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Effect of visible light on malodour production by mixed oral microflora

Nir Sterer and Osnat Feuerstein

The Hebrew University – Hadassah School of Dental Medicine, Department of Prosthodontics, PO Box 12272, Jerusalem 91120, Israel

Correspondence
Nir Sterer
sterer@hadassah.org.il

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Oral malodour is considered to be caused by the proteolytic activity of anaerobic Gram-negative oral bacteria. In a previous study, it was shown that these bacteria were susceptible to blue light (wavelengths of 400–500 nm). In this study, the effect of blue light on malodour production by mixed oral microflora was tested in a salivary incubation assay. Whole saliva samples were exposed to a xenon light source for 30, 60, 120 and 240 s, equivalent to fluences of 34, 68, 137 and 274 J cm^{-2}, respectively. Malodour was scored by two judges. The levels of volatile sulfide compounds (VSC) were measured using a sulfide monitor (Halimeter), the microbial population was assessed using viable counts and microscopy, salivary protein degradation was followed by SDS-PAGE densitometry and VSC-producing bacteria were demonstrated using a differential agar. The results showed that the exposure of mixed salivary microflora to blue light caused a reduction in malodour production concomitant with a selective inhibitory effect on the population of Gram-negative oral bacteria. These results suggest that light exposure might have clinical applications for the treatment of oral malodour.

INTRODUCTION

The oral cavity harbours a large variety of micro-organisms. These micro-organisms include a large group of Gram-positive bacteria, mainly streptococci, and a group of anaerobic micro-organisms such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Prevotella intermedius*, which are Gram-negative oral bacteria whose proteolytic activity is associated with oral malodour and periodontal disease (Berg & Fosdick, 1946; McNamara *et al.*, 1972; De Boever & Loesche, 1995).

These Gram-negative proteolytic micro-organisms break down salivary proteins and desquamative epithelial cell components into amino acids (Kleinberg & Codipilly, 1997). These amino acids are further metabolized, yielding malodorous compounds such as volatile sulfide compounds (VSC; e.g. H_2S and methyl mercaptan) (Persson *et al.*, 1989; Kleinberg & Codipilly, 1997; Tonzetich & Carpenter, 1971).

Bacterial mutagenesis caused by visible light was first reported almost 40 years ago (Webb & Malina, 1967). Later studies suggested an antibacterial effect of visible light in the presence of exogenous photosensitizers or when applied to porphyrin-producing bacteria, such as *Porphyromonas* and *Prevotella* species (Wilson, 1994; Henry *et al.*, 1995, 1996; Konig *et al.*, 2000; Soukos *et al.*, 2005). Recently, Feuerstein *et al.* (2004) showed that both *P. gingivalis* and *F. nucleatum* are more susceptible *in vitro* to blue light (wavelengths 400–500 nm) than are streptococci.

Salivary incubation assays are commonly used as *in vitro* bioassays in oral malodour investigations (Berg & Fosdick, 1946; McNamara *et al.*, 1972; Kleinberg & Codipilly, 1997). In this study a modification of such an assay, previously reported by Goldberg *et al.* (1997), served to test the effect of blue light, commonly used in restorative dentistry, on mixed salivary microflora and malodour production.

METHODS

Filtered saliva. Fresh whole saliva was stimulated by chewing on paraffin wax. The saliva was added to PBS in a 1 : 1 ratio (v/v) and sterilized by filtration through a 0·20 μm vacuum-driven filtration system (Stericup; Millipore).

Light exposure. A non-coherent visible light source was applied, known in dentistry as the plasma-arc, i.e. a xenon light source supplemented with a filter (wavelengths 400–500 nm) (MSq; Caesarea, Israel). The average light power was measured with a power meter (Ophir; Jerusalem, Israel) over a spot 0·7 cm in diameter. To calculate the power density of 1·14 W cm^{-2}, the average power was divided by the area of the light spot.

Whole saliva samples (50 μl) were placed in a 96-well microplate (Nunc) and exposed to light for 30, 60, 120 and 240 s, equivalent to fluences of 34, 68, 137 and 274 J cm^{-2}, respectively.

Abbreviation: VSC, volatile sulfide compounds.

