

## H<sub>2</sub>S in periodontal immune-inflammatory response and bone loss: a study in rats

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### ABSTRACT

Halitosis is highly prevalent in periodontitis and attributed mainly to the presence of volatile sulfur compounds (VSC), where hydrogen sulfide (H<sub>2</sub>S) is the chief culprit in the characteristic malodor of periodontitis and thus may play an active role in its pathogenesis. The aim of this study was to evaluate the effect of H<sub>2</sub>S in the acute, intermediate and chronic immune-inflammatory host response and alveolar bone loss in vivo by using an animal model of induced periodontal disease. Thirty-six rats were divided into 2 groups: test group (n = 18), rats exposed to H<sub>2</sub>S (NaHS - H<sub>2</sub>S donor molecule) and control group (n = 18), rats treated with saline only (Ctrl). All animals had one of their lower second molars ligated to induce periodontal disease (PD). The sound contralateral molar was used as control (H). Each group was subdivided into 3 (n = 6), according to follow-up time (3h, 5 days and 14 days). The gingival tissue was used for mRNA expression analysis (IL-1, IL-6, RANKL, OPG and

SOFAT) by real-time PCR and the mandibles were analyzed morphometrically. Data analysis showed that the ligature promoted alveolar bone loss, observed mainly at 14 days, both in the group exposed to H<sub>2</sub>S and in the Ctrl group. H<sub>2</sub>S administration did not result in additional bone loss. Gene expression showed a significant increase in IL-1, IL-6, RANKL and SOFAT only in the Ctrl-PD group (p<0.05). A significant downregulation in OPG expression was observed over time in the Ctrl-PD group (p<0.05). In conclusion, H<sub>2</sub>S had no effect on alveolar bone loss in the absence of a ligature. In the presence of a ligature, however, exposure to H<sub>2</sub>S had an immunoregulatory effect on the expression of pro-inflammatory and pro-resorptive cytokines.

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**Key words:** Hydrogen sulfide, periodontal disease, alveolar bone loss, halitosis.

## Influência do H<sub>2</sub>S na resposta imunoinflamatória e perda óssea periodontal: um estudo em ratos

### RESUMO

A halitose é altamente prevalente na periodontite e é atribuída principalmente à presença de compostos sulfurados voláteis (CSV), sendo o sulfeto de hidrogênio (H<sub>2</sub>S) o principal gás relacionado ao mau odor e que pode estar envolvido na patogênese da doença periodontal. O objetivo deste estudo foi avaliar o efeito agudo, intermediário e crônico do H<sub>2</sub>S na resposta imuno-inflamatória e na perda óssea alveolar em ratos, com e sem doença periodontal induzida. Trinta e seis ratos foram divididos em 2 grupos: teste (n = 18), ratos expostos ao H<sub>2</sub>S (NaHS - molécula doadora de H<sub>2</sub>S) e grupo controle (n = 18), ratos tratados apenas com solução salina (Ctrl). Todos os animais tiveram um dos seus segundos molares inferiores submetidos à colocação de uma ligadura para o desenvolvimento da doença periodontal (DP), em comparação com o dente contralateral saudável (H). Cada grupo foi subdividido em 3 (n = 6), de acordo com o tempo de eutanásia (3h, 5 dias e 14 dias). Os tecidos gengivais foram utilizados para a

análise da expressão gênica (IL-1, IL-6, RANKL, OPG e SOFAT) por PCR em tempo real e as mandíbulas foram analisadas morfometricamente. Análise dos dados demonstrou que a ligadura promoveu perda óssea alveolar; observada principalmente aos 14 dias, tanto no grupo exposto ao H<sub>2</sub>S quanto no grupo Ctrl. A administração de H<sub>2</sub>S não resultou em perda óssea adicional. A expressão gênica demonstrou aumento significativo de IL-1, IL-6, RANKL e SOFAT apenas no grupo Ctrl-PD (p < 0,05). Uma significativa regulação negativa na expressão de OPG foi observada ao longo do tempo no grupo Ctrl-PD (p < 0,05). Pode-se concluir que o H<sub>2</sub>S não teve efeito adicional na perda óssea alveolar, na ausência de ligadura. Entretanto, na presença de ligadura, a exposição ao H<sub>2</sub>S teve um efeito imunorregulatório na expressão de citocinas pró-inflamatórias e pró-reabsorptivas.

