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Twelve Hour Longevity of the Oral Malodor-Neutralizing Capacity of an Oral Rinse Product Containing the Chlorine Dioxide Precursor Sodium Chlorite

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Abstract

Objectives: The objectives of this investigation were to investigate the effectiveness and longevity of an oral rinse product containing 0.10% (w/v) of the chlorine dioxide precursor sodium chlorite (1) on oral malodor in participants throughout a 12 h daylight diurnal cycle.

Materials and methods: Thirty healthy participants (17 male, 13 female) were recruited to the study. Volatile sulfur compound levels (VSCs: H_2S, CH_3SH and $(CH_3)_2S$) were simultaneously monitored in their oral cavity air samples both before (0 h) and at 0.33, 4, 8 and 12 h after using the above oral rinse, or water as a negative control (participants refrained from oral hygiene measures during this 12 h period). The experimental design for this cross-over investigation was a mixed model ANOVA-based system incorporating treatments, sampling time-points and participants, together with their first-order interactions, as components of variance.

Results: Results acquired demonstrated that the oral rinse formulation effectively suppressed VSC production in the oral environment for 12 h periods ($p<0.0001$, 0.0001 and 0.002 for H_2S, CH_3SH and $(CH_3)_2S$ respectively). Mean 0 *vs* 12 h reductions in oral cavity H_2S and CH_3SH concentrations were much greater than those observed for the H_2O negative control ($p<10^{-8}$), but not so for $(CH_3)_2S$. Principal component analysis (PCA) a H_2S/CH_3SH linear combination and $(CH_3)_2S$ alone significantly loaded on the first and second separate orthogonal components respectively, an observation confirming differing sources for these variable sets.

Conclusions: The oral rinse explored effectively blocked VSC production in the oral cavity for a period of 12 h. This extended efficacy duration is likely to be ascribable to the ability of its active ClO_2^- ingredient to exert a combination of biochemical (direct VSC- and amino acid VSC precursor-consuming) and microbicidal actions *in vivo*.

Clinical relevance: The 12 h longevity of product's[#] oral malodor-neutralizing actions is of much clinical significance in view of the involvements of VSCs, particularly CH_3SH, in the pathogenesis of gingivitis and periodontitis.

[#]Ultradex™ oral rinse, Venture Life Group plc, UK

Keywords: Oral malodor; Volatile sulfur compounds; Oral rinse; Sodium chlorite; Longevity of oral rinse action

Introduction

Oral malodor (halitosis, bad breath) is a common, socially disturbing and recurring condition which primarily affects a large percentage of the adult global population [1]. Cases of this very disturbing disorder are generally ascribable to microbial putrefaction within the oral cavity (usually within anaerobic sites) [2,3], and this process generates malodorous volatile sulfur compounds (VSCs), which are predominantly composed of hydrogen sulphide (H_2S), methyl mercaptan (CH_3SH) and dimethyl sulphide (CH_3SCH_3) [1,4]. Limited salivary flow rates, periodontal diseases, excessive bacterial colonization of the tongue, unclean dentures, and poor or unsuitable dental restorations can trigger halitosis of oral etiology [5-11], although upper and lower respiratory tract conditions, a series of systemic diseases, and gastrointestinal and neurological diseases, together with the therapeutic application of selected drugs, are common non-oral etiologies [12]. Therefore, a broad spectrum of clinical conditions, oral or otherwise, can give rise to oral malodor which can be monitored by a range of strategies.

The above VSCs are derived from the putrefaction of cysteine- and methionine-containing proteins, predominantly by gram-negative micro-organisms. Optimum putrefactive activity occurs in a low carbohydrate environment, at physiological pH and temperature values, and also in anaerobic loci. Salivary sediment containing exfoliated epithelial cells acts as a primary substrate with a predominantly 'oxidized' status (i.e. a high disulphide:thiol concentration ratio). Proteolysis, coupled with a reduction of disulphide bonds, precedes the development of VSC-based oral malodor [1,13].

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Determinations of the nature and magnitude of oral malodor demand reliable, sensitive, accurate and precise experimental techniques, and previously reported methods available for its monitoring include 1) organoleptic (subjective) systems [14,15]; 2) measurement of VSCs via gas chromatography (GC) coupled with flame-photometric detection [16]; 3) a combination of (1) and (2) above [13]; 4) cryo-osmoscopy [17]; or 5) the time-consuming culture of plaque and periodontal pocket exudates in selected bacteriological media [10]. However, to date only a limited amount of experimental data are available on the applications and reproducibilities of such approaches, and considerations including the menstrual cycle, heterogeneity in oral hygiene control, circadian variation, smoking habits and climate may indeed exert an influence on results acquired from such studies [18]. Moreover, following the evacuation of malodorous gases in the oral cavity, the rate and extent of their restoration to this environment are of considerable debate.

Further pioneering reports have outlined the applications of a portable industrial H_2S/CH_3SH-specific VSC monitor (halimeter) [19,20], and highly significant correlations between these measurements and corresponding organoleptic ratings performed by a total of 7 judges have been found [19]. This electrochemical VSC monitor involves a voltammetric sensor which draws a sample of oral gas across an electrocatalytic sensing electrode operating at a potential of +0.50 V, a value sufficient to ensure the complete oxidation of electron-donating thiols, specifically CH_3SH and H_2S (in general, redox potentials (E_o) of thiol/disulphide couples lie in the -0.20 V to +0.40 V range). Such electrochemical reactions generate an electric current, the magnitude of which is directly proportional to the total chemically-reducing, gaseous VSC concentrations. This current is converted to a voltage which, in turn, is then transferred to a meter which provides VSC concentrations in parts-per-billion (ppb) throughout a range of 0 ppb to 1000 ppb. Determinations performed using this device have been shown to be more precise and reproducible than those obtained by subjective, organoleptic panel methods, and more sensitive to decreases in VSC levels arising from treatment with a number of oral healthcare products (OHCPs) [19,21].

Recently, a more specific, portable gas chromatography-based VSC measurement device has been developed (OralChroma™, Abimedical Corporation, Miyamae-ku Kawasaki-shi, Kanagawa, Japan, [22]). This facility has the ability to determine the oral cavity ppb concentrations of H_2S, CH_3SH and CH_3SCH_3 simultaneously in air directly sampled from the oral cavity, and displays each level on a convenient display panel (each of these VSC agents and their oral cavity concentrations may, at least in principle, be correlated with a specific cause of halitosis). Additionally, this VSC monitor offers many bioanalytical benefits over more complex GC methods, and these include substantially lower costings, rapid sample throughput, ready portability, facile point-of-care, 'on-site' use, no major requirements for the involvement of specialist technical staff, and the suitability of the means by which oral cavity air samples are collected.

