Ultrastructural changes in epithelial cells of rats exposed to low concentration of hydrogen sulfide for 50 days

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ABSTRACT

Hydrogen sulfide (H$_2$S) and other volatile sulfur compounds (VSCs) appear mainly in the oral air of patients with halitosis. It seems that VSCs are directly involved in the pathogenesis of gingival diseases. In previous studies, short-term (7 hours-4 days), high concentrations (5-400 ppm) of H$_2$S applications on periodontal tissues have been evaluated in a culture medium.

The aim of the present study was to investigate the potential effects of lower (equivalent to halitosis) concentrations of H$_2$S on rat gingival tissue for longer-term inhalation. The threshold level of pathologic halitosis perceived by humans at 250 ppb of H$_2$S was converted to rat equivalent concentration (4.15 ppm). Rats in the experimental (H$_2$S) group (n=8) were exposed to H$_2$S continuously but not the control rats (n=8).

After 50 days, the gingival sulcular tissue samples of each rat were taken and examined using transmission electron microscope. Ultrastructural changes in the sulcular epithelia of the rat gingiva showed deformation of cellular shape, vacuolization, and disintegrality of intercellular connection by loss of desmosomes and collagen fibrils. No basal membrane damage was observed. Inhalation of low levels of H$_2$S (equivalent of halitosis) in the oral environment causes ultrastructural cellular damages in rat sulcular mucosa.

These results suggest that halitosis may be the potential reason for periodontal destruction in human.

KEYWORDS
Desmosom; halitosis; hydrogen sulfide; sulcular tissue; vacuolization

INTRODUCTION

Poor oral hygiene, existence of bacterial plaque, or tongue coating are the major reasons of halitosis. The gases that contribute to Type 1 (oral) halitosis are (greatest to least) volatile sulfur compounds (VSCs) including hydrogen sulphide (H$_2$S), methyl mercaptan (CH$_3$SH), and dimethyl sulfide ((CH$_3$)$_2$S), volatile organic compounds (VOCs) and nitrogen containing gases (amines) [1]. These compounds are produced through the putrefaction of proteins by mainly anaerobic oral microorganisms. VSCs are not only associated with oral malodor but may also contribute to the etiology of both gingivitis and periodontitis [2].

In vitro studies report that H$_2$S (5 and 10 ng/ mL) inhibits cellular proliferation in gingival epithelial cells [3]. Also 72-hour incubation of H$_2$S (100 ng/mL) induces apoptosis in human gingival epithelial cells and DNA damage in fibroblasts [4] and periodontal cells [5]. VSCs increase permeability in porcine sublingual nonkeratinized mucosa, initiate DNA damage, and cause oxidative stress and p53-mediated apoptosis in keratinocyte stem cells [6]. The role of VSC in periodontal pathogenesis or its destructive mechanism is not clear.

To date, studies have been mostly carried out in culture media with a short-term administration of high concentrations of H$_2$S, such as 5 ppm of H$_2$S for 4 days [7], 50 ppm for 3 days [8], 50 ppm for 2 days [9], 100 ppm for 3 days [4], 100 ppm for 1 day [10], and 400 ppm for 7 hours [11]. It has not been determined whether lower concentration (at halitosis level) and longer-term effect of H$_2$S inhalation on gingival tissues may be destructive.

The aim of the present study was to evaluate ultrastructural changes in the rat gingival sulcular epithelium when animals were exposed to a low concentration (4.15 ppm, human equivalent dose of pathologic halitosis) of H$_2$S via inhalation over a long term.

MATERIAL AND METHODS
Sixteen male Wistar rats (8 weeks old, body weight ranged 245-250 g) were
divided into two groups. The experimental (H₂S) group (n = 8) was exposed to H₂S for 1176 hours (50 days) while the control group was not. Both groups were kept in chambers made of hardened polyfluoro vinyl, which had a 24-hour light-dark cycle, were temperature controlled (25 ± 3°C), and were allowed free access to powdered food and drinking water. Assuming that a = 0.05, fi = 0.20, and 1 - fi = 0.80, it was estimated that eight rats in each group were needed to conduct the study in order to obtain a testing power of \( P = 0.8032 \).

