STRUCTURAL CHANGES IN PERIODONTIUM OF RATS EXPOSED TO A LOW CONCENTRATION OF HYDROGEN SULFIDE FOR 50 DAYS

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Abstract
The aim of the present study was to investigate the effect of H2S inhalation at a low concentration (at human equivalent dose of pathologic halitosis) on rat periodontium over a long term (50 days). The threshold level of pathologic halitosis perceived by humans at 250 ppb of H2S was converted to rat equivalent concentration (4.15 ppm). Rats in the experimental (H2S) group (n = 8) were exposed to H2S continuously but not the control rats (n = 8). After 50 days, periodontal tissue samples were taken from the mandibular first molar region and examined histopathologically to determine inflammatory cell infiltration (ICI), osteoblastic activities, number of osteoclasts, and resorption lacunae. Sulcular epithelium layer destruction was observed in the H2S group. Frequency of ICI was significantly higher in the H2S group compared to the control group (P <0.05). The number of osteoclasts were found significantly higher in the H2S group (34.28 ± 3.28) compared to the control group (8.85 ± 1.85) (P <0.05) and the number of resorption lacunae were also higher in the cementum tissue (6.1 ± 2.4) and alveolar bone (3.8 ± 1.5) versus their corresponding control groups (1.6 ± 0.5 and 1.4 ± 0.5, respectively) (P <0.05). There were no statistically significant differences between the two groups with regard to osteoblastic activity. H2S inhalation induces inflammatory changes in the periodontium as well as resorption of the alveolar bone and cementum tissue in rats. These histopathologic changes in periodontal tissues support the idea that long-term H2S inhalation may have a destructive effect on periodontal tissues.

Keywords
hydrogen sulfide, halitosis, Howship’s lacunae, periodontal tissue

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**Introduction**

Poor oral hygiene, presence of microbial dental plaque, or tongue coating are the major causes of halitosis. Volatile sulfur compounds (VSCs) that contribute to Type 1 (oral) halitosis are (in decreasing order): hydrogen sulphide (H2S), methyl mercaptan (CH3SH), and dimethyl sulfide ((CH3)2S). Many other (organic or nitrogen-based) gases also contribute to oral malodor. These compounds are produced through putrefaction of proteins mainly by anaerobic oral microorganisms. VSCs are not only associated with oral malodor but may also contribute to the etiology of both gingivitis and periodontitis. In vitro studies have reported that H2S (5 and 10 ng/mL) inhibits cellular proliferation in gingival damage, and cause oxidative stress and p53-mediated apoptosis in keratinocyte stem cells. Some studies showed that VSCs increase the permeability of oral mucosa and break down the basement membrane of the gingival sulcus epithelium, thus facilitating the penetration of periodontal-disease causative agents. In other studies, it was demonstrated that the 72-h incubation of H2S (100 ng/mL) induced apoptosis in human gingival epithelial cells and DNA damage in fibroblasts. Furthermore, H2S was also found to inhibit proliferation of osteoblasts at a concentration of 100 ng/mL and cause apoptosis in osteoblasts at 50 ng/mL and has an additive effect on osteoclast differentiation. It is therefore suggested that VSCs are initiators of the periodontal disease.

However, periodontal disease is a result of many processes in vivo. These processes include direct destruction of host tissues by bacterial enzymes and a multitude of other mechanisms of chronic irritation to tissue, and production of proteinases and cytokines by stimulation of the immune system. To date, studies have been mostly carried out in culture media with short-term administration of high concentrations of H2S such as 5 ppm of H2S for 4 days, 50 ppm for 3 days, 50 ppm for 2 days, 100 ppm for 3 days, 100 ppm for 1 day and 400 ppm for 7 h. It has not been investigated whether inhalation of H2S at a low concentration (human equivalent concentration inducing halitosis) over a long term (50 days) has any destructive effects on gingival tissue in rats.

The aim of the present study was to evaluate histological changes in the rat periodontal tissue when animals were exposed to a low concentration (4.15 ppm, human equivalent dose of pathologic halitosis) of H2S via inhalation over a long term (50 days).