**Palavras-chave:** Sulfeto de hidrogênio. Doença periodontal. Perda óssea alveolar. Halitose.

### INTRODUCTION

Periodontal disease may be defined as an infectious condition resulting from the accumulation of bacterial dental biofilm, in which the immune-

inflammatory response plays a crucial role in bone destruction patterns<sup>1</sup>. Several molecules and products are involved in this process, accelerating or preventing bone loss dynamically. The disease

process begins with dysbiosis between the host's immune response and the microbial challenge, where the latter is regarded as a primary etiological factor<sup>2</sup>. Clinically, periodontitis may present as gingival inflammation, decreased tissue resistance to probing, attachment loss, alveolar bone loss, presence of local irritants associated with disease progression<sup>1</sup> as well as a characteristic malodor<sup>3</sup>.

Persistent oral malodor, also known as bad breath or halitosis, is composed of a series of volatile chemicals, mostly sulfur compounds. Sulfur-derived volatile substances, designated by their generic name of volatile sulfur compounds (VSC), include hydrogen sulfide (H<sub>2</sub>S), methyl mercaptan (CH<sub>3</sub>SH) and dimethyl sulfide (CH<sub>3</sub>)<sub>2</sub>S<sup>4</sup>. These compounds are frequently produced in the oral environment as a result of the metabolism of proteins, mainly by anaerobic bacteria<sup>3</sup>.

Evidence has suggested that VSC may be directly involved in the pathogenesis of periodontal disease by increasing epithelial permeability and consequent penetration of lipopolysaccharides and prostaglandins<sup>5</sup>. The presence of VSC in the oral cavity may increase IL-1 and PGE<sub>2</sub> levels, activate collagenase<sup>6</sup>, reduce collagen synthesis and cause apoptosis of gingival fibroblasts<sup>7</sup>. In addition, VSC may act to reduce basement membrane synthesis and proliferation of gingival epithelial cells<sup>8</sup> and osteoblasts<sup>9</sup>.

H<sub>2</sub>S is a gas with multiple tissue-dependent roles, e.g. vasodilation, inflammation, cardiac reaction to ischemic injuries, and is regarded as a Janus-faced molecule because at lower concentrations it causes antioxidant and cytoprotective effects, whereas at higher concentrations it becomes cytotoxic and stimulates oxidative stress<sup>10</sup>. It has been shown that H<sub>2</sub>S accelerates healing in diabetic rats<sup>11</sup> and endogenous levels of H<sub>2</sub>S can preserve the proliferative capacity of stem cells from the periodontal ligament<sup>12</sup>. H<sub>2</sub>S may also lead human gingival fibroblasts to apoptosis and DNA damage with increased levels of reactive oxygen species<sup>7</sup>.

Regarding periodontal disease, H<sub>2</sub>S, which is primarily responsible for halitosis, is a byproduct of bacterial metabolism and is excreted from within the periodontal pocket as well as from other surfaces such as the dorsum of a coated tongue. Therefore, due to its pro-inflammatory properties, H<sub>2</sub>S might play an important role in bacteria-induced inflammatory response in periodontal

diseases<sup>13</sup>. Studies *in vitro* have shown that H<sub>2</sub>S may be involved with the initiation and development of periodontal disease by inhibiting keratinocyte proliferation, reducing protein synthesis by fibroblasts and inhibiting collagen synthesis in the basement membrane<sup>14</sup>. Additionally, a study *in vivo* reported a transient increase in osteoclast differentiation in rats, with up-regulation of RANKL expression in osteoblasts from the periodontal tissues after 3 days of topical application of H<sub>2</sub>S<sup>15</sup>. Clinically, several studies (cited by Basic & Dahlén<sup>16</sup>) have been performed in an attempt to develop methods to measure H<sub>2</sub>S in gingival crevices and correlate its presence with periodontal destruction. Although the studies have shown a positive correlation, the complexity of such methods may have hindered further testing in clinical settings. The fact that the gas is volatile and easily converted to polysulfides has further hampered such endeavors. There is therefore a lack of comprehensive knowledge on the role of H<sub>2</sub>S in bacterial biofilm, the possible effect on local environment and on the host response, as well as on its relationship to clinical parameters in health and disease. In the present study, we hypothesized that H<sub>2</sub>S may have different effects on immunomodulation depending on whether the inflammatory response is acute or chronic with potential consequences to alveolar bone loss.