Both chlorine dioxide ($ClO_2^{\cdot}$) and its precursor chlorite anion (ClO_2^-) are very effective in oxidatively consuming VSCs, and also their sulfur-containing amino acid precursors within the oral cavity [23]. Indeed, the latter is now a key ingredient in selected oral rinse formulations widely available commercially as 'over-the-counter' products such as (1). Two previously conducted investigations focused on an evaluation of the efficacy of an oral rinse product containing 0.10% (w/v) 'stabilized' $ClO_2^{\cdot}$ (predominantly ClO_2^-) on oral soft tissues and gingivitis found that it effectively improved periodontal health. Specifically, this formulation substantially reduced 'bleeding-on-probing' in patients with gingivitis [24], and gave rise to a healing of >67% of periodontal pockets [25]. Moreover, further studies have explored the microbicidal actions of such products [26,27], and Mohammed et al. [28] assessed their effectiveness towards the clinical control of chronic atrophic candidiasis.

Additional studies have clearly demonstrated that such ClO_2^--containing products are efficacious in the treatment of oral malodor *in vivo* [26-30]. Although the VSC-neutralizing activities of ClO_2^- are beyond dispute, both *in vitro* and *in vivo*, considerable debate remains regarding the longevity of these actions. For example, although Shinada et al. [29] monitored the effectiveness of an oral rinse product containing 0.16% (w/v) of this oxyhalogen oxidant, VSCs were only monitored for periods of up to 4 h Therefore, in this investigation, we have explored the clinical effectiveness of an oral rinse product (1), tested against a water placebo treatment, against oral malodor (halitosis) using the above portable gas-chromatographic monitoring system. These VSC determinations were made before, and at selected diurnal time-points after treatment of participants with each of the oral rinse formulations in the recommended manner and compared with corresponding measurements made after they rinsed with a H_2O placebo control in place of the oral rinse formulation. The total (daily) period of each testing was 12 hours in order to determine the capacity of this oral rinse product to combat oral malodor for this prolonged time length.

Materials and Methods

Volatile sulfur compound (VSC) determinations

Measurements of each VSC were made on an OralChroma™ portable gas chromatographic monitoring system. Participants were required to refrain from talking for 5 min prior to measurement, and also to breathe through their noses during the collection of oral cavity air samples via a syringe; a 1 ml volume of air was sampled, and exactly 0.5 ml

of each sample was injected into the OralChroma™ device. The time period between air sampling and gas chromatographic analysis was ≤ 5 seconds. Results were recorded as parts-per-billion (ppb) oral cavity VSC concentrations.

Participant population

This investigation involved 30 non-smoking human volunteers (17 male, 13 female) ranging in age from 24 to 55 years. Written informed consent was acquired from all participants, and this investigation was performed in accordance with the Declaration of Helsinki of 1975 (revised in 1983). It was approved by the Faculty of Health and Life Sciences Research Ethics Committee, De Montfort University, Leicester UK (reference number 1117). During the recruitment stages of the investigation, participants were supplied with a Participant Information Sheet and, if agreeing to take part in the investigation, were subsequently required to sign a University Research Ethics Consent Form. All participants recruited were also required to complete a short questionnaire which requested essential information, including medical history, age, gender, body mass index (BMI), dental treatment history and any current medication that they were receiving.

Exclusion criteria

Participants were excluded from the investigation if they had any serious or chronic medical condition such as diabetes, cardiovascular diseases or cancer, periodontal diseases, or any other condition which precluded their participation in the trial. Those receiving any form of medication during the 7 days prior to the first testing day were excluded from the investigation. All participants were also instructed not to receive any form of medication during the two sampling test days of the trial conducted.

Oral rinse composition

Oral rinse product (1) contained sodium chlorite (Na^+/ClO_2^-) at an added level of 0.10% (w/v), i.e., 1.106×10^{-2} mol.dm^{-3}; 0.20% (w/v) trisodium phosphate, as $Na_3PO_4.12H_2O$ (5.26×10^{-3} mol.dm^{-3}); and 0.079% (w/v) citric acid (4.11×10^{-3} mol.dm^{-3}). The pH value of this product was 6.50.

Evaluations of the abilities of oral rinse products to combat oral malodor

Participants were required to rinse with the oral rinse formulation (15 ml volumes of oral rinse (1)) for a period of 30 seconds. Each participant was also required to rinse with an equivalent volume of tap water on a separate trial day, this treatment serving as a placebo control. Primarily, participants were provided with standard NaF-containing toothpaste (Colgate Triple Cool Stripe, Colgate Palmolive) and allowed to brush with it (each using a standard Colgate Extra Clean toothbrush) as usual in place of their usual oral healthcare regimen for a period of 7 days (2x daily) in order to establish 'baseline' oral cavity VSC data. Participants were randomly allocated to either the primary phase I, pre-crossover oral rinse or negative H_2O control groups using a computerized random number generator. Prior to the testing periods for the oral rinse or H_2O placebo control treatments investigated (12 h in total), each participant was requested to refrain from oral activities (i.e., eating, drinking, tooth-brushing, oral rinsing, etc.) for a period of at least 4 h VSC levels were determined both prior to (0 h) and following oral rinsing episodes with the oral rinse or H_2O placebo control examined (0.33, 4, 8, and 12 h post-administration, together with immediate subsequent measurements made following each of those above, so that there was a total of 9 determinations made per participant per diurnal trial period, i.e. at 0, 2×0.33, 2×4, 2×8, and 2×12 h subsequent to therapeutic application of oral rinse treatments or the H_2O placebo).

The first (baseline) measurement was made at 10.00 AM, and all participants were required to agree to avoid their early morning breakfast meal (and, of course, all further oral activities 4 h prior to the collection of this first (zero control) sample) on each of the two days in which they were involved in the investigation. Administration of the oral rinse or H_2O control to each of the 30 participants was staggered throughout time, and the minimum 'washout' period between the single product administered and the H_2O placebo was 4 days prior to crossing over to the other available treatment regimen. During these 'washout' periods, it was ensured that all participants were maintained on the twice-daily oral healthcare tooth-brushing regimen with the standard, NaF-containing toothpaste. Participants were blinded (i.e., unaware of the nature of the oral rinse or water placebo treatments that they were receiving), since both treatments were provided in coded sterile dispensing containers. Time-dependent VSC determinations were performed on a single participant per day using the same OralChroma™ monitoring device.

As an additional precaution, throughout the 12 h total testing period, participants were instructed to avoid the consumption of foods that have a strong odor such as onions, garlic, selected further vegetables (e.g. chillies and peppers), nuts, cheese, fish, etc., especially spicy foods such as curries, together with certain drinks, especially coffee and alcoholic beverages (the taste and smell of such foods and beverages lingers on the breath long after their consumption), and which may therefore exert effects on the oral cavity VSC measurements made (when consumed and digested, odorous and malodorous agents derived from these foods are absorbed into the bloodstream and then transported to the lungs: this allows the odor associated with them flow from the mouth area during the exhalation process).