**Materials and methods**

Sixteen male, 8-week-old Wistar rats (body weight range, 245–250 g) were divided into two groups. The experimental (H2S) group (n = 8) was exposed to H2S for 1176 h (50 days) while the control group was not. Both groups were kept in temperature-controlled (25 ± 3°C) chambers made of hardened polyfluorovinyl, which had a 24-h light–dark cycle, and allowed free access to powdered food and drinking water. The experimental protocol was approved by the Ethics Committee for Animal Research of Cumhuriyet University Faculty of Medicine (Protocol no. 2012/0297). The study has been carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and applicable local laws and regulations. During the procedure, adequate measures were taken to minimize pain or discomfort in animals.
Figure 1. The H\(_2\)S tube (20,000 ppm 150 bar 10 L) flow rate 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\(_1\) = 8000 \(\text{cm}^3\)). Under these conditions, rats exposed to 4.15 ppm H\(_2\)S in the main chamber (V\(_2\) = 0.1575 \(\text{m}^3\)).

Criteria for setting H\(_2\)S concentrations

There is no consensus on the threshold for pathologic halitosis. Although a socially intolerable level of halitosis is considered as an H\(_2\)S concentration above 700 ppb,\(^2\) pathological levels of 75, 100, 110, 125, 150, and 250 ppb have been established by investigators.\(^2\) A total of 250 ppb (0.25 ppm) of H\(_2\)S was considered as the threshold level for minimal pathologic halitosis. As a rule, a biologic effect occurs when a living organism is exposed to a toxic gas, the extent of which depends on many factors including minute volume and surface area of the extrathoracic region (nose, mouth, nasopharynx, oropharynx, laryngopharynx, larynx). Since respiratory parameters greatly differ between humans and rats, the human threshold concentration for halitosis (250 ppb) must be converted to rat equivalent concentration by using the following formula:\(^22\)

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

where VE is the minute volume and SA(ET) is the surface area of the extrathoracic region for the rat (a) and human (h). VE(a) is 0.275 m\(^3\)/day, VE(h) is 20 m\(^3\)/day, SA(ET)a is 15 cm\(^2\), SA(ET)h is 200 cm\(^2\).

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

When calculating the rat equivalent dose, 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\textsubscript{HEC}].\textsuperscript{22}

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

Eventually, it was found that a H\textsubscript{2}S concentration of 4.15 ppm in rats was equal to the H\textsubscript{2}S concentration Yeler et al. \textsuperscript{95} (250 ppb) that is usually found in the mouth of individuals with minimal pathologic halitosis.

**Experimental design**

An H\textsubscript{2}S cylinder (20,000 ppm) was connected to the gas-mixing chamber (8000 cm\textsuperscript{3}) through gas regulators (Airgas Y14-C445F, USA) and a flow meter (GRV-150 GK0010G, Honsberg, GmbH). Fresh air was obtained from the atmosphere with an air compressor through another flow meter to the gas-mixing chamber (Figure 1). A 1440.3 mL/min (0.3 mL/min of H\textsubscript{2}S + 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, used air in the main chamber (0.1575 m\textsuperscript{3}) 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;\textsuperscript{23} thus, each rat consumes 180 mL/min of air. Eight rats need at least approximately 1440 mL/min of air in the main chamber. Premixing gas ratio, H\textsubscript{2}S, and fresh air concentration and volume were calculated with a computer program written by one of the investigators. H\textsubscript{2}S concentration in the exhaust air was monitored by a gas detector that was capable of measuring a range of 0–500 ppm, with a 0.1 ppm resolution (GasBadge, IndSci, PA) three times per day (a total of 150 measurements in 50 days). Thus, it was possible to confirm the accuracy of actual gas passing throughout the experiment.

**Histopathological evaluation**

Due to similarities in structure and anatomic configuration of first molars in rats and humans,\textsuperscript{24} molar teeth were chosen for histologic examination. Specimens were fixed in a 10% neutral-buffered formalin solution and demineralized in an aqueous 10% formic acid solution. The specimens were then dehydrated, embedded in paraffin, and sectioned along the molars in a mesiodistal plane for hematoxylin-eosin (H&E) staining before examination under light microscopy. Inflammatory cell infiltration (ICI) was scored as 0, not visible; 1, slightly visible; and 2, dense.\textsuperscript{25} Also, the numbers of resorption lacunae and osteoclasts were noted. The presence of osteoblastic activity was determined by the visibility of active bone formation surfaces that were bordered by osteoid and cuboidal osteoblasts. If they were not visible the score was 0, mild to moderate visibility was scored as 1 and existing osteoblastic activity with a dense mass was scored as 2.\textsuperscript{25} The same examiner performed histological evaluation in a blinded manner.