Taking into consideration the evidence on the supposedly positive correlation between H<sub>2</sub>S and periodontal disease as well as the controversial findings reported in the literature, the aim of this study was to investigate the effect of H<sub>2</sub>S on the immunoinflammatory host response and alveolar bone loss in rats with induced periodontal disease at acute (3h), intermediate (5 days) and chronic stages (14 days).

## MATERIALS AND METHODS

### Ethical statement

This study was approved by the research ethics committee for animal use - CEUA / SLMANDIC, protocol #2017/011. This study was designed and executed according to the ARRIVE guidelines.

### Experimental animals

Thirty-six male mice (*Rattus Norvegicus*, *Albinus*, Wistar, SPF) weighing between 200 and 300g were used. Prior to the experimental procedures, the

animals were acclimatized for 30 days in plastic cages with access to food and water *ad libitum* and with light-dark cycles of 12 hours, under controlled humidity and temperature.

### Study Design

The experimental units (rats) were submitted to treatments for a maximum of 14 days. They were divided into 2 groups:

- NaHS group: 18 rats submitted to daily exposure to H<sub>2</sub>S via topical applications of NaHS (H<sub>2</sub>S donor molecule).
- Control group (Ctrl): 18 rats, treated with saline solution and no exposure to NaHS.

In each group, the animals were randomly divided into 3 experimental subgroups according to follow-up period: 6 animals from each group (NaHS and Ctrl) were euthanized 3 hours after exposure to H<sub>2</sub>S (acute response); 6 animals were euthanized after 5 days of exposure to H<sub>2</sub>S (intermediate response) and 6 animals were euthanized after 14 days of exposure to H<sub>2</sub>S (chronic response), as shown in Fig. 1.

### Experimental procedures

#### a) Inducing Periodontal Disease

The animals were weighed and anesthetized accordingly via intramuscular injection in the external region of the thigh using ketamine solution (0.8 ml / kg / IM) (Francotar<sup>®</sup>; Virbac do Brasil Industria e Comércio LTDA, Roseira, SP, Brazil) and xylazine hydrochloride (0.3 ml / kg / IM) (Virbaxil<sup>®</sup>; Virbac do Brasil Industria e Comércio LTDA, Roseira, SP, Brazil). The animals were positioned on a modified Doku apparatus and a silk thread ligature was placed around one of the lower second molars, randomly selected, at the level of the gingival sulcus, in order to favor accumulation of bacterial biofilm and development of periodontal

disease (PD). Sham-operated animals had the ligature immediately removed after the procedure, serving as control (Healthy-H).

#### b) Exposure to H<sub>2</sub>S

Exposure to H<sub>2</sub>S consisted of topical application of NaHS (H<sub>2</sub>S donor) at 5.6mg NaHS (0.9% saline solution) to the gingival sulcus region of the second molars (with PD and H) using a micropipette. In total, 6 applications were performed within 1 hour at 10-minute intervals. The animals from the Ctrl group underwent the same procedures, except NaHS application, which was replaced with 0.9% saline solution.

#### c) Euthanasia

All animals were euthanized according to the follow-up periods for each group via intraperitoneal injection of barbiturate-based anesthetic [Sodium Thiopental (71-73-8) 150mg / kg and Lidocaine (137-58-6) 10mg / ml]. The mandibles were removed following excision of the gingival tissue from around the second molars.

