Two mechanisms of oral malodor inhibition by zinc ions

Abstract

Objectives: The aim of this study was to reveal the mechanisms by which zinc ions inhibit oral malodor. Material and Methods: The direct binding of zinc ions to gaseous hydrogen sulfide (H\textsubscript{2}S) was assessed in comparison with other metal ions. Nine metal chlorides and six metal acetates were examined. To understand the strength of H\textsubscript{2}S volatilization inhibition, the minimum concentration needed to inhibit H\textsubscript{2}S volatilization was determined using serial dilution methods. Subsequently, the inhibitory activities of zinc ions on the growth of six oral bacterial strains related to volatile sulfur compound (VSC) production and three strains not related to VSC production were evaluated. Results: Aqueous solutions of ZnCl\textsubscript{2}, CdCl\textsubscript{2}, CuCl\textsubscript{2}, (CH\textsubscript{3}COO)\textsubscript{2}Zn, (CH\textsubscript{3}COO)\textsubscript{2}Cd, (CH\textsubscript{3}COO)\textsubscript{2}Cu, and CH\textsubscript{3}COOAg inhibited H\textsubscript{2}S volatilization almost entirely. The strengths of H\textsubscript{2}S volatilization inhibition were in the order Ag\textsuperscript{+} > Cd\textsuperscript{2+} > Cu\textsuperscript{2+} > Zn\textsuperscript{2+}. The effect of zinc ions on the growth of oral bacteria was strain-dependent. Fusobacterium nucleatum ATCC 25586 was the most sensitive, as it was suppressed by medium containing 0.001% zinc ions. Conclusions: Zinc ions have an inhibitory effect on oral malodor involving the two mechanisms of direct binding with gaseous H\textsubscript{2}S and suppressing the growth of VSC-producing oral bacteria.

Keywords: Antimicrobial activity. Chemical binding. Hydrogen sulfide. Oral malodor. Zinc ions.
Introduction

Oral malodor is primarily the result of microbial metabolism of amino acids from local debris in the oral cavity. The primary compounds of oral malodor are volatile sulfur compounds (VSCs), such as hydrogen sulfide (H\textsubscript{2}S), methyl mercaptan (\textit{CH\textsubscript{3}SH}), and dimethyl sulfide (\textit{CH\textsubscript{3}SCH\textsubscript{3}}). Periodontopathic anaerobic bacteria, such as \textit{Porphyromonas gingivalis}, \textit{Treponema denticola}, \textit{Prevotella intermedia}, \textit{Fusobacterium nucleatum}, and \textit{Eubacterium} can produce large amounts of H\textsubscript{2}S and \textit{CH\textsubscript{3}SH} from cysteine, methionine, or serum proteins.

Various anti-malodor agents for oral use have been introduced and have proven to be effective in reducing VSC concentration in the oral cavity. Antimicrobial agents such as chlorhexidine, triclosan, and cetylpyridinium chloride can reduce oral malodor by reducing the number of microorganisms present in the mouth. Chlorine dioxide has also been shown to reduce oral malodor by chemically neutralizing VSCs. Natural ingredients, such as hinokitiol, green tea powder, and \textit{Eucalyptus} extract, also reduce oral malodor through various antibacterial mechanisms.

Zinc ions are often found in commercial anti-malodor mouthwashes in combination with other active ingredients. A combination of zinc ions and chlorhexidine or cetylpyridinium chloride was reported to inhibit VSC production synergistically. We considered two mechanisms of oral malodor inhibition by zinc ions. The first is that zinc ions have a strong affinity for the thiol groups present in VSCs. Zinc ions exhibit immediate inhibitory effects on VSC production compared to chlorhexidine, by effectively and directly reducing the activities of VSCs. The second is that zinc ions have an antibacterial effect. Zinc ions can inhibit catabolism by \textit{F. nucleatum} and \textit{P. intermedia}, and acid production by \textit{Streptococcus sobrinus} and \textit{Streptococcus salivarius}. Although these characteristics suggest that zinc ions might be effective oral anti-malodor agents, the majority of studies conducted to date have been based on the results of clinical use in combination with other agents. Therefore, the two mechanisms of action, chemical binding and an antimicrobial property, have not been individually assessed. Furthermore, previous \textit{in vitro} studies examined the inhibitory effects of zinc ions only on the functions of selected targets, and thus the antimicrobial effects of zinc ions on microorganisms related to oral malodor remain unclear. In this study, the direct effects of zinc ions on H\textsubscript{2}S were assessed in comparison with other metal ions. In addition, the inhibitory effects of zinc ions on the growth of microorganisms related to VSC production and those unrelated to VSC production were evaluated.

