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Combined effect of zinc ions and cationic antibacterial agents on intraoral volatile sulphur compounds (VSC)

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Objective: Volatile sulphur compounds (VSC) are major components of oral malodour. As both zinc ions and cationic antibacterial agents inhibit the formation of oral VSC, this study aimed to determine whether these agents combined have synergistic anti-VSC actions. **Methods:** Baseline oral VSC measurements of mouth air from 10 volunteers following cysteine rinsing (6mM, pH 7.2) were obtained using gas chromatography (GC). Subjects rinsed for 1 min with 10ml of the test solutions, 0.3% zinc acetate (Zn), 0.025% chlorhexidine (CHX), 0.025% cetyl pyridinium (CPC), and the combinations Zn+CHX and Zn+CPC. Cysteine rinses were repeated at 1h, 2h and 3h and VSC measurements recorded. Three subjects rinsed with the Zn+CHX combination and fasted for 9h, undergoing cysteine rinses and VSC measurements at 3h intervals. 10µl of the test solutions were also added to 1ml aliquots of human whole saliva (n=8). Following incubation at 37°C for 24h VSC levels in the saliva headspace were measured by GC. Inhibition of VSC formation and the fractional inhibitory index indicating synergy were calculated. **Results:** Zn+CHX mouthrinse had a synergistic anti-VSC effect, and was effective for at least 9h. Zn+CPC mouthrinse was less effective. Both combinations showed a synergistic inhibiting effect *in-vitro*. **Conclusions:** Synergy between Zn and the antibacterial agents confirms different mechanisms of operation.

Key words: Volatile sulphur compounds, oral malodour, zinc ions, cationic antibacterial agents, periodontal disease

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It is well established that the majority of cases of oral malodour are caused by Gram negative, anaerobic bacteria located in the crypts at the back of the tongue, or in periodontal pockets[1-3]. These bacteria are proteolytic and produce volatile sulphur compounds (VSC) by catabolisation of organic substrates, in particular cysteine[4]. The VSC have an unpleasant odour even in extremely low concentrations, and are the major component of oral malodour[1,5,6]. The main VSC are hydrogen sulphide and methyl mercaptan, but small amounts of dimethyl sulphide may also be present[1].

It is also well established that VSC are able to penetrate the tissues in periodontal pockets[7], that they have a direct deleterious effect on the synthesis of proteins in gingival fibroblasts[8], and that for these reasons VSC may be important in the aetiology of periodontal disease[7-10].

It is furthermore known that certain metal ions, in particular zinc, can be used to reduce or inhibit oral malodour, and that along with other metal ions, zinc inhibits the formation of VSC[8,11-13]. There may be several mechanisms involved:

- The zinc ions (in aqueous solutions or as dissolvable tablets) interact with the sulphur in the

substrate or in precursors of VSC, forming insoluble sulphides, since zinc has an affinity for sulphur and oxidises sulphydryl groups[7]

- Heavy metal ions such as zinc directly inhibit thiol proteinase activity related to VSC production[11].

Certain antibacterial agents such as chlorhexidine or cetyl pyridinium may also inhibit oral malodour and VSC formation[14,15]. In beagle dogs, zinc ions have been shown to enhance the plaque-inhibitory effects of cetyl pyridinium chloride[16]. A similar enhancement of the effect of mouthwashing with chlorhexidine was shown with zinc in humans[17]. If zinc ions and cationic antibacterial agents operate by different mechanisms with regard to oral VSC inhibition, it is conceivable that the combination of these agents may also provide an enhanced or synergistic anti-VSC effect. The aim of the present study was to examine this concept. The hypothesis to be tested was thus that zinc and a cationic antibacterial agent have synergistic effects when combined in aqueous solution and used as a mouthrinse to inhibit oral VSC formation.

Materials and methods

Test solutions

The solutions tested included 0.3 per cent zinc acetate 2-hydrate (Zn) (Reidel-deHaën, Germany), 0.025 per cent chlorhexidine diacetate monohydrate (CHX) (Fluka Chemie, Switzerland), 0.025 per cent cetyl pyridinium chloride monohydrate (CPC) (Sigma-Aldrich, Germany) and the combinations: 0.3 per cent zinc acetate + 0.025 per cent chlorhexidine (Zn+CHX), and 0.3 per cent zinc acetate + 0.025 per cent cetylpyridinium chloride (Zn+CPC). All solutions were made with de-ionised water.

