The Effect of Zinc Chloride Mouthwash on the Production of Oral Malodour, the Degradations of Salivary Cellular Elements, and Proteins

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Introduction

Oral malodour, which is caused by volatile sulphur compounds (VSC), appears to be an almost universal problem among adults. It is generally accepted that the oral cavity is the source of both physiological and pathological oral malodour, because VSC originate from the putrefactive activity of micro-organisms on appropriate substrates associated with salivary cellular elements, dental plaque and debris adhering to the mucous membranes of the tongue and other soft tissues.

To date, many methods of inhibiting VSC production in the oral cavity have been developed, such as dentifrices containing chlorhexidine, chewing gum containing flavonoid, mouthwashes, and others. In the USA alone, about $500 million are spent annually for mouthwashes and others. In Japan, about ¥1 billion were spent in the year 1986. At next year, 1987, money spent for mouthwashes increased markedly to ¥3 billion. Accordingly, oral hygiene awareness to this problem seems to have increased in Japan, as in other countries.

However, many of these products have little actual effect, other than masking the malodour for a short period. Even some mouthwashes used in dental clinics do not sufficiently inhibit VSC production.

Tonzetich suggested that zinc ions strongly inhibit VSC production, although the effect and the mechanisms of inhibition have not been elucidated experimentally. Hence, the objectives of this study are to examine the effect of inhibition VSC production by zinc ions, and to determine the effect on degradation of salivary cellular elements since the cell degradation is one of the most important processes of VSC production in the oral cavity.

Materials and Methods

Mouthwashes and subjects

Zinc chloride mouthwash was employed in this study to determine whether zinc ions inhibits production of VSC in the oral cavity. Since more than 0.5% (w/v) of zinc chloride is not soluble completely in distilled water, 10 ml of 0.5% zinc chloride was used to rinse the oral cavity. To compare the effect of zinc chloride, 20 ml Skoal commercial mouthwash (Sunstar Inc., Japan) and 10 ml distilled water were also employed in this study. To select subjects for this study, the evaluations of early morning mouth air were performed at 8:30 AM on male non-smokers who exhibited no evidence of systemic or oral pathology. The smokers were excluded because of their lower odour producing potential and because tobacco smoke itself contains VSC. Female subjects were also excluded because the menstrual cycle affects the concentration of VSC in mouth air. On the basis of the evaluations, five subjects (20-24 years old), who had a higher concentration of VSC in mouth air than the threshold of odour objectionability, were selected to examine the effect of three mouthwashes.

The subjects were instructed to abstain from oral hygiene including oral rinse and ingestion of food and liquid, on the morning of the test until the analyses were completed. Four mouth air analyses, starting at 8:30 A.M. with early morning samples, were performed on each subject. Following the early morning mouth air sampling, the subjects immediately rinsed out the oral cavity for 30 seconds, using one of the mouthwashes. Re-evaluations were performed immediately, 2 hours and 3.5 hours later. Prior to each
analysis, the subjects were instructed to keep the mouth closed and to breathe through the nose for 1 min. Then the subjects were directed to insert a 2.5 mm outside diameter Teflon tube, which was connected to the VSC analysis system. For the evaluation of VSC 13 ml of mouth air was aspirated, then 10 ml of sample was injected into the system. The percent change in H2S, CH3SH, (CH3)2S and total sulphur concentrations were obtained in relation to the early morning values obtained on the same day.

VSC analysis system

VSC analyses were performed according to the method of Yaegaki et al.11). The VSC analysis system was comprised of a GC-8A Gas chromatograph (Shimazu, Japan), a C-R6A Chromatopack (Shimazu, Japan), and an auto injection system for precise injection of samples. H2S, CH3SH and (CH3)2S were assayed, and total sulphur was calculated from sulphur contents of H2S, CH3SH and (CH3)2S[S].

Analysis of head space air of incubated saliva

In order to determine the effect of zinc ion on VSC production through saliva putrefaction, the putrefied saliva systems[12] were employed in this study. Each participant, who exhibited no evidence of systemic or oral pathology (n=5, 22-34 years old), donated 10 ml of paraffin-stimulated saliva, and food debris was removed with through two layers of cheesecloth. Nine hundred microliter saliva aliquots and one hundred µl of zinc chloride-phosphate buffered saline (PBS) of final concentrations of 0.001, 0.01, 0.1 and 0.5 % zinc chloride were dispensed into glass tubes coated with Teflon. The solid rubber liners provided an airtight seal while the adjoining thin Teflon film prevented the rubber liners from coming in contact with head space air. Prior to incubation, head space air in the tube was replaced with nitrogen.

