibits superoxide dismutase in HGFs and significantly increases ROS amount. Hence, an incremental increase in the amount of ROS may start the mitochondrial apoptotic pathway as well as COX inhibition by H_2S. Furthermore, it was implied that increased ROS caused by SOD inhibition with H_2S might be carcinogenic [31].

Recently, many reports have shown a relationship between periodontitis and apoptosis. Ekuni et al. [16] found many apoptotic cells among gingival sulcus keratinocytes and gingival periodontal ligament fibroblasts in experimental periodontitis. It was reported that infection of *Porphyromonas gingivalis* brought out a temporal shift from cellular survival response to apoptosis in HGFs through the activation of pro-apoptotic genes and the deactivation of anti-apoptosis genes [32]. Gamonal et al. [33] examined human gingival tissue accompanied by chronic adult periodontitis and detected DNA fragmentation, active caspase-3-, Fas-, FasL-, and p53-positive cells. Meanwhile, Bulut et al. [34] also found that *bcl-2* expressions were more frequent in generalized aggressive periodontitis than in healthy tissues. The investigators suggested that delayed apoptosis may lead inflammatory cells to stay locally in periodontal tissues; thus, excessive cytokine secretion following the former process may cause progressive periodontal destruction. Jarnbring et al. [35] found reduced proliferation and increased apoptosis in the apical part of the periodontal pocket and suggested that these findings may explain a net loss of keratinocytes in that area. Collectively, these results may imply that the apoptosis occurring in periodontal tissues is one of the etiologic processes of periodontitis and contributes to its destruction.

In this study, we employed 50 ng H_2S/mL air resulting 18 ng H_2S/mL in the culture medium, which is supposed under the realm of pathologic concentration of H_2S in gingival crevicular fluid, since a pathologic concentration was reported as much higher than the concentration employed in this study [22]. Persson [22] also reported the several physiologic cases which demonstrated similar concentrations as utilized in this study. On the other hand, high concentration of H_2S might permanently exist in the gingival crevicular fluid; however, it does not seem like causing a significant damage to gingival tissues in vivo. When H_2S contacts with blood, the compounds are immediately oxidized, i.e., some of H_2S may be detoxified in gingival tissues [36]. Furthermore, the wound-healing mechanism might have an important role in quick recovery from H_2S damages to the tissues. Therefore, if the oxidative or wound-healing mechanisms are deteriorated, H_2S might cause its strong toxicities.

As discussed above, p53 or Bcl-2 family member is implied to be one of the triggers of apoptosis in gingival tissues. Yaegaki et al. [18] reported that genomic DNA damage is caused by H_2S in HGFs. This fact may suggest that the p53 pathway might be involved in the apoptotic process in HGFs, as DNA damage can be a trigger of p53 pathway [33]. To appreciate the relationship between periodontitis and apoptosis by H_2S, other apoptotic molecules, particularly the p53 and Bcl-2 may be investigated.

Conclusion

H_2S induces apoptosis by activating the mitochondrial intrinsic pathway involving mitochondrial depolarization, cytochrome *c* release, caspase-9, and caspase-3. However, the receptor ligand-mediated pathway involving caspase-8 was not triggered by H_2S.

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The authors declare that they have no conflict of interest.

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