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Experimental protocol. Test tubes containing 2 ml decarboxylase medium and 1 ml filtered saliva were supplemented with 50 µl of light-exposed whole saliva samples, non-exposed whole saliva samples or PBS. The test tubes were incubated at 37 °C for 24 h under anaerobic conditions. Malodour levels and VSC production were measured following incubation and samples of the incubation mixtures were taken for microbial analysis, to test degradation of salivary proteins and for pH determination. Experiments were performed in six replicates.

Organoleptic measurements. The test tubes were randomized and malodour production levels were scored by two experienced odour judges blinded to one another's scores. Malodour levels were measured by sniffing the odour emanating immediately after shaking and opening each test tube (Goldberg *et al.*, 1997). The scores were recorded according to a semi-integer scale of 0 to 5 as follows: 0, no appreciable odour; 1, barely noticeable odour; 2, slight, but clearly noticeable odour; 3, moderate odour; 4, strong odour; 5, extremely foul odour.

VSC measurements. Volatile sulfide levels were measured in the test tubes using a Halimeter sulfide monitor (Interscan Corp.) (Rosenberg *et al.*, 1991), as reported previously by Goldberg *et al.* (1997). The monitor was zeroed on ambient air and a 0·25 inch (0·64 cm) diameter disposable plastic straw was attached to the air inlet of the monitor. Test-tube headspace VSC levels were measured by inserting the other end of the straw 2 cm into each test tube immediately after removing the cap and recording the maximum reading in p.p.b. sulfide equivalents.

Microbial counts and microscopy. Viable counts were determined in each test tube by plating 10 µl aliquots of tenfold serial dilutions in PBS onto TSA sheep blood agar plates (Hy-Labs). The plates were incubated at 37 °C for 24 h under anaerobic conditions, obtained by using an anaerobic jar and an AnaeroGen anaerobic kit (Oxoid).

The Gram-positive/Gram-negative ratio was determined by analysing digital images (Olympus Camedia C-4040ZOOM) of incubation mixture samples (10 µl) after Gram staining (Sigma), using Image Pro plus (MediaCybernetics). Six images were analysed for each of the exposure times and the whole saliva control.

An effect of blue light treatment on the bacterial response to Gram staining was ruled out by comparing stained samples of a Gram-positive bacterium (*Streptococcus salivarius*) and a Gram-negative bacterium (*P. gingivalis*) suspended in PBS, before and after exposing the suspension (50 µl) to light for 30, 60, 120 and 240 s.

Salivary protein analysis by SDS-PAGE densitometry. Samples of 40 µl were prepared according to Laemmli (1970) and applied to a 12 % polyacrylamide gel in Tris/glycine/SDS buffer (0·025 M Tris/HCl, 0·192 M glycine, 0·1 % SDS, pH 8·6) followed by electrophoresis (80 mV) using a Mini-PROTEAN 3 electrophoresis minigel cell system (Bio-Rad). The gels were stained with Coomassie brilliant blue (Bio-Rad). Salivary protein levels were determined densitometrically (B.I.S. 202 D Bio imaging system) by comparing the intensities of the stained bands with that of the control.

pH measurements. Incubation mixture samples (10 µl) were spotted on pH indicator strips (Neutralit; Merck). The results were recorded when the colour had stabilized.

Effect of blue light on VSC-producing bacteria. Whole saliva samples (50 µl) were exposed to light as described above. The light-treated saliva samples were added with 50 µl PBS and plated onto a differential agar containing ferrous sulfate and sodium thiosulfate (Hartley *et al.*, 1996). The plates were incubated for 7 days under anaerobic conditions at 37 °C in sealed containers. Following incubation, the black colonies formed on this agar by VSC-producing bacteria were enumerated. The experiment was done in six replicates.

Statistical analysis. To compare the effect of different exposure times on quantitative variables, ANOVA was applied with post-hoc pairwise comparisons according to Scheffe and Dunnett (Dunnett, 1955). Kruskal–Wallis non-parametric ANOVA was applied to compare the effect of exposure time on the rank variables (odour judge scores) as well as on the quantitative variables. For the rank variables, the Mann–Whitney non-parametric test was applied for pairwise comparisons, using the Bonferroni correction for significance level. Spearman's non-parametric correlation coefficient was calculated to estimate the association between pairs of variables. All the tests applied were two-tailed and $P \leqslant 0{\cdot}05$ was considered statistically significant.