Experimental design for the study and statistical analysis of oral cavity VSC concentrations

For each of the above clinical datasets, we employed

analysis of variance (ANOVA)-based experimental designs. These procedures were employed to determine the significance of the 'Between-Treatments' and 'Between Diurnal Time-Points' effects incorporated into the study, and also the further components of variances (CVs) involved, specifically that 'Between-Participants', together with those arising from the Treatment × Diurnal Time-Point, Treatment × Participant and Participant × Diurnal Time-Point interaction effects.

Hence, the overall experimental design for this investigation was classified as a mixed model, 2 factor system with treatments (one oral rinse, together with the water placebo control) and time-points at which the measurements were made being fixed effects at 2 and 5 levels respectively, and participants (n=30 in total) being a random effect. This mixed model component analysis for each VSC determined therefore comprised the 3 main effect factors, their associated interactions, and fundamental error.

However, a total of four different ANOVA-based analysis models were employed. In the first of these models (model 1), the oral rinse (1) and water placebo treatment groups were partitioned, and each 'Treatment' dataset was analyzed separately so that the significance of modifications to oral cavity VSC levels could be evaluated at all post-treatment time-points for each of these treatments (equation 1). The second model (model 2) involved a consideration of the above 3 main factors, but without inclusion of all the above first-order interaction components of variance (equation 2), whereas the third (model 3) was represented by model 2 with the incorporation of all three of these first-order interaction effects (equation 3). In these equations, S_i, P_j, T_j, SP_{ij}, ST_{ik}, PT_{jk} and e_{ijkl} represent the 'Between-Sampling Time-Point', 'Between-Participant', 'Between-Treatment', Sampling Time-Point × Participant interaction, Sampling Time-Point × Treatment, Participant × Treatment interaction and unexplained error sources of variation, respectively.

$$y_{ijl}=S_i + P_j + SP_{ij} + e_{ijl} \quad (1)$$

$$y_{ijkl}=S_i + P_j + T_k + e_{ijkl} \quad (2)$$

$$y_{ijkl}=S_i + P_j + T_k + SP_{ij} + ST_{ik} + PT_{jk} + e_{ijkl} \quad (3)$$

Finally, the fourth model (model 4) featured only the 'Between-Treatments' and 'Between-Participants' main effects, and also the Treatment × Participant interaction one as explanatory variables, and the difference observed between the 0 h control and 12 h diurnal time-points for each participant served as the dependent variable analyzed (equation 4).

$$y_{jkl}=P_j + T_k + PT_{jk} + e_{jkl} \quad (4)$$

For all models, Bonferroni-corrected *post-hoc* ANOVA analysis was performed to test the significance of individual comparisons between pairs of sampling time-points and participants.

Datasets were generalized logarithm (glog)-transformed and normalized (i.e., centered and autoscaled), and these transformed/normalized datasets were analyzed as described above.

Further experimental design models were employed to further explore participant-matched differences between the mean oral cavity VSC levels of the two treatment groups at both the baseline 0 h and final 12 h diurnal trial time-points.

ANOVA of our experimental data according to each of the above experimental designs was performed using XLSTAT2014 software. Pearson and multivariate partial correlations between each of the three VSCs determined were also explored using this software, as was multivariate analysis of variance (MANOVA) and principal component analysis (PCA). Pearson correlations between the participants' baseline 0 h and 12 h diurnal trial time-point VSC concentrations were also investigated with this software package. PCA analysis was employed to investigate inter-relationships between each of the 3 VSCs monitored and featured Varimax PC rotation with Kaiser normalization. A maximum of 2 factors was considered, and a PC loading vector value of 0.40 was considered as the minimum required for a significant contribution towards each PC isolated. Further multivariate analysis was conducted with MetaboAnalyst 3.5 software.

Results

With our model 1 ANOVA analysis (as detailed in "Materials and Methods" section), application of the 0.10% (w/v) sodium chlorite-containing oral rinse formulation (1) as a treatment for oral malodor gave rise to extremely highly significant differences between the mean oral cavity H_2S and CH_3SH concentrations between the 0 h (pre-treatment) time-point and those at 0.33, 4, 8 and 12 h post-treatment ones ($p=1.81 \times 10^{-13}$ and 2.54×10^{-17} for these VSCs respectively), specifically substantial reductions in their post-treatment oral cavity concentrations. For $(CH_3)_2S$, however, there were only highly significant time-dependent decreases from its mean 0 h oral cavity level observed at the 0.33, 8 and 12 h post-treatment time-points ($p=2.57 \times 10^{-8}$). However, no significant differences were observed in the mean concentrations of this VSC between the 0 h and 4 h time-points.

Therefore, for model 1, experimental data acquired clearly confirm that the oral malodor-neutralizing effects of the oral rinse product tested are significantly prolonged to the 12 h post-administration time-point for each VSC, although such alleviations in oral cavity air VSC concentrations are less clear for $(CH_3)_2S$ in this context. The Diurnal Time-Point × Participant interaction effect was also statistically significant for each VSC monitored ($p=4.60 \times 10^{-3}$, 9.23×10^{-8} and 5.10×10^{-3} for H_2S, CH_3SH and $(CH_3)_2S$ respectively), and this confirms that the sampling time-point dependence of the patterns of oral cavity VSC level responses to treatment with oral rinse (1) differed markedly between participants.

For the water placebo treatment, significant differences were found only between the 0 and each of the 4, 8 and 12 h time-point mean values for both H_2S and CH_3SH ($p<4.11 \times 10^{-14}$ and 3.36×10^{-8} respectively), and only the 0 h and 12 h time-point mean values for $(CH_3)_2S$ ($p=1.23 \times 10^{-7}$); there were no statistically significant differences found between the 0, 0.33, 4 and 8 h time-points for this blood source VSC. As expected, the Diurnal Time-Point × Participant interaction effect was again statistically significant for each VSC monitored ($p=9.70 \times 10^{-3}$, 4.97×10^{-4} and 9.07×10^{-2} for H_2S, CH_3SH and $(CH_3)_2S$ respectively), and this provides much evidence for differing time-dependent responses of all determined oral cavity VSCs to the H_2O placebo treatment between our participants.

From this overall mixed model ANOVA analysis, the 'Between-Participants' factor was found to be very highly significant for each VSC monitored ($p<10^{-8}$ for each one).

Figure 1 shows plots of mean (± 95% confidence intervals, CIs) oral cavity VSC level values *vs.* post-treatment time (for H_2S, CH_3SH and $(CH_3)_2S$) for both the oral rinse (1) treatment and the negative water control one. The CIs depicted are those made across all participants, i.e., they arise from the incorporation of both 'Between-Participants' and Error (Residual) components-of-variances, and hence they are much wider than those which are derivable from the latter component alone. These plots confirm that, for H_2S and CH_3SH, the differences observed between the mean 0 h and 12 h time-point oral cavity concentrations observed were much greater for the oral rinse treatment classification than those observed for the H_2O control one.