**Criteria for setting H₂S concentrations:**

There is no consensus on the threshold for human halitosis. Despite socially intolerable level of halitosis has been addressed over 700 ppb of H₂S [12], in the literature, 75, 100, 110, 125, 150, or 250 ppb have been established by investigators [1]. A total of 250 ppb (0.25 ppm) of H₂S was considered as the threshold level for minimal pathologic halitosis. The same extrathoracic effect cannot be observed when human equivalent dose of H₂S is given to rat because of large difference between rat and human coverage of alveolar total area. We needed to convert the human threshold concentration for halitosis (250 ppb) to rat equivalent concentration with a previously described formula [13] to account for the interspecies differences in H₂S sensitivity and toxicity. The regional gas dose ratio of the extrathoracic region [RGDR(ET)] was calculated to find the rat equivalent concentration:

\[
\text{RGDR(ET)} = (\frac{\text{VE}}{\text{SA(ET)}})_{a} - (\frac{\text{VE}}{\text{SA(ET)}})_{h}
\]  

(1)

where VE = the minute volume and \( \text{SA(ET)}_{a} \) = the surface area of the extrathoracic region for the rat (a) and human (h).

- \( \text{VE}_{a} = 0.275 \text{ m}^3/\text{day} \)
- \( \text{VE}_{h} = 20 \text{ m}^3/\text{day} \)
- \( \text{SA(ET)}_{a} = 15 \text{ cm}^2 \)
- \( \text{SA (ET)}_{h} = 200 \text{ cm}^2 \)

\[
\text{RGDR(ET)} = (\frac{0.275}{15}) - (\frac{20}{200}) = 0.18
\]

(2)

When calculating the rat equivalent dose associated with halitosis in rats, an uncertainty factor (UF) of 3 was applied for interspecies extrapolation, because dosimetry adjustment was applied to calculate the human equivalent lowest observed adverse effect level [LOAEL_{HEC}]

\[
0.250 \times \text{UF} - \text{RGDR(ET)} = 0.250 \times 3 - 0.18 = 4.15 \text{ ppm (or 4155 ng/mL)}
\]

(3)

Contrary to what we supposed, according to this calculation, a higher concentration of H₂S was needed to obtain the same effect in rats as in humans. As a result, it was found that rat threshold concentration for halitosis was 16.64-fold greater than the human threshold concentration for halitosis.

**Experimental design**

An H₂S cylinder was connected to the gas-mixing chamber (8000 cm³) through gas regulators (Airgas Y14-C44SF, Radnor, PA, USA) and a flowmeter (GRV-150GK0010G, HONSBERG Instruments GmbH, Reimscheid, Germany). Fresh air was obtained from the atmosphere with an air compressor through another flowmeter to the gas-mixing chamber. (Figure 1) A 1440, 3 mL/min (0.3 mL/min of H₂S + 1440 mL/min of fresh air) gas mixture was sent to the main chamber. To avoid causing negative or positive air pressure in the chamber, the used air in the main chamber was passively exhausted with a pipe without aspirating. Single-aspiration volume of one rat is 0.6-2 mL, and inspiration frequency is 70-90/min [14]; thus each rat consumed 180 mL/min of air. Eight rats needed at least 1440 mL/min of air in the main chamber. Premixing gas ratio, H₂S, and fresh air concentration and volume were calculated with a software computer program written by one of the investigators. H₂S concentration in the exhaust air was monitored by a gas detector that is capable of measure in a range 0-500 ppm, with a 0.1 ppm resolution (GasBadge, IndSci, PA, USA) 3 times per day (a total of 150 measurements in 50 days). Thus, it was possible to immediately confirm the accuracy of actual gas passing throughout the experiment.
Figure 1. The H\textsubscript{2}S flow rate (20 000 ppm 150 bar 10 L) was adjusted to 0.3 mL/min, while the fresh air flow rate was set to 1440 mL/min. Both gases were premixed in a chamber (V\textsubscript{1} = 8000 cm\textsuperscript{3}). Under this condition, the main chamber (V\textsubscript{2} = 0.1575 m\textsuperscript{3}) contained 4.15 ppm H\textsubscript{2}S.

TEM analysis
Gingival samples were taken from the lower first molar gingival sulcular tissue of each rat and were processed for transmission electron microscopy (TEM). Approximately 1mm\textsuperscript{3} samples were dehydrated through a graded ethanol series and embedded in Araldite 502 (PA 19440, USA). Ultrathin sections were mounted on copper grids and double-stained with uranyl acetate and lead citrate. Following that, samples were examined by TEM (Zeiss-Libra120 Germany) and photographed.

The study protocol and experimental design were approved by The Animal Ethics Committee of Cumhuriyet University School of Medicine (Process 2012/0297). The study has been carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations. During the procedure, adequate measures were taken to minimize pain or discomfort.

