A New Mouthrinse Combining Zinc and Chlorhexidine in Low Concentrations Provides Superior Efficacy Against Halitosis Compared to Existing Formulations: A Double-Blind Clinical Study

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Abstract

- Objective: Volatile sulfur compounds (VSC), mainly derived from bacteria located in deep crypts at the back of the tongue and from periodontal pockets, are responsible for approximately 90% of halitosis (bad breath, malodor). The objective of this double blind clinical study was to assess the clinical efficacy of a new formulation for halitosis containing a combination of zinc (0.3% Zn) and chlorhexidine (0.025% CHX) in low concentrations. The new formulation was compared to some widely used and commercially available formulations containing various enzymes and antibacterial agents in a clinical setting under controlled conditions.

- Methodology: Ten healthy volunteers participated in this study (5 female, 5 male, mean age: 46.6, range: 26–79). Each participant served as their own control, and neither the investigator nor the ten test subjects knew which formulation they were testing at any given time (double-blind design). Baseline H2S data were obtained by cysteine rinsing for 30 seconds, 90 seconds mouth closure, and gas chromatographic (GC) analysis of mouth air. On separate days, each participant then rinsed for 60 seconds with 10 ml of each of the eight various formulations. Cysteine rinses were repeated at 1 hour, 2 hours, and 3 hours, and GC measurements of oral H2S levels were again recorded.

- Results: The test rinse (0.3% Zn + 0.025% CHX) reduced the intraoral H2S levels to 0.16% of control (range: 0.01–0.54%) after 1 hour, 0.4% after 2 hours, and 0.75% after 3 hours, providing superior efficacy in reducing H2S compared to the other formulations tested (p < 0.05).

- Conclusion: A combination of Zn and CHX in low concentrations seems to be the most efficient way to remove the VSC that causes bad breath at present. Studies are underway to further explore the extraordinary efficacy of this combination (close to 100%), suggesting a specific mode of action and a synergistic effect of these two components.

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Introduction

Offensive odor emanating from the oral cavity, often termed halitosis, is responsible for approximately 90% of bad breath cases. Halitosis is mainly caused by volatile sulfur compounds (VSC) derived from Gram negative anaerobic bacteria, mostly found in periodontal pockets and in the crypts at the back of the tongue. Hydrogen sulfide (H2S), methyl mercaptan (CH3SH), and, to a lesser extent, dimethyl sulfide (CH3SCH3) are the major components of the VSC that originate from the degradation of the sulfur-containing amino acids, cysteine, and methionine. They have an unpleasant odor, even in extremely low concentrations. In addition to causing halitosis, VSC may play an important role in the etiology of periodontal disease. In particular, methyl mercaptan has been shown to penetrate the various tissues in periodontal pockets, and increase the degradation of collagen, as well as inhibiting the protein synthesis of gingival fibroblasts, thus adversely affecting critical events in the development of periodontitis.

The authors of this paper, and other researchers, have shown that certain metal ions, zinc (Zn) in particular, can be used to inhibit the formation of VSC and subsequently reduce or eradicate halitosis. Moreover, it has been shown that certain antibacterial agents such as chlorhexidine (CHX) or cetylpyridinium chloride (CPC) may also inhibit VSC formation and thus reduce halitosis. If zinc ions and antibacterial agents operate by different mechanisms with regard to oral VSC inhibition, it is conceivable that combinations of two or more of these agents may provide an enhanced or synergistic anti-VSC effect. However, the opposite might also be the case; one or two components might reduce or block the effect of the other. In order to examine this further it was decided to: a) evaluate the clinical effectiveness of a new anti-halitosis formulation (SB12®, Antula AB, Stockholm, Sweden) combining low concentrations of Zn (0.3%) and CHX (0.025%); b) use a double-blind clinical protocol to allow an unbiased comparison with other anti-halitosis formulations containing various enzymes and antibacterial agents, as shown in Table I; c) use a specially modified gas chromatograph particularly suited for measurements of low concentrations of VSC and considered the “gold standard” of halitosis measurements; and d) use cysteine rinsing according to Kleinberg and Codipilli to introduce bad breath in healthy volunteers in order to avoid some of the problems with including “patients,” as well as enabling each participant to serve as his or her own control.