RESULTS

The means (± SD) of the odour judges' scores and the VSC levels emanating from the light-exposed, incubated salivary samples (incubation mixtures) are presented in Figs 1 and 2. An increase in light exposure time resulted in a concomitant decrease in malodour and VSC production compared with

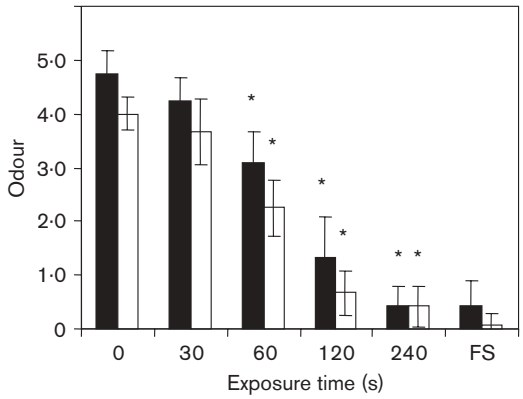

Fig. 1. Effect of light exposure on malodour levels as scored by judge 1 (filled bars) and judge 2 (open bars). Results are presented on a semi-integer scale of 0–5. Asterisks indicate a significant reduction ($P = 0{\cdot}002$) in malodour production compared with that at time 0. FS, Filtered saliva.

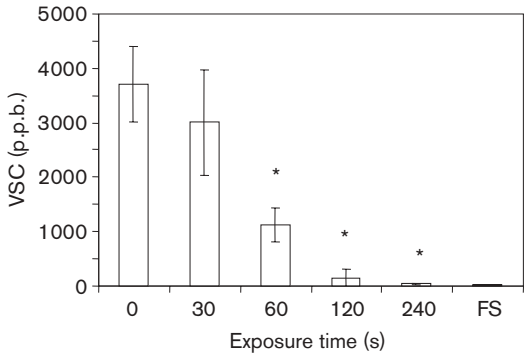

Fig. 2. Effect of light exposure on VSC levels measured, using a sulfide monitor (Halimeter), as p.p.b. sulfide equivalents. Asterisks indicate a significant reduction ($P = 0{\cdot}002$) in VSC production compared with that at time 0. FS, Filtered saliva.

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that in the non-exposed (0 s) control. However, a significant reduction was found only after exposing the samples to light for a period of 60 s or longer ($P = 0{\cdot}002$). Paired comparisons between the various exposure times revealed a significant reduction in VSC production between 30 and 60 s and between 60 and 120 s ($P = 0{\cdot}002$). The odour judges' scores were significantly low between 60 and 120 s ($P = 0{\cdot}004$ and $P = 0{\cdot}002$ for odour judges 1 and 2, respectively) and only moderately significant between 30 and 60 s ($P = 0{\cdot}009$ and $P = 0{\cdot}004$ for odour judges 1 and 2, respectively). No significant reduction in VSC or malodour measurements was observed between 120 and 240 s or between 240 s and filtered saliva samples.

Microbial evaluation of the various incubation mixtures showed no significant difference in total bacterial counts ($1{\cdot}4 \times 10^8 \pm 8{\cdot}2 \times 10^7$ c.f.u. ml^{-1}), except for the 60 s exposure, which showed significantly higher bacterial counts ($2 \times 10^8 \pm 9{\cdot}7 \times 10^7$ c.f.u. ml^{-1}) ($P < 0{\cdot}05$). The ratio between the Gram-negative and the Gram-positive bacteria decreased with the increase in exposure time (Fig. 3). However, a significant reduction in the Gram-negative to Gram-positive ratio ($P < 0{\cdot}007$) compared with that of the non-exposed control was evident only after exposure of the samples to light for 120 s or longer ($P = 0{\cdot}002$).

The strength of the association between the different parameters was evaluated using Spearman's correlation (Table 1). Both odour judge scores and VSC levels were highly correlated ($P < 0{\cdot}001$) with the Gram-positive to Gram-negative ratio as well as with one another.

Densitometric analysis of SDS-PAGE of salivary proteins from the various incubation mixtures showed a decrease in salivary protein degradation with the increase in exposure time, compared with the filtered saliva control (Fig. 4). Whereas 96 % of the protein was degraded in the non-exposed control (lane 2) compared with the filtered saliva control (lane 1), 72 and 43 % was degraded by the 30 and 60 s light-exposed samples, respectively. Protein degradation by the 120 and 240 s light-exposed samples was negligible.