RESULTS
The mean H\textsubscript{2}S concentration of chamber was 4.2 ± 0.98 (mean, ± SD, n = 150) ppm throughout the experiment. To monitorize the H\textsubscript{2}S concentration of the main chamber at the last point (gas exhausting hole) 3 times daily gave the opportunity to confirm validation gas delivery system worked actually. In rats inhaling this dose of H\textsubscript{2}S, significant weight loss was observed (248.3 ± 2.36 vs. 239.5 ± 8.09 (mean ± SD), P < 0.05)), they became anxious, and strove to escape from bght.

In the electron microscopic examination of H\textsubscript{2}S group epithelial tissue, the cells became smaller and rounder (Figure 2B) than those of the control group (Figure 2A). There was intracytoplasmic vacuolization pushing nuclei to periphery.
Roughness of endoplasmic reticulum (ER) (Figure 4C, 5B) and loss of hemidesmosomes appeared (Figure 4B). The intercellular areas between epithelial cells were enlarged (Figure 3B). A decrease in the density of collagen fibrils was seen with disorganization of their structure (Figure 4B, 5B).

**Figure 2.** Control and H\(_2\)S group of sulcular epithelium. TEM images. Bar, 2 pm. (A) In the control group, intact epithelial cells are in polygonal shape and have a euchromatic nucleus (N), surrounded with peripheral heterochromatic regions. Tight interepithelial junctions, desmosomes (d), and tonofilaments (t) are seen. (B) In the H\(_2\)S group, cells have electron-dense cytoplasm (c), and their sizes are smaller and rounder than the control group. Intercellular spaces (s) are expanded with the loss of desmosomes. Nucleus (N).

**Figure 3.** Control and H\(_2\)S group of sulcular epithelium and subepithelial tissues. TEM images. Bar, 2 pm. (A) In the control group, dense collagen fibril (cf) bundles are seen in the dermo-epidermal connection. Basal lamina (bl) and hemidesmosomes (hd) are conspicuous. Tonofilaments (t) observed in the cytoplasm surround euchromatic nucleus (N). (B) H\(_2\)S group cell shows an intracytoplasmic vacuole (V) that pushes a nucleus (N) to the periphery. Extracellular space (S) is enlarged.
Figure 4. Control and H₂S group of sulcular epithelium and subepithelial tissues. TEM images. Bar, 2 μm. (A) In the control group, basal lamina (bl) is definite and hemidesmosomal connections (hd) are in regular intervals. Collagen fibers (cf) are organized regularly in different directions, and fibroblasts (F) are rich in rough endoplasmic reticulum (er) and have normal ultrastructural view. The frame is magnified 2.8 times. (B) In H₂S group, loss of hemidesmosome (black arrows) and collagen fibrils (stars) are seen. (C) The H₂S group shows the loss of collagen fibers and moderate edema (stars) in the dermis. Rough endoplasmic reticulum (er) of fibroblast (F) is slightly dilated. Basal lamina (bl) is seen as intact.
Figure 5. Control and H2S group of connective tissues. TEM images. Bar, 2 μm. (A) In the control group, fibroblast cell (F) with its nucleus (N) and adjacent dense collagen fibrils (cf) are seen in normal appearance. (B) In the H2S group, rough endoplasmic reticulum (er) of fibroblast is slightly dilated and decreased density of collagen fibers (star) (cf) is seen. A macrophage (M) has a normal appearance with its nucleus and cytoplasmic content.
A slight dilation was observed in the ER of fibroblasts (Figure 5B). Moderate edema (Figure 4C) was seen in the subepithelial connective tissue adjacent to the basal lamina; however, basal lamina remained intact. (Figure 4C)

With a separate examination, five histological samples from eight rats of H\textsubscript{2}S group and five samples from eight rats of control group were taken from their gingival tissues near the cross-side lower first molar and then stained with hematoxylin and eosin for light microscopic examination (x40). In the H\textsubscript{2}S group samples, mean lymphocyte infiltration to subepithelial connective tissue was 9 ± 2 cells per every microscope area while in the control group samples, 0 or 1 cell was counted (data not shown).

**DISCUSSION**

To our best knowledge, there are no long-term inhalation studies in animals published in literature. The olfactory mucosa is the first site to be affected by H\textsubscript{2}S exposure [13]. Nasal olfactory lesions were reported in rats exposed to H\textsubscript{2}S at 42 or 110 mg/m\textsuperscript{3} (~30 or 78 ppm, respectively); but no adverse effect was observed at 14 mg/m\textsuperscript{3} (10 ppm) [15]. Human mucosal irritation was reported at concentrations of 2.5-5 ppm over a 15-minute period [16], yet no effects on rats were observed at concentrations even up to 80 ppm for 90 days in rats [17]. One should not assume that an H\textsubscript{2}S concentration toxic for humans is necessarily toxic for rats. In this study, 4.15 ppm was used which was lower than the irritation limit for rats and no visible adverse effects were observed except weight loss in the rats in the experimental group.