The aim of the present study was to examine the effectiveness of a new anti-halitosis formulation combining low levels of Zn and CHX, and to compare it with other widely used formulations
against halitosis in a double-blind clinical design. The hypothesis to be tested was that Zn combined with CHX in low concentrations effectively inhibits H$_2$S production induced in healthy individuals, and moreover, is comparatively more effective than other currently used antibacterial agents and/or enzymes.

**Materials and Methods**

**Oral Rinses**

Eight different oral rinses were included in the study. All the oral rinses were commercially available at the time of the study except SB12$^\circledR$ which was provided free-of-charge by the manufacturer (Antula AB, Stockholm Sweden). This study was performed at the Clinical Research Laboratory, Dental Faculty, University of Oslo, Norway. The following oral rinses were included in the experiment:

A. Zendium$^\circledR$ Munnskölj med Zink (Opus Healthcare, Malmö, Sweden)
B. Listerine$^\circledR$ Natural Citrus (Pfizer Consumer Healthcare, Morris Plains, NJ, USA)
C. Listerine$^\circledR$ Cool Mint (Pfizer Consumer Healthcare, Morris Plains, NJ, USA)
D. Halita$^\circledR$ Chlorhexidine digluconate 0.05%, cetylpyridinium chloride (CPC) 0.05% and zinc lactate 0.14%
E. retarDEX$^\circledR$ Antibacterial agent (cloSYS$^\circledR$)
F. Dentyl$^\circledR$ Refreshing Clove and Clove and Freshening Mint (Fresh Breath Ltd, London, UK)
G. Dentyl$^\circledR$ Smooth Mint (Fresh Breath Ltd, London, UK)
H. SB12$^\circledR$ (Antula Healthcare AB, Stockholm, Sweden)

A summary of the active ingredients of the various rinses, as listed on the bottles, is shown in Table I.

**Table I**

<table>
<thead>
<tr>
<th>kode</th>
<th>Mouth rinse</th>
<th>Active ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Zendium$^\circledR$</td>
<td>Zinc gluconate and various enzymes; amyloglycosidase, glycosidase, and lactoperoxidase</td>
</tr>
<tr>
<td>B</td>
<td>Citrus and Cool Mint</td>
<td>Antibacterial agents: eucalyptol 0.092%, menthol</td>
</tr>
<tr>
<td>C</td>
<td>Halita$^\circledR$</td>
<td>Chlorhexidine digluconate 0.05%, cetylpyridinium chloride (CPC) 0.05% and zinc lactate 0.14%</td>
</tr>
<tr>
<td>D</td>
<td>retarDEX$^\circledR$</td>
<td>Antibacterial agent (cloSYS$^\circledR$)</td>
</tr>
<tr>
<td>E</td>
<td>Denty$^\circledR$</td>
<td>Antibacterial agents: cetylpyridinium chloride, triclosan</td>
</tr>
<tr>
<td>F</td>
<td>Smooth Mint</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>SB12$^\circledR$</td>
<td>Zn acetate 0.3% and chlorhexidine diacetate 0.025%</td>
</tr>
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</table>