For the model 2 ANOVA analysis performed, there were very highly significant differences 'Between-Treatments, -Time-Points and -Participants' ($p=2.58 \times 10^{-4}$, $<10^{-8}$ and $<10^{-8}$ respectively). The significance and magnitude of the 'Between-Treatments' effect is ascribable to the much greater effectiveness of the oral rinse (1) formulation over that of the water control rinse regimen.

An additional statistical analysis of the 0 h baseline time-point VSC levels alone was also conducted in order to determine any 'Between-Treatment' differences between such values, and for this we employed an ANOVA model, incorporating only this and the 'Between-Participants' sources of variation, together with a paired sample t test. Although there were no statistically significant, participant-matched differences found between the mean baseline oral cavity concentrations

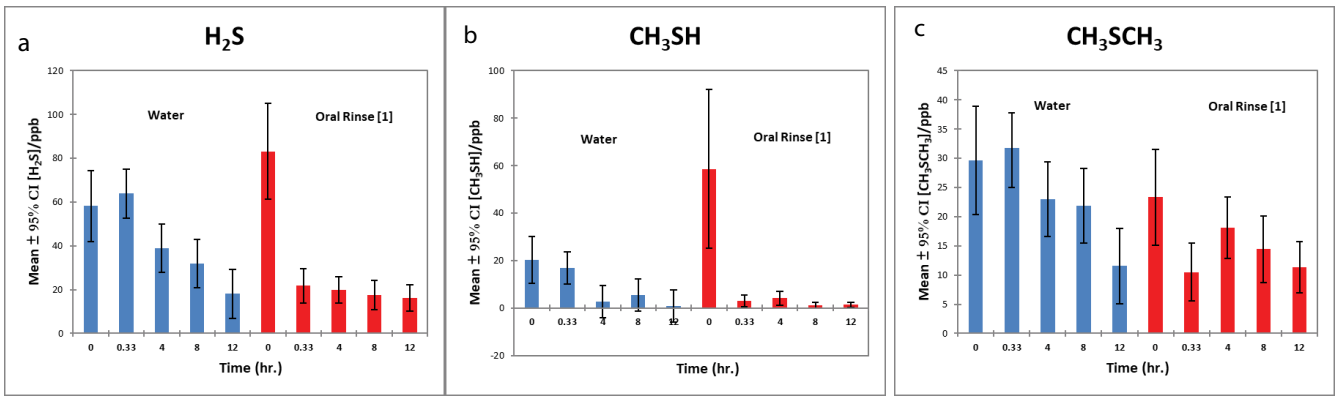

Figure 1: Plots of mean (± 95% CIs) oral cavity (a) H_2S, (b) CH_3SH and (c) $(CH_3)_2S$ concentrations *versus* post-treatment time for both the oral rinse (1) treatment (red) and the negative water control one (blue). The CIs represent those made across all participants, i.e. they arise from the incorporation of both 'Between-Participants' and Error (Residual) components-of-variances. Factorial statistical analysis according to our model 1 ANOVA experimental design revealed that there were extremely significant decreases in the 0.00 hr. baseline time-point concentrations of H_2S and CH_3SH observed at 0.33, 4.00, 8.00 and 12.00 hr. post-rinsing with product (1) ($p=1.81 \times 10^{-13}$ and 2.54×10^{-17} respectively), but only significant reductions in mean baseline $(CH_3)_2S$ levels noted at 0.33, 8.00 and 12.00 hr. post-rinsing with this formulation ($p=2.57 \times 10^{-8}$). For the negative control tap water wash, significant decreases from 0.00 hr. baseline values were observed only at the 4.00, 8.00 and 12.00 hr. post-rinsing time-points for both H_2S and CH_3SH ($p<4.11 \times 10^{-14}$ and 3.36×10^{-8} respectively), and only at the 12.00 hr. time-points for $(CH_3)_2S$ ($p=1.23 \times 10^{-7}$).

A more extensive statistical analysis performed according to our model 3 approach demonstrated that oral rinse formulation (1) was much more effective than the negative H_2O control in reducing oral cavity H_2S and CH_3SH levels ($p=1.34 \times 10^{-5}$ and 4.96×10^{-4} respectively). For (CH3)2S, the only significant difference found 'Between-Treatments' was that at the 0.33 hr. post-rinsing time-point. This analysis model also showed that there were significant Treatment × Time-Point interaction components of variances for H_2S, CH_3SH and $(CH_3)_2S$ ($p<0.0001$, <0.0001 and 0.002 respectively); for H_2S and $(CH_3)_2S$, there were also very highly significant Treatment × Participant interaction effects ($p<0.0001$ for each VSC)

'Between-Treatment' differences observed between the 12.00 hr. time-point values were manifested by those observed only in selected participants, in accordance with the statistical significance of the Treatment × Participant interaction effect noted.

of H_2S and $(CH_3)_2S$ (Bonferroni-corrected p values >0.05), that for CH_3SH was (p=0.015). However, MANOVA analysis found that there were no significant 'Between-Treatment', nor 'Between-Participant' differences between these VSC levels when considered as a multivariate composite (p=0.080 and 0.138 respectively; Hotelling-Lawley's, Pillai's and Roy's tests). Therefore, the ANOVA-detected significant difference observed between the baseline levels of CH_3SH is not simply explicable, but is likely to arise from a type I statistical error, i.e., such differences will occur via chance alone in 5% of such testing for each VSC variable tested at a significance level of 0.05, a value which will increase to an estimate of 15% for a total of 3 such variables included without the incorporation of a false discovery rate correction factor.

As expected, there was also a highly significant random effects 'Between-Participant' random effects component of variance for both H_2S and CH_3SH concentrations (p=6.47 and 1.45×10^{-3} respectively), although not for $(CH_3)_2S$ (p>0.05).

Analysis-of-variance of the datasets using the model 3 ANOVA model revealed that overall, the oral rinse formulation tested was much more effective than the negative H_2O control in diminishing oral cavity H_2S and CH_3SH concentrations (p=1.34×10^{-5} and 4.96×10^{-4} respectively, Figure 2). However, for $(CH_3)_2S$, the only significant difference found 'Between-Treatments' was that at the 0.33 h post-administration time-point, i.e., shortly after their administration, as shown in Figures 1C and 2C.