After 24 hours incubation at 37°C without shaking, head space air was introduced into the VSC analysis system, as follows. A Teflon coated injection needle, which was connected VSC analysis system, was used to remove head space air. This was achieved by means of a 2 mm diameter hole in a plastic cap that permitted insertion of the needle through an airtight seal. Then 4 ml of nitrogen was injected into the tube, and the same volume of head space air was aspirated. Three milliliter of sample was injected into the VSC analysis system.

Separation of salivary cellular elements by percoll density gradient centrifugation

Percoll™ (Pharmacia, USA) density gradient centrifugation[13] was employed to determine the effect of zinc ions on cell degradation of saliva. Human whole saliva (27 ml) was collected through two layers of cheesecloth in ice-chilled tubes under paraffin stimulation. The saliva was divided into 3 test tubes, in 9 ml aliquots. To one of the three aliquots, 1 ml of zinc chloride-PBS was added to make a final concentration of 0.01 % zinc chloride. This sample was incubated for 24 hours as described above. One ml of PBS was added to the other two aliquots, of saliva. One of these two samples was used as a control for the incubation test. The other one was immediately centrifuged at 20,000×g at 4°C for 30 min, and the incubated samples were also centrifuged under the same conditions. The resulting precipitate was suspended in a small amount of 40 % Percoll™,PBS, and was adjusted to 1 ml with the same buffer. After leading this suspension onto 8 ml of 40 % Percoll™,PBS in a tube, the sample was centrifuged in an angle rotor at 20,000×g at 4°C for 30 min. The cell fractions thus obtained were identified by phase-contrast microscopy.

Inhibition of proteinase activity by zinc chloride

Proteinase activity was assayed according to the method of Barret[14]. The freshly collected whole
saliva (0.5 ml) was preincubated for 5 min at 4°C with 1.1 ml 0.14 M KH₂PO₄/Na₂HPO₄ buffer (pH 6.0) together with 0.4 ml zinc chloride at final concentration of 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, and 0.0001 % in the incubation mixture. After preincubation, 50 µl of α-N-benzoyl-DL-arginine-4-nitroanilide HCl solution (40 mg/ml) in dimethyl sulfoxide was added. After 90 min incubation with shaking at 40°C, 2 ml 2 M Tris-HCl buffer (pH 9.0) was added to stop the reaction, and the absorbance was immediately measured at 410 nm.

**SDS polyacryl amid gel electrophoresis (PAGE)**

A whole saliva sample (9 ml) was added to 1 ml of zinc chloride-PBS to make 0.01 % zinc chloride. After replacing head space air with nitrogen the sample was incubated for 24 hours at 37°C. Nine milliliter of whole saliva was incubated with 1 ml of PBS as a control. After incubation, the samples were centrifuged at 20,000×g at 4°C for 30 min, and the supernatants were lyophilized, suspended in 2 ml of 2.3 % sodium dodesyl sulphate (SDS), 5 % β-mercaptoethanol (β-Me), 10 % sucrose, and 0.003 % bromphenol blue in 0.075 M Tris-HCl buffer (pH 6.8), and boiled for 5 min. The precipitates were incubated with 5 ml of 8 M urea, 1 % SDS, 50 mM β-Me in 0.05 M Tris-HCl buffer (pH 9.0) for 4 hours replacing head space air with nitrogen. After incubation the samples were placed in a boiling water bath for 5 min, and then the samples were dialyzed against 1 l of 0.005 M Tris-HCl (pH 8.0), 24 mM β-Me for 48 hours at 4°C, with 3 changes of the buffer. After dialysis, the samples were centrifuged at 20,000×g at 4°C for 30 min. The supernatants were lyophilized, then treated again with SDS mixture as described above.

The samples were subjected to SDS-PAGE. Five and twelve percents acrylamide gels were employed as stacking and separating gels respectively. After electrophoresis, silver stain was performed according to the procedure of Oakley et al.

![Fig. 1](image1.png) ![Fig. 2](image2.png)

**Fig. 1** The change of H₂S concentration in mouth air after rinsing with each mouthwash. The contents of VSC are expressed as %, in comparison with the concentration before rinsing. The average of 5 experiments is shown.

**Fig. 2** The change of CH₃SH concentration in mouth air after rinsing with each mouthwash. The contents of VSC are expressed as %, in comparison with the concentration before rinsing. The average of 5 experiments is shown.
Fig. 3 The change of \((\text{CH}_3)_2\text{S}\) concentration in mouth air after rinsing with each mouthwash. The contents of VSC were expressed as %, in comparison with the concentration of before rinsing. The average of 5 experiments is shown.

Fig. 4 The change of Total sulphur concentration in mouth air after rinsing with each mouthwash. The contents of VSCs are expressed as %, in comparison with the concentration before rinsing. The average of 5 experiments is shown.