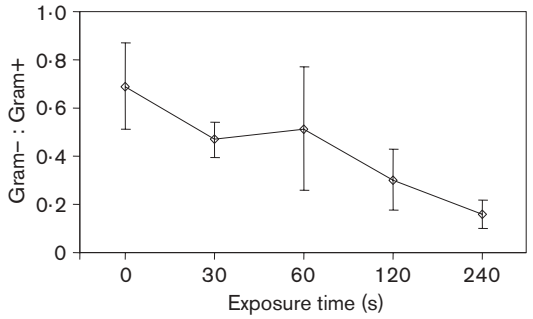

Fig. 3. Effect of light exposure on the Gram-negative to Gram-positive ratio. Results are presented as means ± SD.

The pH recorded in the incubation mixtures was as follows: filtered saliva control, pH 7·0; non-exposed control, pH 6·0; 30 s exposure, pH 6·0; 60 s exposure, pH 5·5; 120 s and 240 s exposure, pH 5·0.

The effect of light exposure on VSC-producing bacteria was demonstrated using a differential agar on which VSC-producing colonies are stained black. There was a significant reduction in the number of black colonies formed by the saliva samples which were exposed to light for a period of 60 s and longer ($P < 0{\cdot}015$) compared with the non-exposed control (Fig. 5).

DISCUSSION

In this study, exposure of mixed salivary microflora to non-coherent blue light resulted in reduced malodour production in a salivary incubation assay. Taken together, the findings suggest that light exposure has a selective inhibitory effect on the Gram-negative oral bacteria found in the mixed salivary microflora, since light exposure did not appear to affect the bacterial response to Gram staining (data not shown). Whereas the total bacterial counts remained virtually unchanged, the Gram-negative to Gram-positive ratio was reduced markedly with the increase in light exposure dose, especially after 120 s. Since Gram-negative oral bacteria are considered the main producers of malodorous compounds in incubated saliva as well as in the oral cavity itself, this might account for the reduction in malodour and VSC production. This premise is further supported by the high association seen in this study between the Gram-negative to Gram-positive ratio and malodour-related variables such as the odour judges' scores and VSC levels (Table 1). Additional evidence for the susceptibility of malodour-producing bacteria to light treatment was demonstrated by the decrease in the number of VSC-producing bacteria resulting from the increase in light exposure times (Fig. 5).

Although salivary incubation assays reflect planktonic cultures which may differ from the *in vivo* environment (El-Maaytah *et al.*, 1996), these assays are commonly used in oral malodour investigations. Earlier studies using such assays demonstrated the ability of Gram-negative, as opposed to Gram-positive, oral bacteria to produce an offensive odour from incubated saliva (Berg & Fosdick, 1946; McNamara *et al.*, 1972; Kleinberg & Codipilly, 1997). Furthermore, a shift towards a higher Gram-negative to Gram-positive ratio was also demonstrated in saliva following incubation, resulting in an elevated proportion of Gram-negative population compared with that in fresh whole saliva (McNamara *et al.*, 1972). In this study, however, the exposure of whole saliva to light resulted in an opposite effect, i.e. an increase in the Gram-positive population following incubation.

The results of a previous investigation showed a higher susceptibility to blue light exposure of the Gram-negative anaerobic bacteria *P. gingivalis* and *F. nucleatum* than streptococci when suspended in broth (Feuerstein *et al.*, 2004). In this study, however, we tested the effect of light

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Table 1. Spearman correlation coefficients between malodour-related parameters

In each case, correlation is significant ($P < 0{\cdot}001$; two-tailed).

Parameter	Sulfide monitor	Odour judge 1	Odour judge 2
Gram-negative : Gram-positive	0·749 ($n = 29$)	0·752 ($n = 29$)	0·735 ($n = 29$)
Sulfide monitor		0·915 ($n = 36$)	0·883 ($n = 36$)
Odour judge 1			0·898 ($n = 36$)

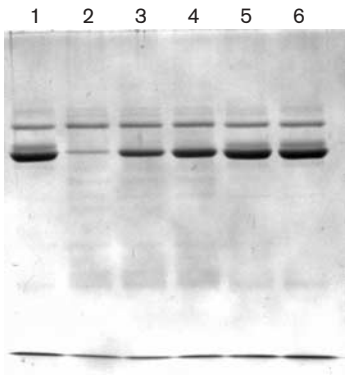

Fig. 4. Effect of light exposure on salivary protein degradation as determined by SDS-PAGE (12 %) analysis. Lanes: 1, filtered saliva (negative control); 2, 0 s exposure (positive control); 3, 30 s exposure; 4, 60 s exposure; 5, 120 s exposure; 6, 240 s exposure.