For each VSC monitored, this analysis also revealed that there were significant Treatment × Time-Point Interaction components of variances for H_2S, CH_3SH and $(CH_3)_2S$ (p<0.0001, <0.0001 and 0.002 respectively), observations which confirm that the nature/magnitude of the time-dependence of the response to treatments was critically dependent on each one investigated (i.e., oral rinse (1) *vs.* the H_2O control, Figure 2). Although no significant 'Between-Treatment' effect was found for CH_3SH in this model 2 analysis, the very highly significant Treatment × Time-Point interaction component of variance for this VSC revealed that such differences were markedly influenced by the time variable, e.g., the much higher and lower levels of it at the zero control and 0.33 h time-points, respectively, for the oral rinse-treatment group. However, corresponding mean 0 h (pre-treatment) and 0.33 h time-point CH_3SH levels for the H_2O negative control group were similar to each other (and also very low when expressed relative to the corresponding zero control value of the oral rinse (1)-treated group), and there was no significant difference between these values (Figure 2B).

These highly significant interaction effects are therefore particularly notable as differences in the mean responses of each VSC between the oral rinse (1) and the water placebo control treatments when expressed as a function of each post-treatment time-point. Indeed, these differences can be

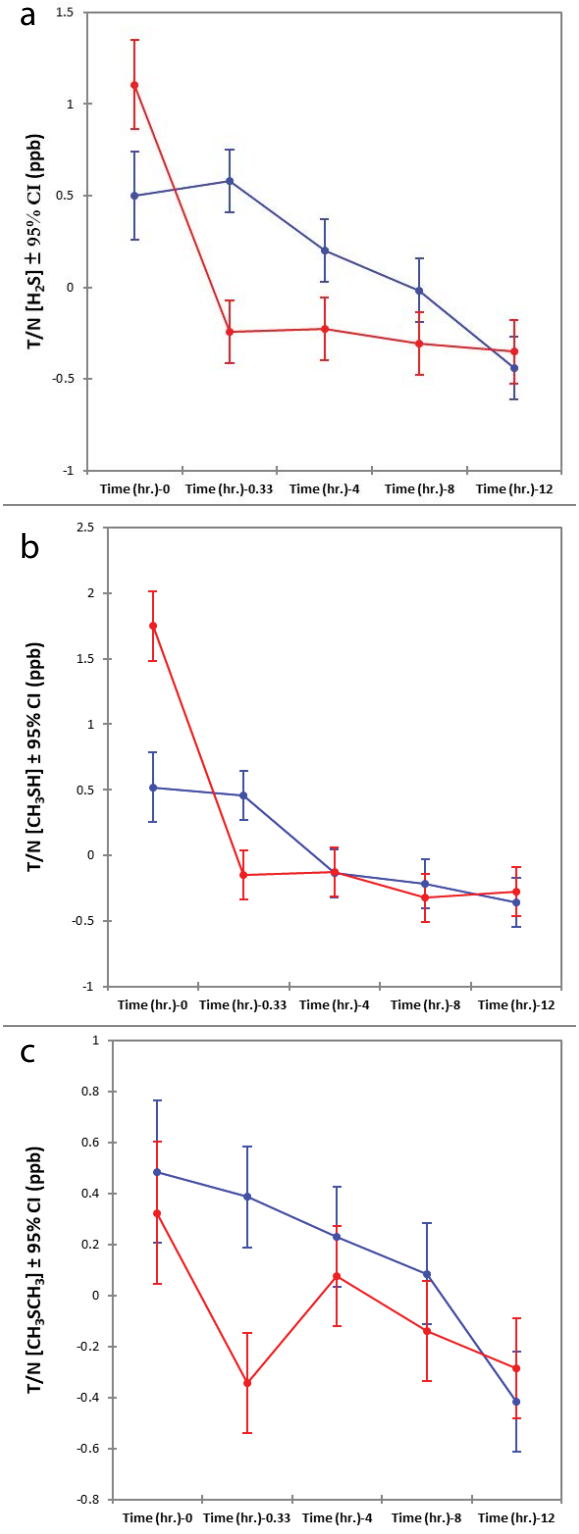

Figure 2: Plots of glog-transformed and normalised mean oral cavity VSC levels (with associated 95% CIs) *versus* sampling time-point for (a) H_2S, (b) CH_3SH and (c) $(CH_3)_2S$ following treatment with the H_2O negative control rinse (blue) and oral rinse (1) (red). 95% CIs were computed according to model 2. The statistical significance of these effects is outlined in the legend to Figure 1.

Treatment	Time (h)	Mean (H_2S) ± 95% CIs/ppb	Mean (CH_3SH) ± 95% CIs/ppb	Mean ($(CH_3)_2S$) ± 95% CIs/ppb
Oral rinse (1)	0.00	100	100	100
	0.33	26.2 ± 9.5	5.26 ± 4.2	45.1 ± 21.2
	4.00	23.8 ± 7.3	7.0 ± 5.1	77.4 ± 22.6
	8.00	21.1 ± 8.0	2.0 ± 6.1	61.9 ± 24.4
	12.00	27.6 ± 14.3	2.5 ± 1.8	48.5 ± 18.9
H_2O control	0.00	100	100	100
	0.33	109.9 ± 33.5	83.6 ± 46.7	107.3 ± 32.2
	4.00	66.8 ± 19.9	13.7 ± 7.7	77.7 ± 21.8
	8.00	58.2 ± 15.0	27.5 ± 41.8	74.0 ± 25.6
	12.00	31.1 ± 15.4	4.6 ± 6.1	38.9 ± 22.5

Table 1: Mean ± 95% confidence intervals (CIs) modifications in oral cavity VSC levels at the 0.33, 4.00, 8.00 and 12.00 hr. time-points expressed as a percentage of those observed at the 0.00 hr. baseline ones for both the oral rinse (1) and H_2O negative control treatment regimens.

clearly visualized as significantly lower VSC concentrations in the oral rinse (1) treatment group at the 0.33, 4 and 8 h time-points than those observed for the negative H_2O control, most especially those at each of these time-points for H_2S, and at the 0.33 h one for both CH_3SH and $(CH_3)_2S$. Moreover, for H_2S and $(CH_3)_2S$, there were also very highly significant Treatment × Participant interaction effects ($p<0.0001$ for each VSC), an observation which provides evidence that the response to each treatment differs markedly for at least some of the study participants (i.e., as expected, there is a non-additive response to treatment). There was no significant contribution of the Treatment × Participant interaction effect towards variation in the CH_3SH VSC parameter.

However, the statistical significance of mean differences in VSC levels at the 12 h diurnal time-point according to our model 3 analysis was manifested by the highly significant interaction sources of variation observed. For example, for H_2S, there were very highly significantly higher levels of this VSC in the H_2O negative control group at this final time-point, but these were only observed for n=3 of the participants (these participants all had very similar baseline 0 h H_2S concentration values at the 0 h baseline time-point for both the oral rinse (1) and H_2O control group regimens). No statistically significant, participant-focused 'Between-Treatment' differences between the 12 h time-points were observed for all the remaining participants.

The Table 1 lists mean percentage changes expressed relative to the baseline 0 h mean concentrations of H_2S, CH_3SH and $(CH_3)_2S$ at increasing trial time-points for both the oral rinse (1) and H_2O control treatment groups. Clearly, these percentage modifications are significantly greater for the oral rinse (1) treatment group for all three VSCs throughout the 0.33 h to 8 h time-points, but less so at the 12 h one.

