

Effects of stress hormones on the production of volatile sulfur compounds by periodontopathogenic bacteria

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Abstract: Little is known about the effects of stress hormones on the etiologic agents of halitosis. Thus, the aim of this study was to evaluate *in vitro* the effects of adrenaline (ADR), noradrenaline (NA) and cortisol (CORT) on bacteria that produce volatile sulfur compounds (VSC), the major gases responsible for bad breath. Cultures of *Fusobacterium nucleatum* (*Fn*), *Porphyromonas endodontalis* (*Pe*), *Prevotella intermedia* (*Pi*) and *Porphyromonas gingivalis* (*Pg*) were exposed to 50 μ M ADR, NA and CORT or equivalent volumes of sterile water as controls for 12 and 24 h. Growth was evaluated based on absorbance at 660 nm. Portable gas chromatography was used to measure VSC concentrations. Kruskal-Wallis and the Dunn post-hoc test were used to compare the groups. For *Fn*, ADR, NA and CORT significantly reduced bacterial growth after 12 h and 24 h ($p < 0.05$). All the substances tested increased hydrogen sulfide (H_2S) production ($p < 0.05$). For *Pe*, all the substances tested reduced bacterial development after 24 h ($p < 0.05$), and NA significantly increased the H_2S concentration after 12 h ($p < 0.05$). In the *Pg* and *Pi* cultures, no effects on bacterial growth were observed ($p > 0.05$). In the *Pi* cultures, ADR, NA and CORT increased H_2S ($p < 0.05$). Catecholamines and cortisol can interfere with growth and H_2S production of sub-gingival species *in vitro*. This process appears to be complex and supports the association between stress and the production of VSC.

Keywords: Halitosis; Catecholamines; Hydrocortisone; Gram-Negative Anaerobic Bacteria.

Introduction

Halitosis, also known as bad breath or malodor, is a term used to describe unpleasant, fetid odors present in air exhaled from the mouth.¹ Being unpleasant, bad breath is a problem that affects both the patient and the family.¹

Studies have indicated that in the majority of cases, malodor is associated with the degradation of sulfur-containing amino acids (methionine, cysteine and cystine) by anaerobic gram-negative bacteria present in the oral cavity, including *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Tannerella forsythia* and *Porphyromonas endodontalis*.^{2,3} Volatile sulfur compounds (VSC), such as hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH) and dimethyl sulfide [$(CH_3)_2S$], are generated as a product of this metabolism.³

Some patients with halitosis do not present clinical evidence of oral pathologies or systemic disorders. Stress, depression and anxiety have been identified as risk factors for a shift in oral homeostasis.^{4,5} It has been proposed that emotional alterations may be a co-factor in halitosis development. Calil and Marcondes⁶ reported increased VSC concentrations in healthy men faced with an anxiety-evoking situation compared with the basal situation, independent of salivary flow.

The relationship between stress and the progression of infectious diseases, including periodontitis, has been the focus of some studies over the last few decades.^{4,7,8,9,10} However, the mechanisms involved in this process are still not clear. Potentially pathogenic microorganisms can recognize stress hormones, a fact that has led to the development of the concept of “microbial endocrinology.”¹¹ These microorganisms could use the hormones produced by the host as ‘environmental trails’ to initiate their growth and pathogenic processes.^{12,13,14,15,16,17} In one study, the growth rate of bacteria commonly found in the oral cavity, such as *Actinomyces naeslundii*, *Eikenella corrodens*, and *Campylobacter gracilis*, was stimulated by exposure to noradrenaline.¹³ In addition, recent studies show conflicting results of noradrenaline and adrenaline on the growth of *P. gingivalis*, with studies showing either no effects of these hormones on growth^{12,17} or demonstrating a reduction in viability.^{13,15} Recently, it was demonstrated that noradrenaline can stimulate the production and activity of arg-gingipain B, an important protease of *P. gingivalis*.¹⁵ Thus, the effects of stress could be mediated by two potentially synergistic pathways involving the down-regulation of the host’s defenses and the up-regulation of bacterial pathogenicity.

Because there is clinical evidence that stress is related to halitosis,⁶ we hypothesized that periodontal bacteria would respond to stress substances by stimulating their growth and their production of VSC. Because there have been no studies on the effects of stress hormones on VSC production, the aim of the present study was to investigate the effects of catecholamines and cortisol on the growth and VSC production of periodontopathogens.

Methodology

Substances Used

The following substances were tested: adrenaline - ADR (epinephrine bitartrate, Sigma Chemical Co.[®], St Louis, USA), noradrenaline - NA (arterenol, Sigma[®]) and cortisol - CORT (hydrocortisone, Sigma[®]). The 50 µM concentration of ADR, NA^{13,18} and CORT¹⁹ was chosen based on previous studies. The solutions were prepared using distilled water as the solvent and were sterilized using Millex[®] (Millipore[®], Billerica, USA) sterile filters with a pore size of 0.22 µm.