Materials and methods

Direct inhibitory effects of metal ions on hydrogen sulfide

Nine metal chlorides, namely, MgCl\textsubscript{2}, AlCl\textsubscript{3}, CaCl\textsubscript{2}, MnCl\textsubscript{2}, FeCl\textsubscript{3}, CuCl\textsubscript{2}, ZnCl\textsubscript{2}, SrCl\textsubscript{2}, and CdCl\textsubscript{2}, and six metal acetates, namely, (CH\textsubscript{3}COO),Ca, (CH\textsubscript{3}COO),Fe, (CH\textsubscript{3}COO),Cu, (CH\textsubscript{3}COO),Zn, CH\textsubscript{3}COOAg, and (CH\textsubscript{3}COO),Cd, were examined in this study. These chemical compounds, except for (CH\textsubscript{3}COO),Cu and CH\textsubscript{3}COOAg, were prepared as 1 M aqueous solutions. The aqueous solutions of (CH\textsubscript{3}COO),Cu and CH\textsubscript{3}COOAg were prepared at concentrations of 0.25 M and 0.0625 M, respectively. Gaseous H\textsubscript{2}S was prepared from a dilute aqueous solution of NaHS.nH\textsubscript{2}S. Two milliliters of aqueous solution containing 10^{-9}\% NaHS.nH\textsubscript{2}S and the appropriate chemical compound was added to individual 15 mL tubes, which were sealed and incubated at room temperature for 5 min. Then, 1 mL of the gas phase was collected and measured by gas chromatography (model GC2014; Shimadzu Works, Kyoto, Japan). To determine which chemical compounds inhibited H\textsubscript{2}S volatilization more strongly, the minimum concentrations of H\textsubscript{2}S volatilization inhibition were determined using serial dilution methods. All test reagents were purchased from WAKO Pure Chemical Industries, Ltd. (Kyoto, Japan). The experiments were repeated at least three times.

Inhibitory effects of zinc ions on the growth of oral bacteria

The bacterial strains used in the study are \textit{P. gingivalis} FDC 381, \textit{P. gingivalis} W83, \textit{P. gingivalis} ATCC 33277, \textit{F. nucleatum} ATCC 25586, \textit{P. intermedia} ATCC 25611, \textit{Streptococcus mutans} JCM 5705, \textit{S. sobrinus} JCM 5176, \textit{S. salivarius} GTC 0215, and \textit{Streptococcus anginosus} FW73. The \textit{S. mutans}, \textit{S. sobrinus}, \textit{S. salivarius}, and \textit{S. anginosus} strains were cultivated in BD Bacto™ brain heart infusion (BHI) medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), while \textit{P. gingivalis}, \textit{F. nucleatum},
and *P. intermedia* were cultivated in BHI medium with hemin (5 µg/mL) and vitamin K (1 µg/mL).

Bacterial cultures were incubated at 37°C anaerobically until full growth, then suspended in fresh BHI medium or fresh BHI medium with hemin and vitamin K to an optical density at 600 nm (OD600) of 0.3. To evaluate the effect of zinc ions on the growth of bacteria, 100 µL of the inoculated medium was cultivated anaerobically in a final volume of 200 µL of ZnCl2-containing BHI medium or BHI medium with hemin and vitamin K. The final concentrations of ZnCl2 in the culture solutions were 0.1% (0.0007 M), 0.01% (0.00007 M), and 0% (control, 0 M). Cultivation was performed in a 96-well (flat-bottomed) microtiter plate (Nunc A/S, Roskilde, Denmark). After suspending the bacterial cells by pipetting, the optical density at 600 nm (OD600) was measured at 0 h, 12 h, and 24 h for Gram-positive bacteria and at 0 h, 12 h, 24 h, and 48 h for Gram-negative bacteria. The experiments were repeated at least three times.

**Statistical analysis**

The Mann-Whitney *U*-test was used to evaluate the direct inhibitory effects of metal ions on gaseous *H2S* compared with 10–5% NaHS·nH2S solution, and the inhibitory effects of zinc ions on the growth of oral bacteria compared with that in the 0% ZnCl2 medium. Differences were considered to be significant when *P*<0.05. Statistical evaluations were carried out using the R software package, ver. 3.4.0 (http://www.R-project.org).