Mouth rinse experiments

Collection of samples and VSC analysis of mouth air

Test subjects consisted of ten volunteers (4 males, 6 females, aged 30 to 72 years) recruited from the staff at the Dental Faculty, University of Oslo. The volunteers did not complain of oral malodour and had no obvious medical history that could relate in any way to oral malodour. All test subjects took part in the experiments with informed consent, after having received an explanation of the protocol approved by an ethics committee. On test days, the subjects were instructed to refrain from their normal oral hygiene routine following breakfast and present at the clinical research laboratory at 9.00am. The cysteine challenge model according to Kleinberg and Codipilly[18] was used for inducing oral malodour in the subjects. This involved the subjects rinsing for 30s with 5ml of 6mM L-cysteine solution (pH 7.2) (Sigma Chemicals, USA). Subjects then kept their mouth closed for 1 min 30s, after which mouth air samples were taken (baseline/control measurements).

Mouth air samples were aspirated using a 10ml syringe connected to the outlet of the auto-injector, and analysed for VSC directly in a gas chromatograph as described below. Immediately after this procedure, the subjects rinsed for 1 min with 10ml of one of the test solutions. Cysteine rinsing and mouth air analyses were repeated at 1h, 2h and 3h after rinsing with the respective solutions. Although the study was not double blind, the different test solutions were given at random to the test subjects on different days without the subjects knowing which mouth rinses they were using.

In a second mouth rinse experiment, three healthy persons (one female aged 41 years and two males aged 45 and 73 years) were used as test subjects to examine the long-term effect of a single mouth rinse containing 0.3 per cent zinc acetate and 0.025 per cent chlorhexidine acetate. The cysteine challenge model was used as described above. Subjects rinsed for 1 min with 10ml of the test solution. Cysteine rinsing and mouth air analyses were repeated at 3h, 6h and 9h. Subjects did not eat or drink during the entire test period. Due to the length of fasting, the number of test subjects was limited.

Gas chromatography

The VSC analysis system included a GC-14B gas chromatograph (Shimadzu, Japan) equipped with a flame photometric detector, a 12-ft x 1/8 inch Teflon column packed with 5 per cent polyphenyl ether-0.05 per cent phosphoric acid on 40/60 mesh Chromosorb T, and an auto-injection system with a 3ml sample loop. Column conditions were column temperature 70°C, nitrogen gas flow rate 32 ml/min, hydrogen gas flow rate 125ml/min, air flow rate 43ml/min, according to Yaegaki and Sanada[2].

Salivary putrefaction experiments

Eight of the volunteers participating in the clinical experiments also provided saliva samples (2 males and 6 females, age range 30 to 46 years). Between 9.00 and 10.00am, 1-2h following normal daily dietary intake and oral hygiene routines, each subject chewed a paraffin wax tablet for 1 min while swallowing normally, before collection of 10ml whole saliva. Saliva samples were shaken thoroughly following collection prior to 1ml aliquots being pipetted into separate test tubes with screw lids. 10µl aliquots of the test solutions were added to the 1ml saliva aliquots. The samples were incubated overnight at 37°C. Two untreated saliva samples were included for each test subject.

After 24–30 h incubation the tubes were shaken for 15s according to Kleinberg and Codipilly[18], a sample of the saliva headspace was withdrawn from the test tube using a 10ml syringe, and the sample was analysed for VSC directly in the gas chromatograph.

Calculation of synergy

Data from the mouth rinsing experiments were calculated as percentage of the original concentration of H_2S (control), at each of the measurement times for each test subject. Data from the salivary putrefaction experiment was also calculated as percent of the control VSC. The mean values for the results for all test subjects were used to evaluate possible synergistic effects according to the fractional inhibitory index (FIC index) as described by Berenbaum[19] and used in similar studies[20,21]:

$$\text{FIC index} = \frac{A + B}{A} + \frac{A + B}{B}$$

A = effect of antibacterial agent, B = effect of zinc, A + B = effect of the combination of an antibacterial agent and zinc. A FIC index of <1 indicates a synergistic (or complimentary) effect, FIC =1, an additive effect, and FIC >1 an antagonistic effect.