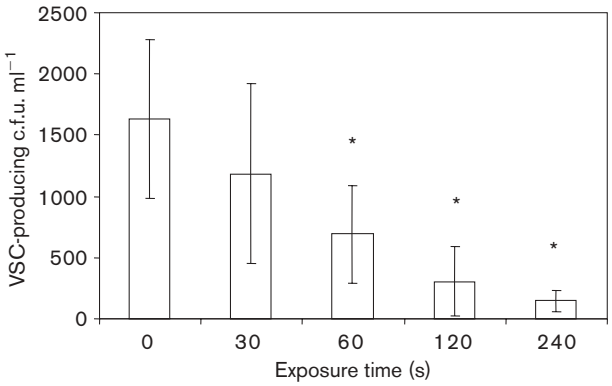

Fig. 5. Effect of light exposure on VSC-producing bacteria as measured by using a differential agar on which VSC-producing colonies are stained black. Expressed as VSC-producing c.f.u. (ml saliva)$^{-1}$. Asterisks indicate a significant reduction ($P < 0{\cdot}015$) in VSC production compared with that at time 0.

exposure on whole saliva, a complex system that more closely resembles the natural oral environment and contains a mixture of micro-organisms.

The suspension medium may play a crucial role in the effect of bacterial exposure to light (Komerik & Wilson, 2002). In a previous experiment, for example, we showed a decrease of 99·6 % in the viable count of *P. gingivalis* suspended in clear broth, following only 60 s (~69 J cm^{-2}) exposure to plasma-arc curing (PAC) light (Feuerstein *et al.*, 2004). A similar decrease in survival (1·48 %) of suspended *P. gingivalis* was found by Soukos *et al.* (2005) after exposure to blue light at a lower power density for a longer duration of 10 min (~42 J cm^{-2}). In this study, exposures of 30 and 60 s (equivalent to fluences of ~34 and ~69 J cm^{-2}, respectively) had a minor to moderate inhibitory effect on malodour-related parameters. Only after 120 s exposure (~137 J cm^{-2}) was there a significant reduction in these variables, including a decrease in the Gram-negative to Gram-positive ratio and complete inhibition of protein degradation. The lower phototoxic effect in the presence of saliva and serum could be attributed to the presence of proteins which, by absorbing the light, decrease damage to the bacteria (Wilson & Pratten, 1995). Since the phototoxic effect may be mediated by the formation of reactive oxygen species (Gourmelon *et al.*, 1994), the presence of antioxidants in saliva could hinder this process. The latter might also account for the selective effect of light exposure on anaerobic bacteria.

The inhibition of protein degradation, as measured in this study, could be explained by the reduction in the Gram-negative proteolytic bacterial population, by the inhibition of proteolytic enzyme activity or by a combination of the two. However, a direct effect of light on protein degradation can be ruled out, since the amount of exposed saliva was negligible compared with the total amount of saliva in the incubation mixture. Furthermore, as mentioned above, an increase in exposure time resulted in a decrease in protein degradation.

The findings of this study suggest that non-coherent visible light sources such as xenon and halogen lamps or a light-emitting diode (LED), which are often used in restorative dentistry, may have clinical applications in the treatment of oral malodour. However, blue light exposure may have disruptive effects on cell survival, activity and growth in soft tissues (Aggarwal *et al.*, 1978; Gorgidze *et al.*, 1998; Pflaum *et al.*, 1998; Wataha *et al.*, 2004), possibly mediated by light-induced reactive oxygen species (Massey, 2000). In a clinical setting, light parameters such as power density and exposure time should be optimized to minimize damage to the soft tissues and tooth demineralization. Further investigations *in vivo*, using animal models, are warranted in order to determine the effectiveness and safety of this treatment modality compared with that of existing treatments. In addition, due to the importance of the tongue microflora

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in oral malodour production, additional investigations regarding the effect of light on tongue-coating samples are currently under way.

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