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RESEARCH LETTER

The influence of hydrogen sulfide on gingival wound healing: An in vitro study

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1 | INTRODUCTION

Halitosis is a highly prevalent condition, affecting roughly 30% of the world population.[1] Approximately 80% of halitosis cases has an intra-oral origin, mostly due to factors like tongue coating or periodontal diseases.[2]

Halitosis is caused by the release of volatile sulfur compounds (VSC), being hydrogen sulfide (H_2S) the most often found VSC in oral cavity.[3] Recent studies have shown deleterious effects of high concentrations of H_2S to periodontal tissues, and more specifically to gingival epithelial cells (GECs).[4]

Wound healing processes are of paramount importance for the achievement of favorable treatment outcomes of periodontitis and peri-implantitis,[5] and GECs are known to play a key role in this process.

The prevalence of halitosis on periodontal patients is high.[6] Therefore, the aim of this in vitro study is to investigate the effects of different clinically relevant concentrations of H_2S on GECs. We hypothesize that the presence of H_2S at concentrations observed in intra-oral halitosis patients will impair the migration, viability, and function of GECs.

2 | METHODS

GECs of a human gingival epithelial line (Ca9-22) were plated in a migration insert (migration-insert2 Well24, ibiTreat, Martinsried, Germany) to create a fixed gap between GECs fronts after its retrieval. Therefore, this device allowed to mimic a wound.

H_2S was produced in a Kipp's apparatus and subsequently transferred to the GECs in a container. GECs were incubated for 8 h with various ranges of clinically relevant concentrations of H_2S (control group: 0 ppb, low: 90–299 ppb, medium: 300–999 ppb, and high: ≥1000 ppb).[7,8]

H_2S concentration (ppb) inside each container was determined with a gas chromatograph device OralChroma2, at two time points: 15 min after H_2S insufflation (T_0) and after 8-h incubation (T_1) to ensure the H_2S level in the container.

Microscopy analyses of the gap were performed at T_0 and T_1 by using a converted microscope at 100× and 400× magnification. Subsequently, the cells were trypsinized and prepared for further assessment of cell viability and qPCR analysis for *ICAM-1*, *e-cadherin*, *TNF-α*, *COX IV*, *Ki67*, and *caspase 3*.

The study was approved by the Local ACTA Ethical Committee (020.277).

3 | RESULTS

3.1 | Less GEC migration with increasing H_2S concentration

A significant difference in gap closure was found between the control and medium H_2S groups (mean Δ95.37%, $p < 0.0001$) and between the control and high H_2S groups (mean Δ97.12%, $p < .0001$) (Figure 1A,B). Similarly, the low H_2S group showed significant differences in gap closure compared to the medium (mean Δ81.63%,

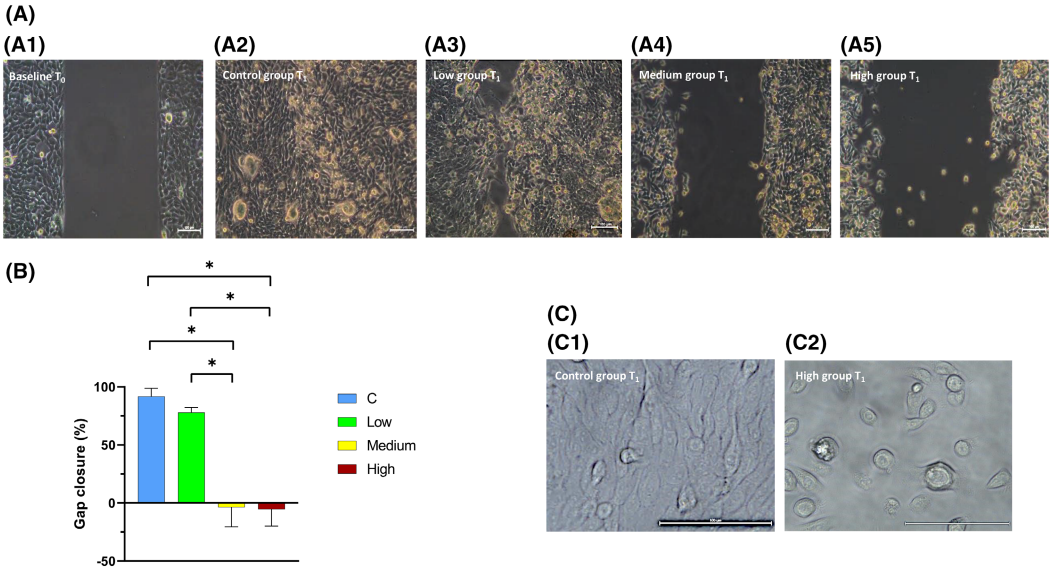

FIGURE 1 (A) Micrographs of the epithelial cell gap closure: (A1) The baseline (T_0), (A2) Control group (0 ppb H_2S) shows a complete closure of the gap after 8-h incubation (T_1 mean 91.64% ± 7.17), (A3) Low concentration group (90–299 ppb H_2S) shows almost a complete closure of the gap after 8-h incubation (T_1 mean 77.90% ± 4.38), (A4) Medium concentration group (300–999 ppb H_2S) shows minimum-to-no closure of the gap after 8-h incubation (T_1 mean 3.73% ± 16.70), (A5) High concentration group (≥1000 ppb H_2S) shows no closure of the gap and even a widening of the gap surface area after 8-h incubation (T_1 mean closure −5.48% ± 14.43). Magnification ×100, Bar = 100 μm. (B) Between-group comparison of the gingival epithelial cell gap closure (T_0–T_1). Mean ± SD. *$p < .0001$ C, Control group (0 ppb H_2S), Low: Low H_2S concentration group (90–299 ppb H_2S), Medium: Medium H_2S concentration group (300–999 ppb H_2S), High: High H_2S concentration group (≥1000 ppb H_2S). (C) Micrographs of gingival epithelial cells (GECs) after 8-h exposure (T_1) to H_2S: (C1) GECs from the Control group (0 ppb H_2S) at T_1. (C2) GECs from High H_2S concentration group (≥1000 ppb H_2S) at T_1. Magnification × 400. Bar=100 μm.