Results

Figure 1 shows the results of mouth rinsing with the different test solutions. 0.3 per cent Zn had a very strong anti-VSC effect after 1h, but this effect diminished relatively fast. 0.025 per cent CHX had only a moderate anti-VSC effect after 1h, but this effect diminished only slightly with time. A similar result was seen for 0.025 per cent CPC after 1h, but the effect of this agent deteriorated more rapidly. The anti-VSC effect of the combination of Zn and CHX was surprisingly marked and long lasting. Scarcely any reduction in anti-VSC effect was observable after 3h, in contrast to the effect of Zn alone. The combination of Zn and CPC had a good anti-VSC effect after 1h, but minimal anti-VSC effect above that of Zn alone at all three measurement times. *Synergy:* The FIC indices for the mouth rinse combination Zn + CHX were 0.56, 0.35 and 0.38 at 1, 2 and 3h respectively, the combination thus having a synergistic effect (see * in *Figure 1*) at all three measuring periods. The Zn + CPC mouth rinse combination had FIC indices of 1.45, 0.88 and 1.13 at 1, 2 and 3h respectively, thus only synergistic at 2h.

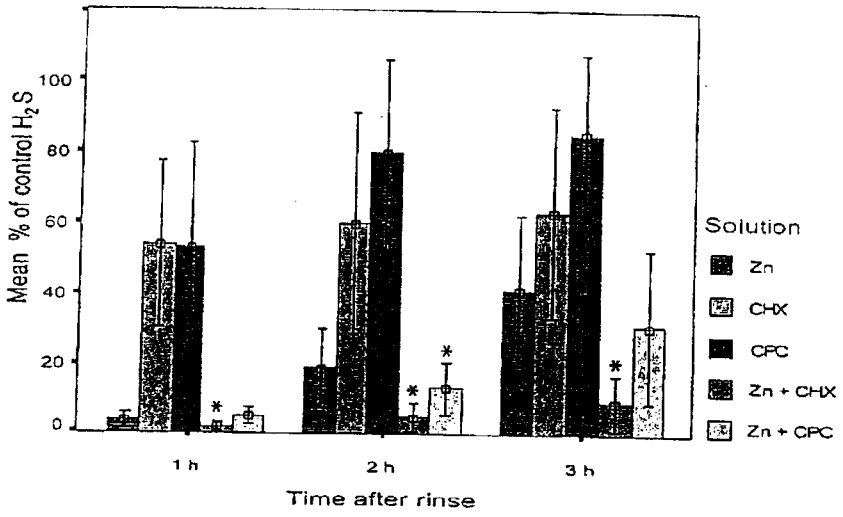

Figure 1. Inhibition of hydrogen sulphide production (mean per cent of control H_2S, ±2 s.d.) at 1, 2 and 3h after mouth rinses with individual test solutions and combinations. Test solutions: 0.3 per cent zinc acetate (Zn), 0.025 per cent chlorhexidine diacetate (CHX), 0.025 per cent cetyl pyridinium chloride (CPC) and combinations of Zn + CHX and Zn + CPC. A lower per cent of control value indicates a more effective anti-VSC agent (control value = 100 per cent). Synergistic anti-VSC effect, * FIC < 1.

The results of the salivary putrefaction experiment for methyl mercaptan are shown in *Figure 2*. A similar pattern of VSC inhibition could be observed as for the mouth rinsing, whereby Zn was the most effective of the individual agents, and both combinations (Zn + CHX and Zn + CPC) were more effective than the individual agents. The results for inhibition of hydrogen sulphide production were in line with the clinical results and are not shown. *Synergy*: Both mouth rinse combinations showed synergistic anti-VSC effects, showing FIC indices of 0.63 and 0.26, for Zn+CHX and Zn+CPC, respectively (see * in *Figure 2*).

Figure 3 shows the results of the extended mouth rinse experiment. The combination of 0.3 per cent zinc acetate and 0.025 per cent chlorhexidine diacetate had a marked anti-VSC effect even after 9h. It should be noted that the mouth rinsing experiments, performed using cysteine rinses according to the cysteine challenge model[18], provided information almost exclusively related to one VSC component, hydrogen sulphide.

Discussion

Previous studies have shown a correlation between oral malodour measured organoleptically and measurements of VSC by gas chromatography[5,22] thereby indicating the relevance of VSC measurements to oral malodour. Similar correlation has been shown using the portable sulphide monitor[23–26].