On test days, the subjects refrained from their normal oral hygiene and presented at the laboratory at 9:00 a.m. The participants rinsed for 30 seconds with 5 ml of a 6 mM solution of L-cysteine (Sigma Chemical Co., St Louis, MO, USA) at pH 7.2. Subsequently, they kept their mouths closed for 90 seconds, after which mouth air samples were aspirated into a 3 ml sample loop connected to the auto injector of a gas chromatograph (Shimadzu, Kyoto, Japan), modified for this purpose as previously described. The obtained mouth air samples were thereafter analyzed directly by separation in the gas chromatograph using a Teflon column (3.66-mm x 0.32 cm, temperature 70°C, nitrogen gas flow 32 ml min$^{-1}$, hydrogen gas flow rate 125 ml min$^{-1}$ and airflow rate 43 ml min$^{-1}$) packed with polyphenol ether (5%)-phosphoric acid (0.05%) on 40/60 mesh Chromosorb T and a flame photometric detector. The standardized H$_2$S formation in the mouth that was obtained after the cysteine rinsing constituted the baseline as a control for each tested subject. Immediately following, each subject rinsed for 30 seconds with one of the eight test solutions (A-H). Thereafter, cysteine rinses followed by mouth air analyses were repeated at 1, 2, and 3 hours. The H$_2$S levels were subsequently compared with the baseline levels for each subject. At least one non-test day between uses of the different test solutions was introduced to avoid a putative cross-over effect between the different test solutions.

**Statistical Analyses**

Concentration of H$_2$S in breath samples from the control measurement, and from measurements taken 1, 2, and 3 hours after treatment were obtained from gas chromatograph software (EZStrat 7.2) as AUC (area under the curve) for the chromatogram peak. Those raw data were furthermore calculated as a % of control for each of the test subjects.

Differences between the examined mouthrinses were statistically tested by one-way ANOVA and LSD multiple comparisons. These tests were performed on both AUC (presented in Table II) and % of control (Figures 1, 2, and 3). The outcomes of the statistical analyses were similar in both cases. It was further investigated whether different active ingredients have or do not have an inhibitory effect on oral H$_2$S formation; results greater than 100% were considered as “not having” inhibitory effect. The reason for those results greater than 100% needs closer investigation.

**Results**

A significant inhibition of H$_2$S production was observed in mouth air samples taken 1, 2, and 3 hours after the rinse with a combination of Zn and CHX in low concentration (H) compared to the H$_2$S baseline in all the 10 subjects tested. A great inter-individual variation in H$_2$S levels was observed between the different test subjects. The results are summarized in Table II.

A great variation in effectiveness among the various formulations was observed, ranging from virtually no observed effect (A, F) to almost 0% of control (H) over the whole testing period (3 hours). The results of the rinsing experiment (AUC) comparing the eight different anti-halitosis formulations are summarized in Table II and illustrated as % of control in Figures 1–3.
Discussion

Given the design of this study with each test subject serving as their own control, the great inter-individual variation in H₂S levels that was observed did not adversely influence the overall quality of the results. Moreover, by inducing halitosis in healthy volunteers, the difficulty with putative interference with various diseases and medication (drugs) that might influence H₂S production was avoided. Halitosis is a symptom and not a disease, that often occurs in otherwise healthy individuals mainly due to local conditions in the mouth; i.e., putrefaction of anaerobic bacteria in crypts at the back of the tongue and in periodontal pockets.1,4-6,8 The choice of test subjects thus seemed appropriate. The subjective nature of bad breath *per se*, as well as rather subjective (organoleptic, nasopalatinal index)23 and less sensitive and specific measurement methods (i.e., portable sulfide monitor, e.g., Halimeter®),24 further complicate this picture making it more difficult to perform reliable comparative studies, as well as

Table II

Comparison of Oral H₂S Formation Before and After Treatment with the Different Mouthrinses