H\textsubscript{2}S is methylated to CH\textsubscript{3}SH and (CH\textsubscript{3})\textsubscript{2}S by organisms [11,18,19]. If H\textsubscript{2}S is present in a biological medium, methylated sulfides are also found there. H2S is inhaled to the lungs or it is swallowed by dissolving in the saliva [20] due to high solubility of H\textsubscript{2}S (1 gr /242 ml water at 25°C). Throughout the experiment, it dissolved in the oral or nasal secretion and tear, finally reaching the gingiva of the rats passing their nasopharynx or through the nasolacrimal canal. On the other hand, H\textsubscript{2}S may induce systemic effects, secondarily affecting the gingival tissue. The present study focused on the link between volatile gases responsible for halitosis and sulcular tissue changes in rat’s oral environment. The exact concentration of H\textsubscript{2}S which influenced the rat gingival epithelia remains unknown.

In the literature, VSC application was shown to result in inhibition of cell growth and proliferation and increased permeability [21] as well as cell cycle arrest and decreased DNA synthesis in oral epithelial cells [3] and apoptosis of periodontal ligament cells [5]. Parallel to previous findings, this study revealed that H\textsubscript{2}S-exposed cells were rounder and smaller compared to those of the control group.

Desmosomes contribute to tissue integrity by means of intercellular connections in the epidermis to obtain resistance to mechanical forces. Desmosome connections between cells in the stratum spinosum, granulosum, and basal layer were disassociated and damaged in patients with periodontitis [22]. The findings of the present study, especially those regarding loss of desmosomes between stratum spinosum cells and expansion in the intercellular area, can be evaluated as indicator of disintegration, or inflammation in epithelial tissue. Further, the present study demonstrates that H\textsubscript{2}S induced vacuolization in epithelial cells; it has already been reported that vacuolization was one of the most important alterations occurring in chronic inflammation of gingiva [23]. Loss of tonofilaments’ clarity and electron-dense cytoplasm were also observed in the desmosome-deficient cells. These findings may be indicative of impairment of the cytoskeletal structure of epithelial cells.
H$_2$S was shown to induce apoptosis in epithelial cells derived from human gingiva [4] and increase collagen solubility in human gingival fibroblasts [24]. Damage in collagen fibril bundles was seen in the H$_2$S group (Figure 4B, 5B). However, there was no evidence for apoptosis in epithelial cells or visible damage to basal lamina. These effects may be dose dependent. Basal lamina would be disintegrated when higher concentration of H$_2$S is applied or when tissue is exposed to H$_2$S for a longer period. In the experiment group, there was slight dilatation in the cisternae of rough endoplasmic reticulum (RER) compared to control group. RER is an organelle of fibroblasts playing an important role in collagen synthesis and it contains electron-rich material. This may be related to ER stress. In ER stress, folding process of proteins produced is adversely influenced and in consequence, unfolded or misfolded proteins accumulate in ER lumen [25,26]. Domon et al. [27] reported that the expression levels of UPR (unfolded protein response)-related genes were significantly higher in periodontal lesions compared with gingival lesions due to ER (endoplasmic reticulum) stress. These accumulated proteins are the result of proteotoxic threat to cellular viability and may lead the cell to undergo apoptosis [25]. Recently, degenerative changes in sulcular epithelium and periodontal tissues near the basal layer-subepithelial connective tissue junction have been observed in rats. Also, massive inflammatory cell infiltration in the subepithelial connective tissue and periodontal membrane and enlarged Howship’s lacunae in the alveolar bone and cementum tissue have been demonstrated histo- pathologically in rat periodontal tissues [28]. The current results confirm that patients with halitosis may have a risk of degeneration in their periodontal tissues due to inhaling H$_2$S self- produced over their lifetime.

This experiment builds a model for inhaled H$_2$S at a level of halitosis. These results demonstrate potential effects of halitosis on gingival tissues. Serum creatine kinase-MB, aspartate aminotransferase, lactate dehydrogenase, and alkaline phosphatase levels were found to be elevated when compared to the control group (data not shown). All of these changes support the idea that halitosis may have deleterious effects not only locally but also systemically.

**Conclusion**
Low levels of H$_2$S (equivalent of halitosis) inhalation may cause ultrastructural morphological cellular deformation, vacuolization, and disintegrity of intercellular connection by loss of desmosomes and collagen fibrils in rat sulcular mucosa.

**Clinical implications**
Results of this study suggest that halitosis patients may have a low level of intoxication from chronic inspiration of self-produced H$_2$S.

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