Cadaverine and putrescine have not been demonstrated as components of oral malodour *per se*[27]. Previously, Tonzetich[28] did not find any non-sulphur compounds involved in oral malodour. More recently Tonzetich[29] suggested that

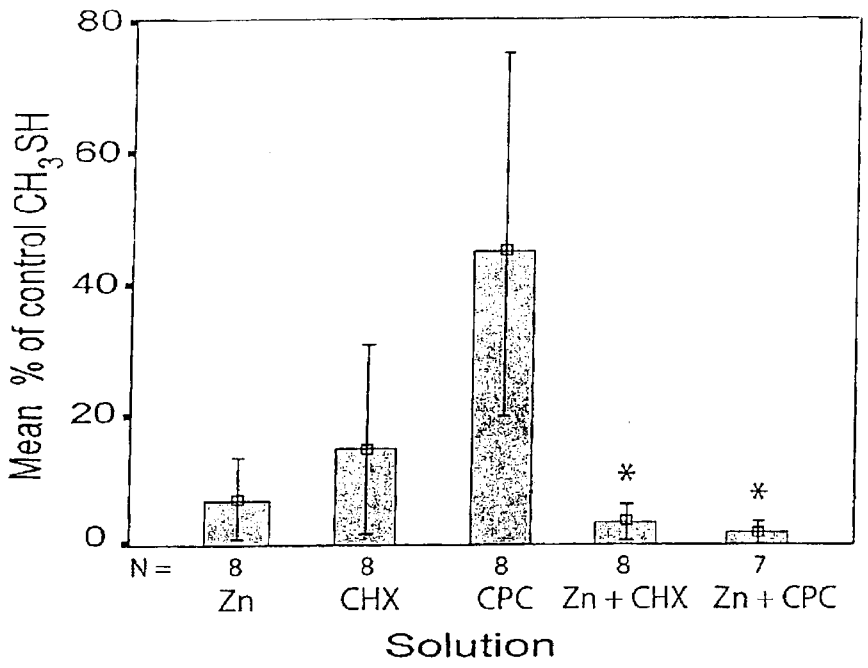

Figure 2. Inhibition of methyl mercaptan production in incubated saliva (mean per cent of control CH_3SH, ±2 s.d.) by test solutions and combinations. Test solutions: 0.3 per cent zinc acetate (Zn), 0.025 per cent chlorhexidine diacetate (CHX), 0.025 per cent cetyl pyridinium chloride (CPC) and combinations of Zn + CHX and Zn + CPC. A lower per cent of control value indicates a more effective anti-VSC agent (control value = 100 per cent). Synergistic anti-VSC effect, * FIC < 1.

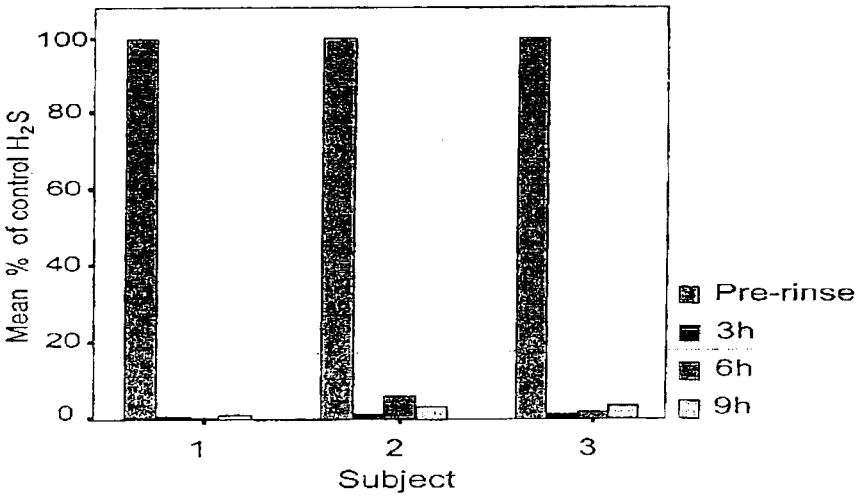

Figure 3. Inhibition of hydrogen sulphide production in three test subjects (mean per cent of control H_2S) over 9 h, by the combination of 0.3 per cent zinc acetate and 0.025 per cent chlorhexidine diacetate. Control =100 per cent.

minor components that are unable to produce oral malodour themselves, occurring in amounts below the threshold of organoleptic perception can still alter the intensity and quality of oral malodour in combination with VSC. The ratio of H_2S and CH_3SH is known to contribute to the quality of oral malodour, at least in periodontal patients[2]. It is also known that zinc ions that inhibit oral malodour also inhibit VSC, as mentioned above. The experimental data cited above indicates that the method involving the use of oral VSC measurements by gas chromatography can be considered as valid and directly related to oral malodour.