#### d) Sample collection for analysis

Gingiva: the marginal portion of the gingiva from the ligated teeth was collected along with their respective contralateral controls, using a scalpel and a micro chisel. The tissue samples were stored in Eppendorf tubes containing RNA Later solution (Ambion Inc., Austin, TX, USA). The samples were frozen for subsequent quantitative evaluation of the expression patterns of the following genes related to the immune-inflammatory response and bone metabolism: Interleukin-1 $\beta$  (IL-1 $\beta$ ), Interleukin-6 (IL-6), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and secreted osteoclastogenic factor by activated T cells (SOFAT).

Mandibles: After collecting the gingival tissue, the mandibles were completely removed and stored in 3% hydrogen peroxide overnight followed by staining with 1% methylene blue. Horizontal bone loss was quantified morphometrically by measuring the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest as described by Casati et al.<sup>17</sup>. Measurements were performed by a trained, calibrated (Intraclass correlation = 93%) and treatment-blind examiner (A.J.S.N.), using the public domain software ImageJ (NIH).

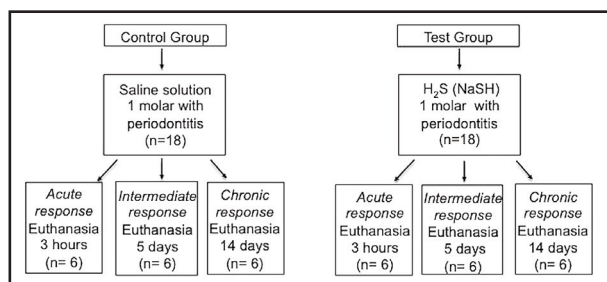


Fig. 1: Experimental design.

### e) Polymerase chain reaction

Total RNA was isolated from the gingival tissue using Trizol reagent (Life Technologies). Briefly, the tissue was collected and homogenized with 1 ml of Trizol, and the aqueous and organic phases were separated by addition of 0.2 ml of chloroform followed by centrifugation (12000 g, 15 minutes, 4°C). RNA was precipitated from the aqueous phase with 0.5 ml of isopropanol (12000 g, 15 minutes, 4°C), washed with 75% ethanol and resuspended in water. One µg samples of RNA were treated with 1U of DNase I and reverse transcriptase cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) reagent according to the manufacturer's instructions. Briefly, reactions occurred from 1 µg of RNA, 0.5 µg of oligo (dT) 18, 1 mM of the dNTP mix, 200 U RevertAid H Minus M-MuLV transcriptase and 20 U of RiboLockRNase Inhibitor at 42° C for 60 minutes. The reactions were then terminated by heating at 70° C for 5 minutes. The amplification reactions occurred from 40 ng of cDNA and 0.3 µM primer pairs (Table 1), added to the Maxima SYBR Green qPCR Master Mix (Thermo Scientific). Reaction conditions were as follows: 10 minutes at 95° C, followed by 40 cycles at 95° C, 15 seconds at 60° C, 1 minute. The 7500 Fast Real Time PCR System (Life Technologies) was used. Expression levels were quantified using the SDS System program (Life Technologies), and the relative expression between

the samples was calculated according to the Ct (threshold cycle) comparison method, based on the formula  $2^{-\Delta\Delta Ct}$ . Normalization of expression levels was carried out based on the endogenous GAPDH gene.

### Statistical analysis

Sample size calculation was based on the findings from a pilot study. It was based on the equation for a finite population<sup>18</sup> and indicated a sample size of 6 animals per group, providing 80% power with  $\alpha = .05$ . A bifactorial experimental design was considered for statistical calculations, in which the study factors were treatment (NaHS and Ctrl) and time (3h, 5d and 14d). The response variables were alveolar bone loss and mRNA expression of interleukins. Statistical tests were applied to study the significance of the factors as well as their interaction on the independent variables, namely two-way ANOVA) followed by the *post-hoc* Tukey test for multiple comparisons., The paired Student t-test was used for the intragroup comparisons between ligated and non-ligated teeth. All calculations were performed at a significance level of 5% on GraphPad Prism program, version 6.0.