Finally, for our model 4 analysis, mean ± 95% CI decreases in the H_2S, CH_3SH and $(CH_3)_2S$ VSC concentrations between the 0 h control and 12 h post-administration time-points were 67 ppb ± 6.0 ppb, 57 ppb ± 0.9 ppb and 12 ppb ± 3.7 ppb, respectively, for the oral rinse treatment, and 39 ppb ± 6.0 ppb, 20 ppb ± 1.0 ppb and 18 ppb ± 3.8 ppb, respectively, for the negative water placebo control (Figure 3). Moreover, 'Between-Treatment' differences between these mean decreases were extremely significant at the $p<10^{-8}$ level for both H_2S and CH_3SH. However, that observed for CH_3SH is at least partially explicable by the significantly higher 0 h baseline concentration value observed for the oral rinse (1) treatment regimen. Moreover, the corresponding 'Between-Treatment' difference observed for reductions observed in mean $(CH_3)_2S$ concentrations was found not to be significant. For H_2S, the above difference corresponds to a 12 h time-point longevity reduction of >80% for treatment with oral rinse (1); the corresponding 12 h decrease observed for the negative water control group was only 55%.

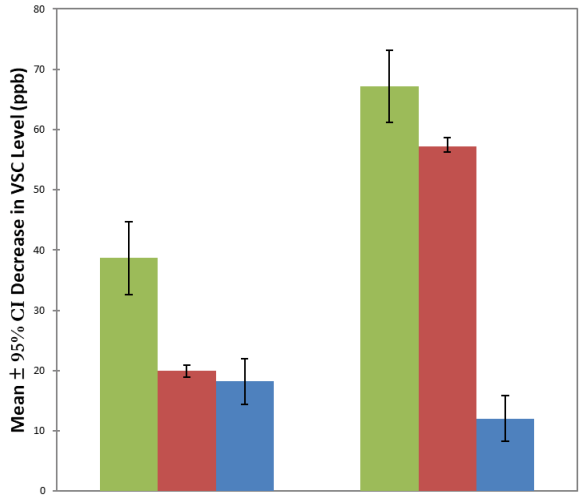

Figure 3: Plot of mean (± 95% CIs) differences in oral cavity H_2S (green), CH_3SH (brown) and $(CH_3)_2S$ (blue) concentrations between the 0.00 hr. pre-treatment and 12.00 post-treatment time-points observed for the negative water placebo control (left-hand side) and oral rinse (1) (right-hand side). Between-Treatment' differences between these mean decreases were extremely significant for both H_2S and CH_3SH ($p<10^{-8}$), but were not significant for $(CH_3)_2S$.

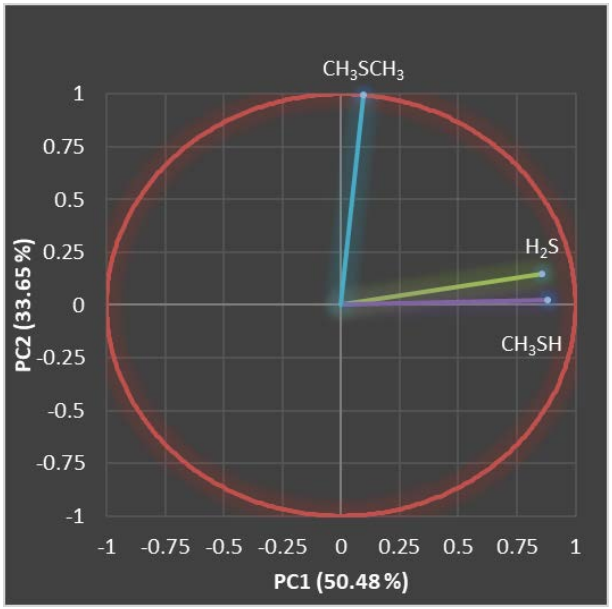

Figure 4: PCA scores plot of PC2 versus PC1 for the 0.00 hr. baseline VSC concentration dataset (involving two treatment regimen groups each containing n=30 matched participants). PC loadings vectors were 0.856 and 0.879 for H_2S and CH_3SH, respectively, on PC1, and 0.994 for $(CH_3)_2S$ on PC2. Eigenvalues (i.e. the mean number of variables loading on a PC) for the corresponding unrotated PCA performed were 1.61 and 0.91 for PC1 and PC2 respectively. PCs 1 and 2 accounted for 50.48 and 33.65% of the total model variance respectively. Line color codings: green, H_2S; purple, CH_3SH; blue, $(CH_3)_2S$.

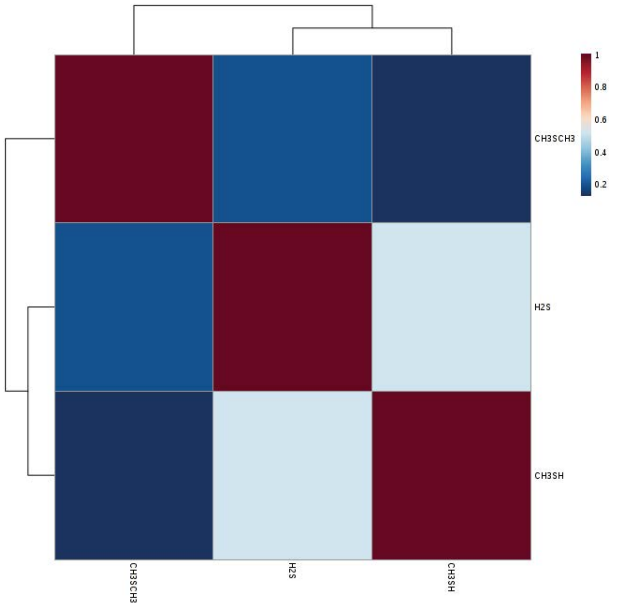

Figure 5: Correlation matrix diagram displaying a moderate but highly significant correlation between 0.00 hr. time-point morning baseline oral cavity H_2S and CH_3SH concentrations (n=60 in total, 30 in each of the two treatment groups), but not between those of H_2S and $(CH_3)_2S$, nor CH_3SH and $(CH_3)_2S$. The left-hand and top axis tab displays results arising from agglomerative hierarchal clustering (AHC) analysis of this dataset, which confirms the significant clustering of H_2S and CH_3SH levels, and also the independence of those of $(CH_3)_2S$.

Multivariate analysis of our experimental dataset at the baseline 0 h (control) time-point via PCA demonstrated that the 3 VSC variables were effectively segregated into two clear orthogonal (i.e., uncorrelated) principal components (PCs), the first containing H_2S and CH_3SH (loading vectors 0.86 and 0.88 respectively), the second $(CH_3)_2S$ alone (loading vector 0.99). These results are fully consistent with the sources of these malodorous agents, i.e., H_2S and CH_3SH arise from the oral environment, whereas $(CH_3)_2S$ has a non-oral source (predominantly blood).