Mouth rinses with cysteine to enhance formation of oral VSC in the test panel were used in the present study[18]. This method lends itself to clinical testing of inhibitors of oral VSC production and oral malodour, as discussed by these authors. The reduction in VSC formation subsequent to a single rinse with an inhibiting agent is compared with the original VSC value observed, and any reduction taken as caused by the inhibitor. Additional cysteine rinses at an hourly interval, challenge the effect of the inhibitor and provide data concerning the duration of the effect. A limitation of this method is that only hydrogen sulphide is formed in the oral cavity immediately following cysteine rinses, as mentioned previously[4,13,17]. However, supplementing the clinical experiments with salivary putrefaction experiments can compensate for this limitation. This involves measurement of the effect of an oral malodour inhibitor on the production of both hydrogen sulphide and methyl mercaptan in incubated human saliva. The results for methyl mercaptan can be seen in *Figure 2*.

A comparison of the anti-VSC results for the individual agents and those for the combinations, showed that the combination of zinc ions and chlorhexidine had a better anti-VSC effect than that of zinc ions or chlorhexidine alone (*Figure 1*). The hypothesis to be tested in the present study was thus supported for this combination. This was confirmed by the calculated FIC index, demonstrating a synergistic effect at 1, 2 and 3h after rinsing. That result was further supported by the findings from the salivary putrefaction experiment (*Figure 2*) demonstrating that the combination of zinc ions with both chlorhexidine diacetate and cetylpyridinium chloride showed synergistic anti-VSC effects. In a recent study on effect of antibacterial agents on cariogenic organisms, a different interpretation of drug interactions was used[30]. According

to Isenberg[31], if the FIC index = 0.5 > x <1.0, this is described as partial synergy. Using this interpretation in the present study, the mouth rinse experiments showed that the combinations Zn + CHX at 1h, and Zn + CPC at 2h were partially synergistic, while the *in vitro* experiments demonstrated partial synergy for the Zn + CHX combination.

In the long-term experiment the combination of zinc and chlorhexidine provided a reduction of more than 95 per cent of the baseline VSC level even 9h after rinsing (*Figure 3*). This result should be considered very satisfactory especially taking into account the low concentration of the ingredients. Each mouth rinse with cysteine appears likely to consume any zinc retained in the mouth. Under normal conditions without cysteine challenges it may be safe to conclude that the mouth rinse as tested in the long-term experiment could be effective for 12 hours or more.

The moderate clinical anti-VSC effect of the cationic antibacterial agents alone was most likely due to the low concentrations used in order to avoid untoward side effects. Zinc has a metallic taste. The 0.1 per cent and 0.3 per cent zinc acetate solutions used in this experiment are dilute compared with concentrations used in some earlier clinical experiments (4 per cent or more). Pilot experiments by the current authors have shown that even a combination of as low as 0.1 per cent zinc and 0.01 per cent chlorhexidine had an anti-VSC effect, though this was not as long lasting as the presently used concentrations.

However, despite the relatively low concentrations involved, it was surprising to observe how much the combination of the antibacterial agents improved the effect of zinc ions, both in the mouth rinsing and salivary putrefaction experiments. It appears likely that the specific effect of zinc against sulphur and the unspecific antibacterial effect against the bacterial membranes, in particular related to chlorhexidine, are the mechanisms behind the synergism of the combination. However, previous experiments have indicated that antibacterial agents, and in particular chlorhexidine, can split disulphide bonds[32]. Chlorhexidine is known to be a strong denaturing agent. A splitting of disulphide bonds would be beneficial, as oral bacteria mainly contain desulphydrases, as demonstrated by the results of the present experiment. The splitting of disulphide bonds could provide an explanation for the observed marked and long lasting effect of the mouth rinse containing an aqueous combination of chlorhexidine and zinc ions.

It may be speculated that a further beneficial effect of the antibacterial agents could be to inhibit any additional foul, volatile non-sulphur bacterial products in the oral cavity. Zinc ions alone would have negligible effect on the formation of such products.

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