## RESULTS

### Morphometric evaluation

The morphometric readings revealed that the ligature alone promoted significant bone loss from day 5, both in the Ctrl group and in the H<sub>2</sub>S group

**Table 1: Primers for real-time q-PCR analyses.**

Gene name	Gene Symbol	Accession number (NM)	Sequences
Interleukin 1 beta	<i>IL1B</i>	031512.2	F 5'- CGACAAAATCCCTGTGGCCT -3' R 5'- TGTTTGGGATCCACACTCTCC -3'
Interleukin 6	<i>IL6</i>	012589.2	F 5'- CTGGTCTTCTGGAGTTCGGT -3' R 5'- TGCTCTGAATGACTCTGGCT -3'
TNF superfamily member 11**	<i>TNFSF11</i>	057149.1	F 5'- GAAACCTCAGGGAGCGTACC -3' R 5'- ACCAGTTCTTAGTGCTCCCC -3'
TNF receptor superfamily member 11B†	<i>TNFRSF11B</i>	012870.2	F 5'- GTATCAGGTGCACGAGCCTT -3' R 5'- AGCCAAGTCTGCAACTCGAA -3'
Threonine synthase-like 2‡	<i>THNSL2</i>	001009658.1	F 5'- GCAGCCCAGTAGCATCCC -3' R 5'- CATTGGGGTACAGCGTGTCT -3'
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>	017008.4	F 5'- CGCCTGCTTACCACCTTC -3' R 5'- GACTGTGGATGGCCCTC -3'

\* Also known as RANK

\*\* Also known as RANKL

† Also known as OPG

‡ Also known as SOFAT

(Table 2). No significant difference ( $p > 0.05$ ) was observed between the groups with PD, i.e.,  $H_2S$  neither increased nor prevented bone loss ( $p > 0.05$ ).

### mRNA expression

Regarding the proinflammatory cytokines IL-1 $\beta$  and IL-6, no significant difference was observed between groups at 3h ( $p < 0.05$ ). Significant upregulation was observed at days 5 and 14 ( $p < 0.05$ ) for the Ctrl-PD group only, as described in Fig. 2, A-B. The two-way ANOVA test detected a significant influence of “time” on the expression of IL-1 $\beta$  and IL-6, which increased in the Ctrl-PD group from day 5 to day 14 ( $p < 0.05$ ). No such pattern was detected in the other groups ( $p > 0.05$ ). In terms of bone metabolism markers (Fig. 3, A-C), RANKL was upregulated in the Ctrl-PD group ( $p < 0.05$ ) at 3h, 5 days and 14 days ( $p < 0.05$ ), with no significant difference between the remaining treatments ( $p > 0.05$ ). The “time” factor had no influence on the remaining treatment groups (two-way ANOVA  $p > 0.05$ ). OPG expression did not differ between treatments at any given time ( $p > 0.05$ ). The two-way ANOVA test, however, detected an influence of the factor “time” in OPG expression only for the Ctrl-PD group ( $p < 0.05$ ). SOFAT was upregulated in the Ctrl-PD group ( $p < 0.05$ ) at 3h, 5 days and 14 days. The comparisons over time within the same treatment group revealed no influence of the factor “time” in the expression of SOFAT (two-way ANOVA  $p > 0.05$ ).

### DISCUSSION

In addition to the evidence that halitosis is a characteristic sign of periodontal disease<sup>3</sup>, it is reasonable to suggest that because VSCs are produced by proteolytic activity of periodontopathic bacteria<sup>19</sup>,  $H_2S$  could contribute to periodontal destruction, as reported in several studies<sup>5-7,15,20</sup>. The aim of the present study was to investigate the effect

of exogenous  $H_2S$  on periodontal tissues with and without ligature-induced periodontitis. In general, the results demonstrated that the presence of a ligature induced alveolar bone loss and that exogenous exposure to  $H_2S$ , despite having an impact on tissue immunomodulation, had no effect on periodontal bone destruction.