Bacterial Samples and Culture Conditions

The growth patterns and VSC production of four bacterial species (*Porphyromonas endodontalis* from clinical specimens - *Pe*, *Porphyromonas gingivalis* W83 - *Pg*, *Fusobacterium nucleatum nucleatum* ATCC 25586 - *Fn* and *Prevotella intermedia* ATCC 25611 - *Pi*) were examined. These species were selected based on their ability to produce VSC constitutively. The bacteria were grown statically in brain heart infusion broth (BHI; Difco Co., Detroit, USA). To cultivate *Pg* and *Pe*, 5 µg/mL hemin (Sigma[®]) and 1 µg/mL menadione (Sigma[®]) were added to the BHI. Bacterial cultures were grown at 37 °C under anaerobic conditions (10% CO₂, 10% H₂ and 80% N₂; MiniMacs Anaerobic Workstation, Don Whitley Scientific[®], Shipley, UK).

Study design and Experimental Groups

Cultures of *Pg*, *Pe*, *Pi* and *Fn* were exposed to 50 µM ADR, NOR and CORT for 12 and 24 h (early and mid-logarithmic growth phases). Tubes containing bacteria without the tested substances but containing equivalent volumes of distilled water (vehicle) were used as positive control for growth. Negative controls contained only the tested substances and the culture medium. After incubation, bacterial growth and VSC production were assessed as follows. At least two separate experiments were performed for each hormone tested. In each experiment, 8 replicates were tested for each group (ADR, NA, CORT and controls).²⁰

Susceptibility Tests (Macrodilution Broth Tests)

Susceptibility was assayed using a macrodilution broth test as previously described.²¹ Tubes containing

5 mL of BHI broth medium (plus 5 µg/mL hemin (Sigma®) and 1 µg/mL menadione (Sigma®) for the *Pg* and *Pe* cultures) were prepared for each experimental group. A standardized bacterial suspension was prepared in sterile saline solution (0.9% NaCl) and adjusted with a spectrophotometer (Unico 1100 RS;Unico®, Dayton, USA) to an optical density of 0.5 (8×10^6 CFU/mL). An inoculum of 500 µL was added to each tube to give a final concentration of 4×10^6 CFU/mL. The tubes were then incubated at 37°C in anaerobic conditions for 12 or 24 h. After these periods, bacterial growth was assessed spectrophotometrically at a wavelength of 660 nm.

Bacterial VSC Production Tests

This experiment was conducted as previously described, with some modifications.²⁰ For bacterial VSC production analyses, the experimental groups and inocula were prepared as described above. After incubation at 37 °C in anaerobic conditions, 0.1 mL of air from the tubes was aspirated using a 1 mL syringe (BD®, Franklin Lakes, USA) coupled to a needle (BD®) (Figure 1) and injected into Oral Chroma® (Abimedical, Osaka, Japan). For a period of 8 minutes, the apparatus performed molecule counts in parts per billion (ppb) of hydrogen sulfite (H₂S) and methylmercaptan (CH₃SH). VSC analyses were performed at two different time intervals (12 and 24 h). These incubation lengths were chosen because at these periods, the bacteria are growing exponentially, and there are more viable cells than dead cells.

Gaseous standards of the VSC (H₂S and CH₃SH) were prepared as described previously²² by White Martins (Campinas, Brazil) (RBC-INMETRO n M-32433/10), and the device was calibrated every three months according to Tangerman and Winkel.²³

Statistical Analysis

Levene's test was used to test the homogeneity of variance, and the normality of distribution was tested by the Shapiro-Wilk's test. Kruskal-Wallis and the Dunn post-hoc test were used to compare the groups (ADR, NA and CORT compared to control for each bacteria and each time interval separately). Statistical analysis was performed using BioEstat 5.0 (Fundação Mamirauá, Belém, Brazil) at a significance level of 5%.

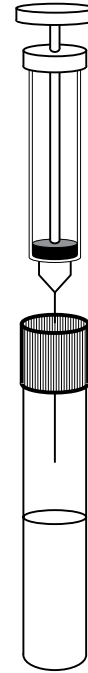


Figure 1. Disposition of the tube, syringe and needle used in the Bacterial VSC Production Tests.

Results

Effects of Adrenaline, Noradrenaline and Cortisol on Bacterial Growth

Figure 2 summarizes the effects of these substances on bacterial growth (optical density, 660 nm). For the *Fn* cultures, ADR, NA and CORT significantly reduced bacterial growth after 12 and 24 h ($p < 0.05$, Kruskal Wallis test). For *Pe*, the tested substances reduced bacterial growth, but only after 24 h of exposure ($p < 0.05$). No statistically significant differences in optical density ($p > 0.05$) were observed for the *Pg* and *Pi* cultures in any of the time periods evaluated.