**Statistical analysis**

Assuming that $\alpha = 0.05$, $\beta = 0.20$, and $1-\beta = 0.80$, it was estimated that eight rats in each group were needed to conduct the study in order to obtain a testing power of $P = 0.8032$. The frequency of ICI and osteoblastic activity were analyzed using a Chi-square test. Osteoclast number and resorption lacunae formation were analyzed using a Mann–Whitney U test. Data were presented as mean ± standard deviation or as percentages, as appropriate. A P value below 0.05 was considered statistically significant and the data were analyzed with SPSS 22.0.

**Results**

The mean H\textsubscript{2}S concentration of chamber was $4.2 \pm 0.98$ (mean ± SD) ppm throughout the experiment. A significant weight loss was observed in rats inhaling this H\textsubscript{2}S dose (mean ± SD; 248.3 ± 1.17
versus 239.5 ± 2.5, respectively, P <0.05). In the H2S group, each layer of sulcular epithelium showed degenerative changes (Figure 2b, 2d). Polymorphonuclear cell infiltration was observed in the intercellular space (Figure 2b) and epithelial basal layer showed degeneration and irregularities (Figure 2b, 2d). Figures 2a, 2c, 2e are from the control group. ICI was observed in the subepithelial connective tissue (SEC) and periodontal membrane (PM) in both groups but the percentages of ICI with scores of 1 and 2 were higher in the experimental group versus the control group (P <0.05) (Figures 2d, 2f) (Table 1). Figures 2f and 2g present the number of Howship’s lacunae in the cementum tissue and alveolar bone in the study groups. Howship’s lacunae were found in all rats but the H2S group showed significantly higher numbers of resorption lacunae in comparison to the control group. Also, the resorption lacunae were found to be denser and larger in the cementum tissue (6.1 ± 2.4) (Figure 2g) than the alveolar bone (3.8 ± 1.5) (Figure 2f) while their controls were 1.6 ± 0.5 and 1.4 ± 0.5, respectively (P <0.05) (Figure 2h). Osteoclasts were found in all rats; however, osteoclast numbers were significantly higher (34.28 ± 3.28) in the H2S group compared to the control group (8.85 ± 1.85) (P <0.05). Osteoblastic activity was not significantly different between both groups (P >0.05).

**Figures 2:**
Figure 2. Histopathology of the mandibular first molar in all groups. (a) In the control group, sulcular epithelium (SE) and epithelial cells of basal layer (BL), connective tissue (C) seem normal (hematoxylin-eosin (H&E) stain; bar = 100 µm). (b) In the experimental H2S group, degenerative changes and polymorphonuclear cell infiltration (PNL) are seen in the gingival epithelium (asterisk) along with degeneration and irregularities in stratum basale (BL) (H&E stain; bar = 100µm). (c) In the control group, sulcular epithelium (SE) and subepithelial connective tissue (C) seem normal (H&E stain; bar = 100 µm). (d) In the H2S group, degenerative changes in sulcular epithelium (asterisk) and nearby basal layer (BL)–subepithelial connective tissue junction are observed. There is also massive inflammatory cell infiltration (ICI) in the subepithelial connective tissue (C) compared to the control group (H&E stain; bar = 100 µm, second image inside the first one, has been magnified ×2.3). (e) In the control group, alveolar bone (A), periodontal membrane (P), and cementum tissue (C) seem normal (H&E stain; bar = 100 µm). (f) In the H2S group, ICI is seen in the periodontal membrane (P) and several enlarged Howship’s lacunae (HL) in the alveolar bone (A) (H&E stain; bar = 100 µm). (g) In the H2S group, the arrows indicate Howship’s lacunae in the cementum tissue (C). Periodontal ligament (P) (H&E stain; bar = 100 µm). (h) Numbers of Howship’s lacunae in the alveolar bone and cementum tissue (P <0.05).

Table 1 Frequency (%) of inflammatory cell infiltration (ICI) in the both groups.