![Figure 1](image.png)

*P*<0.05 compared with the 10–5% NaHS·nH2S solution (Mann-Whitney *U*-test).

**Figure 1** - The direct inhibitory effects of aqueous solutions of metal chlorides (A) and metal acetates (B) on gaseous *H2S*
Results

Direct inhibitory effects of metal ions on hydrogen sulfide

The inhibitory effects of metal chlorides on gaseous H₂S are shown in Figure 1A. Aqueous solutions of ZnCl₂, CuCl₂, and CdCl₂ almost entirely inhibited H₂S volatilization. The CaCl₂ and FeCl₂ solutions moderately inhibited H₂S volatilization, at 72.7% and 45.0%, respectively. There was a statistically significant difference between each of these five metal chlorides and the control (P<0.05). Aqueous MgCl₂, AlCl₃, MnCl₂, and SrCl₂ solutions did not inhibit H₂S volatilization. For metal chlorides that directly inhibited gaseous H₂S, as well as hydrophobic silver chloride, we assessed the inhibitory effects of the metal acetates of the same ions on gaseous H₂S (Figure 1B). The (CH₃COO)₂Zn and CH₃COOAg solutions inhibited H₂S volatilization entirely (100% and 100%) (P<0.05), while (CH₃COO)₂Fe, (CH₃COO)₂Cu, and (CH₃COO)₂Cd inhibited it almost entirely (97.0%, 99.8%, and 99.9%) (P<0.05). Overall, four ions, zinc, copper, cadmium, and silver, were considered to have excellent inhibitory effects on H₂S volatilization. Serially diluted aqueous solutions of ZnCl₂, CuCl₂, CdCl₂, and CH₃COOAg were assessed to determine the minimum concentration that inhibits H₂S volatilization (Figure 2). The CH₃COOAg solution exhibited the strongest effect, as it inhibited H₂S volatilization entirely at 4⁻⁷ M. Comparing the strength of H₂S volatilization inhibition among metals, the order was as follows: Ag⁺ > Cd²⁺ > Cu²⁺ > Zn²⁺.

Inhibitory effects of zinc ions on the growth of oral bacteria

Among the tested oral bacteria, F. nucleatum ATCC 25586 was the most sensitive, as it was suppressed by 0.001% zinc ions in the medium (Figure 3). The growth of S. sobrinus JCM 5716, S. salivarius GTC 0215, P. gingivalis ATCC 33277, P.gingivalis W83, and P. intermedia ATCC 25611 was suppressed in medium with 0.01% zinc ions. For P. intermedia ATCC 25611, medium with 0.001% zinc ions suppressed bacterial growth for the first 24 h. The growth of S. mutans JCM 5705 was suppressed in medium with 0.1% zinc ions, but not in medium with 0.01% zinc ions. Although the growth of S. anginosus FW73 was suppressed in media with 0.1% and 0.01% zinc ions, the inhibitory effect of 0.01% zinc ions was not complete. P. gingivalis FDC 381 increased in the first 12 h in medium with 0.1% zinc ions as well as 0.01% zinc ions, but exhibited no further growth at 24 h and 48 h.

Discussion

Among the nine metal ions examined in this study, calcium, iron, zinc, cadmium, copper, and silver had direct inhibitory effects on H₂S volatilization. These metals have been widely used in dental materials,
except for cadmium, which can have harmful effects on health. Iron, zinc, copper, and silver are not only used alone in tooth restorations but are also mixed into alloys due to their antimicrobial nature. Calcium and silver are also used as antibacterial fillings for intractably infected root canals. Iron ions have an inhibitory effect on glycosyltransferase enzymes of S. mutans, and a previous study reported that iron-containing sucrose reduced the amount of mutans streptococci in biofilm compared with sucrose. For inhibition of odor in the oral cavity, zinc has been extensively studied and developed, whereas other metal ions have not been evaluated for this purpose. However, these metal ions exhibited direct inhibitory effects on H\textsubscript{2}S volatilization in this study, in addition to their previously reported antimicrobial effects. In particular, the inhibitory effects of copper and silver on H\textsubscript{2}S volatilization were excellent compared with that of zinc. The effects of these metal ions released from dental materials might thus contribute to reduce oral malodor due to antimicrobial effects and inhibition of H\textsubscript{2}S volatilization. Liu, et al. (2016) reported that Ti-Cu alloy implants released about 0.014 µg/day of Cu\textsuperscript{2+} ions, which is considerably lower than the minimal inhibitory concentration of Cu\textsuperscript{2+} for Staphylococcus aureus and Escherichia coli. The authors also explored the antibacterial activities of Ti-Cu alloy against P. gingivalis and S. mutans, and postulated that Ti-Cu alloys might exhibit antimicrobial activity markedly even if low levels of Cu\textsuperscript{2+} ions are released.