Results

Effect of zinc chloride mouthwash on the production of VSC in mouth air

As shown in Fig. 1-4, zinc chloride rinse indicated that \(\text{H}_2\text{S}, \text{CH}_3\text{SH}, (\text{CH}_3)_2\text{S}\) and total sulphur contents in mouth air were 10.1%, 5.6%, 50.6%, and 9.0%, respectively, at 2 hours after rinsing, in comparison with each concentration before rinsing. At 3.5 hours after rinsing, the contents of \(\text{H}_2\text{S}, \text{CH}_3\text{SH}, (\text{CH}_3)_2\text{S}\) and total sulphur were 17.7%, 12.2%, 68.7%, and 12.5%, respectively. The inhibition of VSC production remained at 3.5 hours after zinc chloride rinsing.

In contrast, the VSC contents were 96.1%, 54.5%, 102.4%, and 74.0% of \(\text{H}_2\text{S}, \text{CH}_3\text{SH}, (\text{CH}_3)_2\text{S}\) and total sulphur at 3.5 hours after rinsing with Skoal™. In the water rinse experiment, VSC contents at 2 hours after rinsing were 122.7% 108.2%, 108.3% and 120.6% of \(\text{H}_2\text{S}, \text{CH}_3\text{SH}, (\text{CH}_3)_2\text{S}\) and total sulphur, respectively. Results showed that, Skoal™ rinse lost the effect of suppressing odour production by 3.5 hours after rinsing and water rinse lost the effect by 2 hours after rinsing.

On the basis of these results, zinc chloride rinse was demonstrated to strongly inhibit production of VSC in the oral cavity.

Effect of zinc chloride on VSC production of saliva putrefaction

Since the putrefied saliva systems produce VSC in head space, \textit{in vitro} experimentations of saliva putrefaction were employed in this study to examine the effect of zinc ions on VSC production.

The reduction of VSC by zinc chloride was observed at concentrations of more than 0.0073 mM zinc chloride, corresponding to a 1/5,000 concentration of original mouthwash, as shown in Fig. 5. At 0.073 mM zinc chloride, 53.7%, 35.9%, 103.0% and 39.5% of \(\text{H}_2\text{S}, \text{CH}_3\text{SH}, (\text{CH}_3)_2\text{S}\) and total sulphur
Fig. 5 The effect of zinc chloride on VSC production in the 24-hour putrefied saliva systems. The contents of VSC are expressed as %, in comparison with the concentrations in the control. The average of 5 experiments is shown.

Fig. 6 The effects of Skoal™ on VSC production in the 24-hour putrefied saliva systems. The contents of VSC were expressed as %, in comparison with the concentrations in the control. The average of 5 experiments is shown.

Fig. 7 Percoll density gradient centrifugation of salivary cellular elements I: fresh saliva, II: 24 hours incubated saliva, III: 24-hour incubated saliva in 0.01% zinc chloride. a: upper fraction (density ≥1.051), b: middle fraction (density: 1.051–1.076), c: lower fraction (density: ≤1.076).

Effects of zinc chloride on degradation of salivary cellular elements

Percoll™ density gradient centrifugation was employed to determine whether zinc ions inhibit the cell degradation during 24 hours incubation. As shown in Fig. 7, the upper fraction (density ≥1.051 g/ml) from fresh saliva contained a thick layer composed of mostly intact cells. After 24 hours incubation, the cell layer disappeared in the upper fraction, and another thick layer appeared in the middle fraction (density: 1.051–1.076 g/ml), although incubation in 0.01% zinc chloride showed that the thick layer still remained in the upper fraction. Microscopically, the upper fraction from the zinc chloride experiment contained mostly intact cells, as in the fresh saliva sample, but the control saliva contained many degraded cells, as shown in Fig. 8–10. These findings showed that zinc chloride inhibits cell degradation in saliva.
The effect of zinc chloride on proteinase activity in salivary cellular elements

Since salivary cellular elements, which involve microorganisms and protein substances, are VSC sources in the oral cavity, the effect of zinc ions on proteinase activity was determined.

Although the method of Schwert\textsuperscript{18} using N-benzoyl-L-arginine ethylester as substrate could not show any proteinase activity in salivary cellular elements, the method of Barrett\textsuperscript{14} using α-N-benzoyl-DL-arginine-4-nitroanilide HCl showed that the proteinase activity existed in salivary cellular elements. Hence, the inhibiting effect of zinc chloride on proteinase activity in salivary cellular elements was determined using a modified procedure of Barrett\textsuperscript{14}. As shown in Fig. 11, complete inhibition was observed at the concentration of 3.7 (0.05 %) and 37 (0.5 %) mM zinc chloride. At 0.73 mM (0.01 %) zinc chloride, 32.8 % inhibition of proteinase activity was observed in salivary cellular elements. Under 0.37 mM zinc chloride concentration, no inhibition was observed.