<table>
<thead>
<tr>
<th>Mouthrinse</th>
<th>Control ± Std. Error</th>
<th>1 h ± Std. Error</th>
<th>2 h ± Std. Error</th>
<th>3 h ± Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10526730 ± 1996725</td>
<td>*10399169 ± 1642165</td>
<td>*11016940 ± 1893715</td>
<td>*11062133 ± 1777625</td>
</tr>
<tr>
<td>B</td>
<td>8034153 ± 2261068</td>
<td>*6952575 ± 1469653</td>
<td>*6806082 ± 2025882</td>
<td>*7630772 ± 2105447</td>
</tr>
<tr>
<td>C</td>
<td>9393820 ± 2207629</td>
<td>*4727536 ± 2138393</td>
<td>*5526099 ± 1870641</td>
<td>*8212024 ± 2650699</td>
</tr>
<tr>
<td>D</td>
<td>8659070 ± 1343685</td>
<td>1130869 ± 878992</td>
<td>1477441 ± 1133042</td>
<td>146372 ± 597131</td>
</tr>
<tr>
<td>E</td>
<td>6915213 ± 165857</td>
<td>2985235 ± 673863</td>
<td>4028744 ± 1090056</td>
<td>3211370 ± 9375354</td>
</tr>
<tr>
<td>F</td>
<td>8303359 ± 2418222</td>
<td>*9731853 ± 1689299</td>
<td>*9476981 ± 1916136</td>
<td>*8223760 ± 1427925</td>
</tr>
<tr>
<td>G</td>
<td>7758629 ± 2341766</td>
<td>*6585337 ± 233692</td>
<td>*8376508 ± 2750260</td>
<td>*7402215 ± 1680240</td>
</tr>
<tr>
<td>H</td>
<td>13677005 ± 5266525</td>
<td>12213 ± 5013</td>
<td>48234 ± 23533</td>
<td>87059 ± 41391</td>
</tr>
</tbody>
</table>

One-way

ANOVA p > 0.05 p < 0.05 p < 0.05 p < 0.05

LSD p > 0.05 p < 0.05 p < 0.05 p < 0.05

* Significantly different from test rinse, H—p < 0.05

Figure 1. Box plot of the results showing the inhibition of oral H₂S formation (percentage of control baseline H₂S) obtained 1 hour after mouth rinse. The lines within the boxes indicate the medians. Top and bottom boundaries of each box show 75th and 25th percentiles, respectively. Whiskers indicate the maximum/ minimum points.

Figure 2. Box plot of the results showing the inhibition of oral H₂S formation (percentage of control baseline H₂S) obtained two hours after mouth rinse.

Figure 3. Box plot of the results showing the inhibition of oral H₂S formation (percentage of control baseline H₂S) obtained three hours after mouth rinse.
assessing the relative amounts (and contribution) of H₂S and CH₃SH to halitosis. The introduction of the gas chromatograph, with further modifications of this equipment to suit this purpose (separate and measure VSC obtained directly from the mouth in vivo), has greatly improved the quality of data, and allows direct comparison of various mouth rinses and combinations of active ingredients used to inhibit bad breath. These modifications include changing the sample injection system to allow application of air samples directly from the mouth to the GC, and a specially made Teflon column to allow better separation of large samples and higher sensitivity readings for low concentrations of sulfur gases which smell badly at extremely low concentrations, particularly CH₃SH.

Although the results supported our original hypothesis that a combination of Zn and CHX in low concentrations was the most efficient way to inhibit H₂S formation and thus halitosis, the degree of effectiveness was surprising (almost 0% of control in H₂S even after 3 hours). Additional studies are underway to further explore this effect, as well as its apparent long-lasting effectiveness. It moreover supports a previous pilot study indicating some H₂S inhibitory effect even after 12 hours, and given the low concentration of the active components (Zn and CHX), suggests a synergistic effect of the two. It further indicates that Zn and CHX in low concentrations have specific mechanisms of action, separate binding sites, and might even work in a different way than when applied in concentrations most widely used (and significantly higher; Zn 0.3 % vs. 2-5 % and CHX 0.025 % vs. 0.2 %). No side effects have moreover been observed when they are used in such low concentrations compared to some reported side effects (such as discoloration, metal taste, mucosal desquamation, and possible disturbance of the normal micro flora of the mouth) of current formulations.