Regarding the relationship between  $H_2S$  and periodontitis, studies show discrepant results depending on the  $H_2S$ -donor used, the administration route and dosage<sup>20</sup>. Since  $H_2S$  is a highly volatile gas,  $H_2S$ -donor molecules should be used to allow for optimized contact between the periodontal tissues and the substance, in order to simulate the situation observed within the oral cavity. Several  $H_2S$  donor molecules have been tested, such as  $Na_2S$ , NaHS or saturated solutions of  $H_2S$  gas<sup>21</sup>. Following methods used in other studies<sup>15,22</sup>, NaHS administration was chosen to deliver  $H_2S$  to the target tissues. In order to interpret the findings thereof, it is necessary to consider the limitations involved in making exogenous  $H_2S$  available, since bacteria within the periodontal pocket produce the gas continuously and at lower concentrations compared to exogenous topical application. Such differences could be crucial for the outcomes, so there is a need to develop novel  $H_2S$  donors or even slow, sustained delivery methods in order to approach true likeness with *in vivo* conditions as well as reproducibility across studies.

The morphometric findings from the present study demonstrated that at 14 days, alveolar bone loss was significantly higher in the ligature groups than in the control groups, though no significant difference in alveolar bone loss was observed between the treatment groups (with or without NaHS). These findings are comparable to those reported by Toker et al.<sup>23</sup>, who evaluated the effect of systemic administration of three different doses of NaHS to

**Table 2: Mean  $\pm$  standard deviation of the alveolar bone loss measurements in micrometers ( $\mu m$ ), for the groups exposed to  $H_2S$  (NaHS), control (Ctrl), with and without periodontal disease.**

Ligature	Ctrl			NaHS		
	3 h	5 d	14 d	3h	5 d	14 d
No (H)	370 $\pm$ 62 Aa	302 $\pm$ 51 Aa	383 $\pm$ 49 Aa	340 $\pm$ 39 Aa	324 $\pm$ 29 Aa	415 $\pm$ 82 Aa
Yes (PD)	368 $\pm$ 48 Aa	610 $\pm$ 86 *Ab	660 $\pm$ 85 *Ab	356 $\pm$ 86 Aa	564 $\pm$ 69 *Ab	672 $\pm$ 134 *Ab

(\*) indicates significant difference between the groups with and without PD (unpaired t-test). Different uppercase letters indicate significant intergroup differences (Ctrl x NaHS). Different lowercase letters indicate significant intra-group statistical differences (different times). (two-way ANOVA, Tukey's post hoc test,  $p < 5\%$ ).

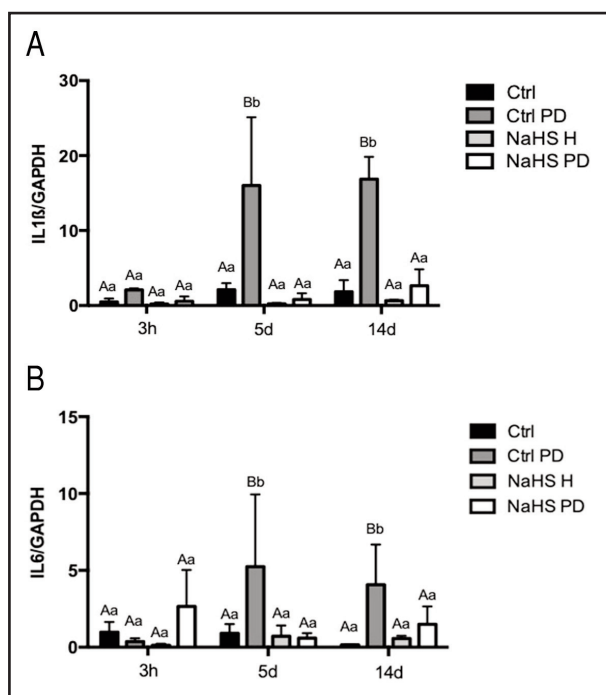


Fig. 2: Mean ( $\pm$  standard deviation) of mRNA expression of the proinflammatory cytokines IL-1 $\beta$  (A) and IL-6 (B) for the different treatments and times evaluated.

Different capital letters indicate significant intra-group differences (different times). Different lowercase letters indicate significant intergroup differences (different treatment, within a same period) (two-way ANOVA criteria, Tukey's post hoc test, 5% alpha).