Degradation of proteins in incubated salivary samples

SDS-PAGE was employed to examine protein degradation in both saliva supernatant and salivary
cellular elements during 24 hour incubation. In saliva supernatant, 66.2 K protein band and 20.7 K~14.4 K of bands nearly disappeared after incubation as shown Fig. 12. The saliva incubated with 7.3 mM zinc chloride, however, involved 66.2 K and 14.4 K protein bands. In salivary cellular elements, 66.2 K, 84.4 K, 27.2 K, 24.6 K and 15.3 K protein bands disappeared after incubation. The other hand, the result of incubation study with zinc chloride indicated that those bands still remained after incubation. Also, strongly stained bands under 13.5 K appeared in the zinc chloride experiment. The findings implied, zinc chloride inhibits protein degradation during incubation, specially in salivary cellular elements.

Discussion

Volatile sulphur compounds (VSC) are the principal components of oral malodour\(^2\). Other molecules such as fenol or indole were reported to modify the quality and intensity of malodour\(^2\). Therefore, inhibiting production of VSC seems to be the most effective method for prevention or reduction of oral malodour.

Recently, cytotoxity of VSC to gingival tissues was demonstrated. Tonzetich \textit{et al.}\(^{19,20}\) reported that VSC strongly suppressed protein, and collagen synthesis in human gingival fibroblasts. VSC are also reported to cause an increase of the degradation of newly synthesized collagen in human gingival fibroblasts\(^{19}\), and to enhance the penetration of oral mucosa by PGE\(_2\) and endotoxin\(^{21}\). By reason of these findings, Tonzetich \textit{et al.}\(^{21,21}\) strongly suggested that, VSC are the promoting factors of periodontal disease. Accordingly, inhibiting production of VSC not only prevents oral malodour, but may also reduce periodontal disease.

Since a commercial mouthwash containing zinc chloride as stabilizer was reported to affect production of VSC\(^3\), zinc chloride mouthwash was examined in this study. The effect of 0.5 % zinc chloride mouthwash was compared with a commercial product (Skoal\textsuperscript{TM}) and water rinse as shown in Fig. 1-4. The results demonstrated that zinc chloride has strongly inhibits production of VSC in the oral cavity. The putrefied saliva systems of the \textit{in vitro} study also indicated that zinc chloride strongly inhibits VSC production.
To date, the mechanism of inhibiting VSC production has not been reported. Therefore, Percoll™ density gradient centrifugation was employed in this study to examine the effect on cell degradation in the putrefied saliva systems, because cell degradation involving proteolysis has an important role in VSC production. This study demonstrated that zinc chloride inhibits cell degradation during 24 hours incubation. Also, we showed that zinc chloride inhibits proteinase activity in salivary cellular elements from fresh whole saliva, as shown in Fig. 11. The results of SDS-PAGE demonstrated that many protein bands remained in the cellular elements after 24 hours incubation with 0.01 % zinc chloride, whereas the control sample lost many bands after incubation. And some protein bands in the saliva supernatant, i.e., the 66.2 K and 20.7 K~14.4 K bands, remained after incubation with 0.01 % zinc chloride. The results of electrophoresis showed that proteolysis in saliva is inhibited by zinc chloride.

Zinc chloride reduces the enzyme activity related to cell degradation. Heavy metal ions, such as zinc ions and mercury ions, act as potent inhibitors of thiol enzymes, thus supporting the assumption of a thiol group as the active centre of the enzymes21). Therefore, zinc chloride might directly inhibit thiol proteinase activity related to VSC production in saliva. Also, the vitality of microorganisms which degrade the cells or proteins might be reduced because thiol enzymes supporting cell metabolisms are inhibited by zinc chloride. Although the effects on microorganisms were not examined in this study, it is reasonable to speculate that an inhibition of thiol enzymes or a reduction of bacterial vitality causes a decrease in oral malodour.

Conclusion

The 0.5 % zinc chloride mouthwash was demonstrated to inhibit production of VSC in the oral cavity. At 3.5 hours after rinsing, the contents of H2S, CH3SH, (CH32)2S and total sulphur were 17.7 %, 12.2 %, 68.7 % and 12.5 % respectively in comparison with each concentration before rinsing. Also strong inhibition of VSC was observed in the putrefied saliva systems.

The results of Percoll™ density gradient centrifugation of putrefied saliva indicated that zincs ion inhibited the degradation of salivary cellular elements during incubation. Also, this study showed that protein degradation and proteinase activity in saliva were inhibited in the presence of zinc chloride.

On basis of these findings, zinc chloride was shown to be one of the most effective mouthwashes to reduce oral malodour.

References

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