<table>
<thead>
<tr>
<th>Inflammatory cell infiltration score</th>
<th>Subepithelial connective tissue</th>
<th>Periodontium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H2S group</td>
<td>Control group</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>14.3</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>85.7</td>
<td>0</td>
</tr>
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</table>

H2S, experimental group; ICI, inflammatory cell infiltration; PM, periodontal membrane; SEC, subepithelial connective tissue.
Discussion
In this study, 4.15 ppm was used, which is the rat equal concentration of the threshold for perceivable (minimal) halitosis in humans. Thus, results of the current study can be used to estimate any potential damage in human gingival tissues of halitosis patients who continuously inhale orally produced halitosis gases. Since this concentration (4.15 ppm) was lower than the irritation limit for rats, no visible adverse effects were observed except weight loss in the rats in the experimental group. H₂S was administered via the oro-nasal airway in the present study. H₂S is inhaled to the lungs or it is swallowed by dissolving in the saliva due to high solubility of H₂S (1 g /242 mL water at 25°C). Throughout the experiment, it dissolved in the oral or nasal secretion and tear, eventually reaching the gingiva of the rats after passing through nasopharynx or nasolacrimal canal. On the other hand, H₂S may induce systemic effects, secondarily affecting the periodontal tissue. The present study focused on the link between volatile gases responsible for halitosis and periodontal changes in the rat’s oral environment. The exact concentration of H₂S associated with adverse effects on the rat gingival epithelia remains unknown.

H₂S is methylated to CH₃SH and (CH₃)₂S by microorganisms. When H₂S is present in a biological medium, methylated sulfides are also found there. Thus, cellular effects demonstrated in this study may be related to H₂S or its methylated forms. However, in the literature, administration of VSCs (including CH₃SH and (CH₃)₂S) to oral epithelial cells was demonstrated to increase permeability of oral mucosa, inhibit cell growth and proliferation, induce cell cycle arrest, and decrease DNA synthesis significantly in a large number of impaired periodontal cells. In an additional study, deformation, vacuolization, and disintegration of intercellular connection by loss of desmosomes and collagen fibrils were ultrastructurally demonstrated in the rat sulcular mucosa (data not shown). Inflammatory cells increase in the gingival epithelium and connective tissue following topical application of NaHS (H₂S donor drug) to rat gingival sulci. Further, it was demonstrated that H₂S enhanced neutrophil migration by increasing their rolling, adhesion, and locomotion. In this experiment, the massive increase in inflammatory cells may have resulted from the effect of H₂S which enhanced vascular permeability and serum diapedesis. Bone metabolism is tightly regulated by osteoclastic bone resorption and osteoblastic bone formation. In pathologic conditions such as periodontal disease, the balance is shifted toward bone resorption. The degenerative changes in periodontal tissue are attributable to a combination of the presence of microorganisms and the host immune factors. In addition to the direct effects of microorganisms, microbiota damages the host cells indirectly by activating the host immune response. Bacteria release lipopolysaccharides (LPS) and other bacterial products to the sulcus, affecting the immune cells in the connective tissue as well as osteoclasts. In the immune cells, these products induce local factor production, such as interleukin-1 (IL-1), prostaglandin E2 (PGE2), and tumor necrosis factor- alpha (TNF-α). These factors can stimulate osteoclast differentiation. It has been shown that H₂S leads to significant increases in the production of cytokines TNF, IL-1, and IL-6. In addition, Irie et al. found that sodium hydrogen sulfide and LPS have an additive effect on differentiation of osteoclasts. Moreover, it has been shown that topical H₂S application enhances production of receptor activator of nuclear factor-κB ligand (RANKL) to trigger osteoclast differentiation. The enlarged and increased resorption lacunae in the H₂S group can be explained by the effects of H₂S on osteoclast differentiation and...
cytokine production in line with the literature findings. In the H₂S group, increased resorption area of the cementum tissue and alveolar bone are consistent with previous short-term effects as observed in other H₂S studies. A previous culture study has also shown that H₂S induces apoptosis in the osteoblasts and inhibits osteoblast proliferation. Unlike the previous studies, we examined the effects of H₂S on osteoblastic activity and no differences were found in the H₂S group.

This experiment builds a model for inhaled H₂S at a level associated with halitosis. These results provide further evidence for potential effects of halitosis on periodontal tissues. In this study, serum levels of creatine kinase-MB, aspartate aminotransferase, lactate dehydrogenase, and alkaline phosphatase were found to be elevated in experimented rats 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.

In conclusion, H₂S inhalation at a rat equivalent dose of human pathologic halitosis level induces inflammatory changes in the periodontium, and resorption of alveolar bone and cementum tissue in rats. These histopathological changes in periodontal tissues support the idea that long-term H₂S inhalation may have a destructive effect on periodontal tissues. While it can be speculated that higher H₂S concentrations might cause greater destruction in periodontal tissues, the physiological level of halitosis associated with such destruction could not be clearly defined.

Declaration of conflicting interests
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