We speculate that the mechanism of action is mostly a direct inhibition of the gas per se (H₂S) and, to a lesser extent, the antibacterial effect that is well known for both CHX and Zn in higher concentrations. We suggest there is a two-step mechanism where CHX initially splits SH bindings, rendering S⁻ available for positive Zn⁺ ions to bind, resulting in the formation of insoluble non-odorous Zn-sulfides that are passed through the GI tract and eventually excreted. Further studies of this hypothetical mechanism of action are clearly needed, and the potent inhibitory effect of this new formulation may also include other mechanisms working in parallel. Clearly, more information is needed to better understand how CHX and Zn work in such low concentrations.

The results from comparing various commercially available and widely used oral rinses against halitosis were rather surprising. Our working hypothesis that CHX and Zn, taken in combination and in low concentrations, was the most efficient way to inhibit halitosis, was substantiated by the finding that the two most efficient oral rinses (D and H) contained such a formulation. The combination of CHX, Zn, and cetylpyridinium chloride (CPC; D) seemed to be less effective that CHX and Zn alone (F). This might be due to some unwanted inhibitory effect, the most likely being Cl⁻ in CPC binding to the positively charged CHX as we have previously shown. The origin of the active ingredient (kind of salts added) differs and might also account for some of these differences. The active ingredients of H are chlorhexidine diacetate and Zn acetate, compared with D, chlorhexidine digluconate and Zn lactate, with slightly different concentrations involved. No side effects have been reported with either formulations (D and H), except for a slight discoloration of the tongue in some individuals after using D, and the effect of both on halitosis, as well as other relevant parameters, seems well documented.

The clinical effectiveness of B and C, particularly as antibacterial agents, is also well documented. This effect was supported by our comparative study; B and C had a H₂S inhibitory effect ranging from 20–0% of control, depending on the exact formulation (taste and color) and time (1–3 hours). However, B and C are mainly prescribed as plaque and gingivitis inhibitory agents, and are significantly less effective against bad breath than D and H. The halitosis-inhibitory effect is probably secondary to an inhibition of the oral microflora, including some anaerobic sulfur-producing species. Some H₂S-inhibitory effect was also observed after rinsing with E (50% of control after 3 hours) as well as G (90–100% of control), whereas A and F did not show any effect after 3 hours. Formulation A contains Zn in addition to enzymes, and although Zn has been shown to have an effect against VSC, it does not work against H₂S in this formulation. F and G both show very little effect against H₂S, although one of its active ingredients (CPC) has been shown to be active against VSC. Moreover, they contain triclosan which is known as a potent plaque inhibitor. The conclusion to be drawn from these observations is that even if a rinse contains ingredients previously shown to be active against VSC, this does not necessarily mean that they work against VSC in the present formulation. Most of the active ingredients are charged molecules, easily neutralized by other components of the rinse. It furthermore suggests that all new formulations or changes in old ones should be thoroughly tested for anti-halitosis effect, preferably applying the more sensitive and reliable GC method before introduction to the market. B, C, E, F, and G contain antibacterial agents as their active ingredients (CloSYS II® and CPC + triclosan in combination) and probably work mainly through inhibiting the oral micro flora, the anti-halitosis effect being secondary to an inhibition of sulfur-producing bacteria. D and H seem to be more specifically addressing the responsible gases given the low concentrations of both Zn, CPC, and CHX used, suggesting that the antibacterial component of these formulations seems to be less dominant.

In conclusion, given the important role of the oral microflora in preserving oral health and protecting against foreign intruders, including infectious micro-organisms, food proteins, and other potentially immune-activating substances, it seem logical to recommend cautious use of local antibacterial agents in general. When the indication is clear, the most efficient and specific formulations (i.e., a patented combination of CHX and Zn in low concentrations) targeting the VSC that are major components in bad breath should be preferred. This formulation is also less likely to cause unwanted side effects.

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References