Consistent with these results, a Pearson correlation analysis of the untransformed baseline 0 h time-point VSC concentrations confirmed that there was a highly significant, albeit moderate, linear correlation between oral cavity H_2S and CH_3SH levels (r=0.52, $p=2.07 \times 10^{-5}$), but not between H_2S and $(CH_3)_2S$ (r=0.19, ns), nor CH_3SH and $(CH_3)_2S$ (r=0.14, ns) concentrations (Figure 4), data consistent with the above PCA analysis and also indicating an independent (non-oral) source for $(CH_3)_2S$ (corresponding partial correlation coefficient values for these data were 0.51, 0.15 and 0.04 respectively).

Cross-over correlations between participants' 0 h baseline VSC concentration data of the oral rinse treatment sampling group with those of the H_2O control group at this time-point were strong for both H_2S (r=0.44, p=0.014) and CH_3SH (r=0.66, $p=7.94 \times 10^{-5}$), but much less so for $(CH_3)_2S$ (r=0.25, p=0.046), as might be expected from its non-oral source.

We also performed a full correlation analysis of our datasets in order to determine if there were any significant relationships between the concentrations of each VSC at the zero baseline time-point and that at the 12 h trial completion one, and this confirmed that for both treatment groups combined, there were weak but nevertheless significant correlations between these time-points for oral cavity H_2S (r=0.250 $p=5.91 \times 10^{-3}$) and $(CH_3)_2S$ (r=0.315, $p=4.58 \times 10^{-4}$) levels, but not for those of CH_3SH (r=0.00, ns) at these two extremes, and this demonstrates at least some consistency in these H_2S and CH_3SH concentrations between participants recruited to the study.

Pearson correlation coefficients for these relationships were also determined for each treatment group, and found that both time-points sets of oral cavity H_2S and $(CH_3)_2S$ concentrations were significantly related (r=0.26 and 0.37, p=0.045 and 3.46×10^{-3} respectively) for the negative H_2O control group, and also for the oral rinse (1)-treated group (r=0.29 and 0.22, p=0.025 and 9.69×10^{-2} respectively). There were no significant correlations for CH_3SH levels in either of these groups.

Discussion

For H_2S, CH_3SH and $(CH_3)_2S$, the oral rinse formulation (1) explored here exerted very highly significant VSC-neutralizing activities which were of a significantly greater magnitude than those observed with the water placebo control rinse (especially for H_2S and CH_3SH). Since reported threshold concentrations of malodorous objectionabilities (TCMOs) are 95, 12 and 24 ppb for H_2S, CH_3SH and $(CH_3)_2S$ respectively [31], it is clear from the investigations described here that application of this oral rinse product successfully retains the mean level of each of these VSCs below these objectionable threshold values for periods of up to 12 h post-application. Indeed, the mean 12 h time-point reductions in oral cavity VSC concentrations observed in participants using this oral rinse formulation were 70 and as much as 77% of the above TCMO values for H_2S and CH_3SH respectively; this clearly is a very significant observation regarding the longevity of the VSC-neutralizing activities of this product.

It should also be noted that the overall, total zero pre-treatment time-point mean oral cavity air H_2S, CH_3SH and $(CH_3)_2S$ concentrations of our randomly selected 24-55 year age participant population were 71, 39.5 and 26.5 ppb respectively, values which are either close to (H_2S) or exceed (CH_3SH and $(CH_3)_2S$) these TCMOs. These data clearly indicate that oral malodor has a high incidence within the human population sampled.

The reductions, albeit lower ones (with also smaller numbers of statistically significant ones) recorded in oral cavity VSC levels subsequent to participants receiving the water placebo treatment were only observed at or subsequent to the 4 h post-application time-point, and are not unexpected. Indeed, these differences are likely to arise from diurnal variation in these values, which represents a significant source of variation for oral cavity VSC concentrations [32]. Indeed, although saliva effectively serves to remove oral cavity bacteria, the production of this biofluid is greatly diminished during the night, and therefore there are corresponding increases in the numbers of such residual microbes, together with their metabolic rates [33,34]. Hence, tongue biofilm- and plaque-harboring bacteria generate higher concentrations of VSCs throughout the night, and this, in turn, leads to 'morning bad breath'. Oral hygiene regimens instigated in the morning will primarily reduce oral cavity VSC levels which then begin to increase again prior to meals [35]; surprisingly, it has been reported that such eating episodes serve to either decrease VSC levels, or alternatively exert little or no effect [35]. However, oral cavity VSC levels increase between eating and/or drinking episodes, but such levels rarely exceed those developed overnight.

Notwithstanding, despite the markedly higher VSC level reductions observed in the oral rinse (1) treatment group over those of the H_2O control one, it should be noted that the only significant 'Between-Treatment' differences between the mean 12 h time-point oral cavity concentration values of these for all VSCs evaluated were those observed in small numbers of participants, a consequence of the significant Participant × Treatment interaction effect in our Model 3 analysis, and this may be explicable by their diurnal variation and potential reductions in their concentrations induced by the consumption of an evening meal at a time-point close to the final 12 h sampling and testing one (exactly 10.00 PM) by a significant or even substantial proportion of them.

A further consideration is that all VSC level values were virtually zero at this 12 h time-point in both treatment groups for CH_3SH. Again, this observation may be ascribable to diurnal variation, possibly featuring meal consumption activities close to the final 12 h VSC measurement time-point. However, the percentage reduction in the oral cavity concentration of this VSC observed in the matched oral rinse treatment group over that of the negative H_2O control one (according to our model 4 experimental design) was resoundingly significant ($p<10^{-8}$).

The oxidative consumption of the VSCs H_2S, CH_3SH and $(CH_2)_2S$, together with their essential amino acid precursors L-cysteine and L-methionine, serves as a major mechanism of action for the chlorine dioxide precursor chlorite anion present in the oral rinse formulation investigated here (displayed for CH_3SH and $(CH_3)_2S$ in equations 5 and 6 respectively). Indeed, orally-generated H_2S and CH_3SH are produced from these amino acids in key metabolic pathways operating in gram-negative bacteria.

$$4CH_3SH + ClO_2^- \rightarrow 2CH_3S\text{-}SCH_3 + Cl^- + 2H_2O \quad (5)$$

$$2CH_3SCH_3 + ClO_2^- \rightarrow 2CH_3SOCH_3 + Cl^- \quad (6)$$

Our group has previously employed a less specific halimeter monitoring device to evaluate the relative effectiveness of 6 oral healthcare products in diminishing oral cavity VSC concentrations [36]. This study involved a mixed model 3-factor factorial experimental design involving 6 volunteers, 7 treatment regimens (including a water placebo), and 5 VSC monitoring time-points (0 h to 5.29 h), and from the results acquired it was concluded that oral rinses containing oxyhalogen oxidants such as chlorite anion, and, in principle, also chlorine dioxide ($ClO_2^{\bullet}$) derived therefrom *in vivo*, may indeed provide a useful therapeutic strategy for the treatment of oral malodor.