$p < .0001$) and high groups (mean Δ83.38%, $p < .0001$). All other comparisons were nonsignificant.

3.2 | Less viability and changed morphology of GECs at high H_2S concentration

Viability of GECs in the medium and high H_2S groups decreased significantly compared to the control group (mean Δ4.97%, $p = .0300$ and 5.32%, $p = .0193$, respectively). No other significant differences in viability were observed between groups.

Morphometric analysis disclosed a round morphology along with broad intercellular spaces for GECs exposed to high H_2S concentrations. In contrast, GECs from the control group displayed an elongated morphology, with tight intercellular spaces (Figure 1C).

3.3 | E-cadherin, TNF-α, ICAM-1, and Ki-67 are downregulated, whereas caspase 3 is upregulated by high H_2S concentration

Gene expression of *e-cadherin* ($p = .009$) and *TNF-α* ($p = .043$) were significantly downregulated in the high H_2S group (Figure 2, Figure S2). Gene expression of *ICAM-1* and *Ki-67* was significantly downregulated, while *caspase 3* significantly upregulated in medium ($p = .099$, $p = .015$ and $p = .010$, respectively) and high ($p = .099$, $p = .034$, and $p = .015$, respectively) H_2S groups. All other comparisons in gene expression were not statistically significant.

4 | DISCUSSION

The current study indicates that H_2S concentrations ≥300 ppb (Figure S1) may impair gingival wound healing due to (1) impaired gingival epithelial cell migration; (2) reduced cell viability, which may lead to inadequate tissue regeneration and repair; (3) down-regulation of genes involved in cellular functions, such as *TNF- α* (inflammation), *e-cadherin* (intercellular junction), *ICAM-1* (cellular adhesion), and *Ki-67* (nuclear function); and (4) upregulation of apoptotic genes (*caspase-3*) leading to an increase in cellular damage to GEC.[9] Furthermore, previous studies have demonstrated that high concentrations of H_2S can also disrupt the intestinal epithelial layer by decreasing mitochondrial energy metabolism.[10]

There are several limitations given the current study. First, the use of gingival fibroblasts in addition to GECs could increase the understanding of gingival wound healing. Also, the in vitro nature of this study may prevent drawing solid conclusions at clinical level. For example, GECs were continuously exposed to only H_2S, while in vivo H_2S concentrations in the oral cavity fluctuate. Furthermore, other VSCs such as methyl mercaptan and dimethyl sulfide can be found in the oral cavity.

Based on the results of this study, we speculate that early diagnosis and treatment of halitosis may play a role in the treatment and prevention of periodontal and peri-implant diseases. Although speculative, it underscores the potential importance of addressing halitosis as part of comprehensive periodontal and peri-implant care. Early halitosis intervention, for example through tongue cleaning and anti-halitosis mouthwashes, could potentially improve outcomes in these patients.

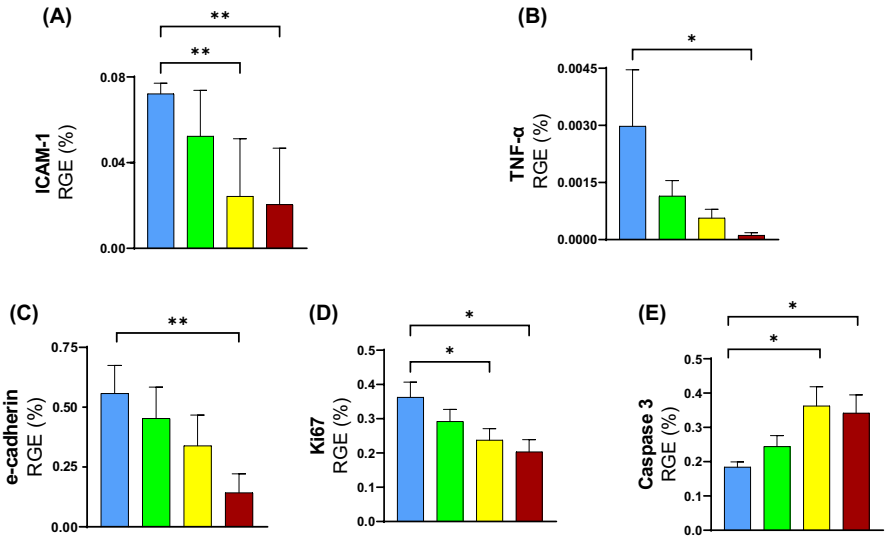

FIGURE 2 Between-group comparison of relative gene expression (%) of (A) *ICAM-1*, (B) *TNF- α*, (C) *e-cadherin*, (D) *Ki-67*, and (E) *caspase 3* at T_1: Mean ± SD are shown. $^*p < .05$, $^{**}p < .01$, NS, No statistically significant difference. C, Control group (0 ppb H_2S), Low: H_2S concentration 90–299 ppb, Medium: H_2S concentration 300–999 ppb, High: H_2S concentration ≥1000 ppb.

AUTHOR CONTRIBUTIONS

J. Villalba-Recuerda contributed to the conception, design, data acquisition, analysis and interpretation, drafted and critically revised the manuscript. I.D.C. Jansen contributed to conception, design, data analysis and interpretation and critically revised the manuscript. M.L. Laine contributed to conception, design, data analysis and interpretation and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

No conflicts of Interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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