Ctrl - control group: no exposure (NaHS) and no periodontitis; Ctrl PD: no drug exposure, with periodontitis (PD); NaHS H: drug exposure and no periodontitis (H); NaHS PD: drug exposure and periodontitis.

rats (with and without ligature-induced periodontal disease), suggesting that systemic NaHS neither prevented nor increased alveolar bone loss. The present study intended to simulate a clinical scenario in which the periodontal pockets of individuals with periodontitis and halitosis experience high H<sub>2</sub>S concentration locally. Although the ligature-induced periodontitis groups showed greater bone loss than the controls, topical administration of H<sub>2</sub>S did not influence such tissue loss. This might be explained by the fact that periodontitis is a chronic disease characterized by several episodic cycles of acute-chronic inflammation over a long period of time<sup>2</sup>. Therefore, in such a context, it may be speculated that a relatively short, high-dose exposure to H<sub>2</sub>S in a single episode of acute-chronic cycle might be insufficient to draw definitive conclusions regarding alveolar bone loss.

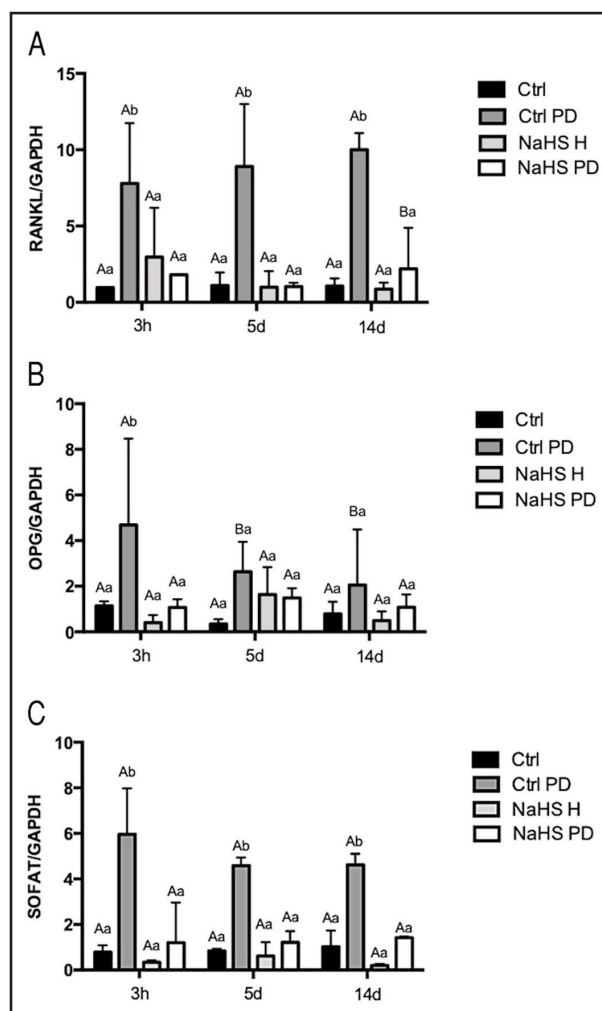


Fig. 3: Mean ( $\pm$  standard deviation) of RANKL (A), OPG (B) and SOFAT (C) mRNA expression in the different groups and at different times.

Different capital letters indicate significant intra-group differences (different times). Different lowercase letters indicate significant intergroup differences (different treatment, within a same period) (two-way ANOVA criteria, Tukey's post hoc test, 5% alpha).

Ctrl - control group: no exposure (NaHS) and no periodontitis; Ctrl PD: no drug exposure, with periodontitis (PD); NaHS H: drug exposure and no periodontitis (H); NaHS PD: drug exposure and periodontitis.

Periodontal bone loss is a result of osteoclastic activity, which is orchestrated by a series of cytokines, where pro-inflammatory cytokines may be inactivated by anti-inflammatory cytokines to achieve balance<sup>2</sup>. It is likely that an imbalance in favor of inflammation would therefore determine the level of periodontal loss<sup>2,24</sup>. In the present study, H<sub>2</sub>S seems to have interfered with such mechanism, based on mRNA expression of several cytokines for which exposure to H<sub>2</sub>S cancelled out their

upregulation, placing them back at basal levels, as observed for IL-1, IL-6, RANKL and SOFAT, which were significantly upregulated in the groups with periodontitis (PD) but not in those exposed to H<sub>2</sub>S (Ctrl). The findings from the present study corroborate the association between overexpression of proinflammatory cytokines and osteoclastogenic factors, with consequent periodontal bone loss<sup>2,24-27</sup>. mRNA expression was analyzed in an attempt to elucidate some of the mechanisms potentially involved in periodontal bone loss and its relationship with H<sub>2</sub>S and, interestingly, downregulation of proinflammatory and pro-resorptive cytokines did not translate into less bone loss.