Earlier evidence for the oral malodor-neutralizing properties of chlorite anion/chlorine dioxide, and any of the latter derived from the former *in vivo*, has been provided by Tozentich [1], who revealed that the therapeutic application of an oral rinse formulation containing only 0.01% (w/v) of these agents significantly decreased VSC levels in early morning mouth air samples collected from human participants with highly objectionable concentrations of these malodorous agents.

However, one limitation of our experimental design was the exclusion of other oral rinse formulations, including a positive control product formulation. However, the oxyhalogen oxidant present in oral rinse product (1) has already been proven to be effective in combating oral malodor. Indeed, Shinada et al. [29] compared the effectiveness of two oral rinses against oral cavity VSC levels, one containing ClO_2^-, the second without this active ingredient. These researchers found that the former product significantly reduced mouth air H_2S, CH_3SH and $(CH_3)_2S$ levels and hence improved oral malodor, and that such effects were prolonged, but only for a 4 h period. In a related study, the ability of another ClO_2^--containing mouth rinse product to combat oral malodor for periods of up to 96 h post-rinsing were evaluated [37], and results arising therefrom revealed that VSC concentrations, as monitored by organoleptic measurements and an early total sulphide and thiol monitoring device, showed that VSC levels in the test (oral rinse receiving) group attained minimal levels at the 8 h post-rinsing time-point, and these observations are consistent with ours, although such minimal levels were maintained up to a 12 h time-point in this study.

Therefore, the VSC-neutralizing capacity of the oral rinse product tested here can be rationalized with special reference to its chemical composition, e.g., chlorine dioxide and its chlorite anion precursor, which are both highly cidal towards odorigenic micro-organisms, and/or have the ability to directly oxidize VSCs to non-malodorous products.

The somewhat weaker VSC-neutralizing actions of the oral rinse (1) formulation towards dimethyl sulphide, $(CH_3)_2S$, are presumably ascribable to the source of this VSC, i.e., its origin is outside of the mouth, and predominantly arises from blood [1]. Hence, the capacity of the active oral rinse agent (1) evaluated here (specifically chlorite at a level of 0.10% (w/v)) to react with and hence modulate or attenuate oral cavity levels of this particular VSC will be expected to be less so than those with H_2S and CH_3SH, which arise from the bacterial degradation of both cysteine and methionine within the oral cavity.

These results are comparable to those achieved in a study featuring an alternative oral rinse product containing low levels of chlorhexidine and zinc ions (Zn^{2+}), and in which morning breath odor was successfully suppressed throughout a 12 h period, both with and without a challenge with oral cavity VSC-promoting L-cysteine [38]. The duration of the efficacy of this product observed was attributed to the involvement of a synergistic effect between the two active agents therein. However, this investigation focused on the actions of this oral rinse overnight during sleep episodes, and VSC measurements (limited to those of H_2S and CH_3SH) were only made at the zero control and post-12 h time-points. In contrast, our study was targeted on daily diurnal VSC measurements, and a total of 9 oral cavity VSC determinations were made on each participant at increasing time-points (0 h to 12 h) for each treatment tested, i.e., oral rinse (1) *vs.* the negative H_2O control. Moreover, our experiments also featured the simultaneous measurement of 3 rather than 2 VSCs.

A recent study [39] explored the long-term activities of an oral rinse formulation containing a mixture of 0.30% (w/w) zinc acetate and 0.025% (w/w) chlorhexidine, against intra-oral malodor. This double-blind, controlled cross-over study, which involved three treatments administered 12 h apart, i.e., both morning and evening on consecutive days, and with a 5 day washout period between such treatments, found that mean H_2S, CH_3SH and $(CH_3)_2S$ concentrations were significantly reduced by the administration of this product for evaluations conducted either overnight or during daylight hours. This effect prevailed throughout a 12 h time-point. Moreover, this oral rinse was also found to exert a significant reduction in the mean organoleptic score values of participants during the overnight monitoring period.

The results acquired here also have a high level of clinical significance, since there is now much evidence available that VSCs, which are extremely toxic to tissues at very low concentrations, are involved in the pathogenesis of periodontal diseases and further inflammatory conditions [40]. Moreover, the generation of high concentrations of CH_3SH appears to be restricted to periodontal pathogenic bacteria. Protein biosynthesis by cultured human gingival fibroblasts is also inhibited by these VSCs, and CH_3SH has been found to enhance the permeability of the intact mucosa and promote the generation of cytokines, which are clearly linked to periodontal diseases. Additionally, further *in vitro* investigations have revealed that exposure of cells to CH_3SH gives rise to a diminished level of collagen biosynthesis, and also a higher level of its degradation, together with the accumulation of poorly-cross-linked collagen precursors, the latter also being particularly susceptible to proteolysis. Hence, these malodorous VSCs have the capacity to exert clinically significant adverse effects on the local immune response of periodontal tissues towards plaque antigens, and also on extracellular matrices [40].

Indeed, periodontal diseases give rise to elevated VSC concentrations in mouth air, and those of CH_3SH have been found to be significantly enhanced in patients with periodontal disease over those of an orally healthy control group [41]. Although the current investigation was performed with orally healthy participants, results acquired indicate that the ClO_2^--containing oral rinse formulation tested here will also successfully exert such VSC-neutralizing actions in periodontal disease patients.

Finally, to the best of our knowledge, this is the first study reporting a multivariate statistical analysis of baseline oral cavity VSC levels collected from pre-fasted human participants, and this involved a full PCA strategy performed

on a trivariate VSC dataset. Results from this analysis were fully consistent with the differing biological sources of a composite H_2S- and CH_3SH-loaded multivariate PC arising from the oral environment, and which was shown to be clearly distinct from a second PC containing blood source $(CH_3)_2S$ alone.

Conclusions

An oral rinse formulation containing the chlorine dioxide precursor sodium chlorite at a concentration of 0.10% (w/v) serves as a very effective intra-oral neutralizer and/or consumer of VSCs, an observation which strongly supports its employment for controlling oral malodor. This efficacy is prolonged for periods of 12 h, and the mechanisms involved in this process are likely to feature 1) the direct chemical consumption of VSCs and their salivary sulfur-containing amino acid precursors, and/or 2) the bactericidal actions of chlorite anion against gram-negative bacteria responsible for VSC generation. These results have a high level of clinical significance in view of the established highly toxic actions of VSCs, and their striking relationships to the pathogenesis of periodontal diseases.

Acknowledgements

We are very grateful to all the participants who took part in the study.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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