The ZnCl\textsubscript{2} solution inhibited H\textsubscript{2}S volatilization entirely at 4 × 10\textsuperscript{-4} M (0.053%) in the current in vitro experiment. Young, et al. (2001) reported approximately 80% reduction of oral hydrogen sulfide after 1 h in a clinical trial of rinsing with 0.1% ZnCl\textsubscript{2} solution. In other previous studies, zinc ions were usually combined with other antimicrobial agents, such as chlorhexidine, cetylpyridinium chloride, or triclosan, and the concentrations of zinc compounds were in the range of 0.14% to 0.4%, which were effective for direct inhibition of H\textsubscript{2}S volatilization in an in vitro study. The recommended intake and tolerable intake of zinc established by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) are 14–20 mg/day and 0.3–1 mg/kg body weight/day, respectively, and therefore effective and

![Figure 3](https://example.com/figure3.png)
safe concentrations of zinc ions can be incorporated into mouthwash. On the other hand, Japanese law requires the concentrations of some synthetic antimicrobial agents combined with dentifrice or mouthwash to be lower than their effective concentrations due to occurrences of anaphylactic shock. For example, chlorhexidine and cetylpyridinium chloride are limited to 0.05% and 0.1%, respectively, although foreign studies examining the effectiveness of these compounds on oral malodor have been performed using 0.1%-0.2% chlorhexidine and 0.05%-0.07% cetylpyridinium chloride. Interestingly, it has been reported that mouthwash containing 0.3% zinc acetate and 0.025% chlorhexidine showed a synergistic anti-VSC effect. By combining multiple components, the concentration of each can be kept low. Furthermore, this synergistic effect indicates different mechanisms of VSC inhibition by each component.

The effect of zinc ions on the growth of oral bacteria was strain-dependent. *F. nucleatum, P. intermedia, and P. gingivalis* have been recognized as VSC-producing organisms that are important to oral malodor. *F. nucleatum* and *P. intermedia* were especially sensitive to zinc ions compared with other bacteria assessed in this study. The growth of *F. nucleatum* ATCC 25586 and *P. intermedia* ATCC 25611 was suppressed entirely and for 24 h, respectively, with 0.001% ZnCl$_2$ and 0.01% ZnCl$_2$, whereas strain FDC 381 increased for the first 12 h at both 0.01% and 0.1% ZnCl$_2$, and then stopped growing. Gram-negative strict anaerobes have been identified as the main organisms capable of producing H$_2$S, but *S. anginosus*, which is a Gram-positive microaerophilic anaerobe, has a greater capacity to produce H$_2$S and CH$_3$SH. Only the effect of zinc ions on H$_2$S production was evaluated, and the effect on CH$_3$SH should also be assessed. Second, no sustainability assessment of the inhibitory effect of zinc ions on H$_2$S volatilization was performed in this study. A previous clinical trial reported that the inhibitory effect of rinsing with 0.1% ZnCl$_2$ on H$_2$S continued for only 1 hour, less than those of SnF$_2$ and CuCl$_2$. The longitudinal evaluation of the binding reaction between zinc ions and H$_2$S should be performed in vitro. Finally, the antimicrobial mechanism of zinc ions has been compared to the characteristics of silver ions, which have been studied well. The reason why the effect of zinc ions is weaker than those of silver and copper ions remains unclear. Further investigations into the chemical reactivity between zinc ions and thiol groups as well as the specific active sites of antimicrobial activity of zinc ions are necessary.

In conclusion, zinc ions, which exhibit an inhibitory effect on VSCs and are incorporated into products for oral malodor prevention, employ the two mechanisms of direct binding with gaseous H$_2$S and antimicrobial activity. In particular, the growth of bacteria related to VSC production was inhibited at a lower concentration of ZnCl$_2$ compared with bacteria not related to VSC production.
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