Although the mechanism of bone destruction is not yet well understood, evidence suggests that the RANKL/OPG axis plays an important role in it<sup>28</sup>. In the present study, RANKL expression was significantly higher in the groups with periodontitis throughout the 14 days, except for the H<sub>2</sub>S group, as previously discussed, whereas OPG was upregulated only at 3h. According to Chun-Mei et al.<sup>22</sup>, the H<sub>2</sub>S-related mechanism of tissue damage and repair may vary considerably from tissue health through to acute and chronic inflammation. Furthermore, periodontitis is a chronic disease with peaks and troughs as it alternates between periods of aggressiveness and calm<sup>1,2,24</sup>. This is why we performed evaluations at three times: at an acute phase of bacterial accumulation (3 hours), at an intermediate phase (5 days) and at a chronic phase (14 days), where a cumulative effect of bacterial biofilm was observed, although no clear role of H<sub>2</sub>S could be established. Morphometric evaluation is a validated and widely used method of analysis<sup>17,29</sup>, though its detection requires bone loss to have occurred already, which is an important limiting factor. Further studies with more sensitive techniques and longer follow-up may aid in understanding the process between intracellular signaling following H<sub>2</sub>S exposure and the expression phenotype of bone loss.

Halitosis may be regarded as a public health problem because it has high prevalence in the population – approximately 32%, according to a recent systematic review with meta-regression<sup>30</sup>. Additionally, individuals with periodontitis were 3.16 times more likely (OR 3.16; 95% CI: 1.12-8.95) to have halitosis<sup>3</sup>. Despite the widespread incidence in the general population, it is a subject with little scientific

research and somewhat neglected by health professionals. Considering that halitosis may exert a negative impact on social interactions and quality of life, a better understanding of its complex etiology is of utmost importance, especially as it may be associated with health issues such as diabetes, depression, lupus erythematosus, Sjogren's syndrome and periodontitis<sup>21</sup>. In the present study, we sought to investigate the effects of H<sub>2</sub>S because it is the main halitosis-related gas, has dubious role in the inflammatory response and may affect bone loss in periodontitis<sup>10,21</sup>. Understanding such effects both locally and systemically might assist in the development of preventive and therapeutic approaches, not only for halitosis but also for other related systemic problems.

In view of the different effects attributed to H<sub>2</sub>S regarding cytoprotection and/or cytotoxicity<sup>10-13,20,23</sup>, it is assumed that some factors may contribute to the paradoxical findings reported in the literature, such as the different concentrations and delivery methods of the drug, the presence or absence of biofilm accumulation as well as tissue sensitivity at varying degrees of inflammation. There may be a concentration threshold for topical application of H<sub>2</sub>S that distinguishes between beneficial and harmful effects. The findings from the present study suggest that in an infectious condition such as periodontitis, there are self-sufficient factors to regulate bone loss, either by direct bacterial aggression, or indirectly by host defense mechanisms. Within the morphometric parameters investigated, exposure to H<sub>2</sub>S did not influence periodontal bone loss during the 14 days of the experiment. Regarding the cytokines studied, however, the presence of H<sub>2</sub>S caused an immunomodulatory effect on the mRNA expression of important pro-inflammatory and pro-resorption proteins. Extrapolating such findings to a clinical scenario, individuals with periodontitis and halitosis (CSV, especially H<sub>2</sub>S) would not appear to have greater short-term bone loss compared to individuals with periodontitis alone.

## CONCLUSION

H<sub>2</sub>S had no synergistic effect to ligature (biofilm accumulation) on alveolar bone loss, though exposure to appears to have an immunoregulatory effect on the expression of pro-inflammatory and pro-resorptive cytokines.

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