Effects of Noradrenaline, Adrenaline and Cortisol on Bacterial VSC Production

The median values and interquartile deviations of H₂S and CH₃SH concentrations (in ppb) for *Fn*, *Pe*, *Pg* and *Pi* cultures exposed for 12 and 24 h to ADR, NA and CORT are presented in Figures 3 and 4, respectively.

In *Fn*, only CORT caused a significant increase in H₂S levels at 12 h (Kruskal-Wallis, $p < 0.05$), while no changes in H₂S concentration were observed at 24 h

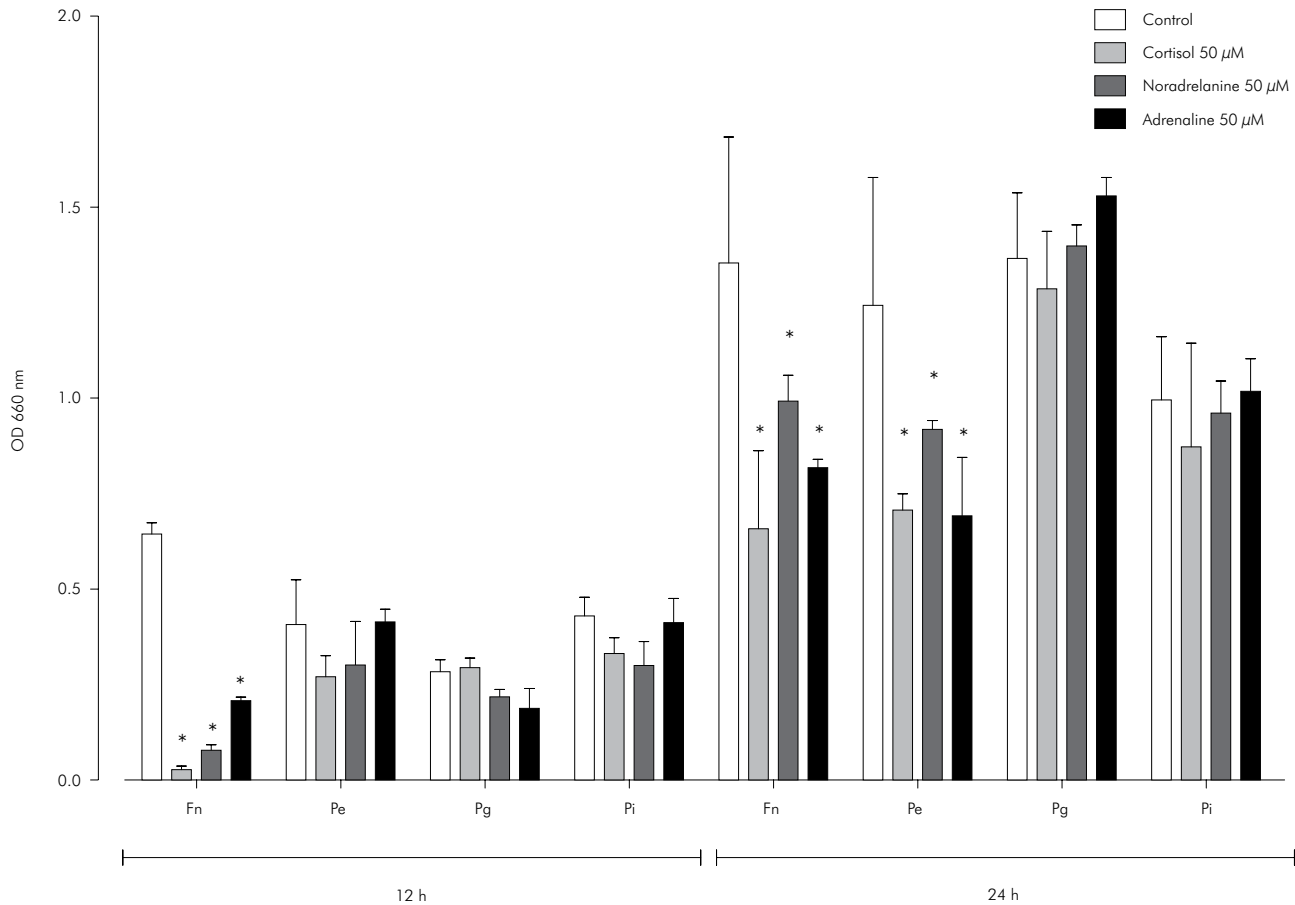


Figure 2. Mean and standard deviation of optical density (660 nm) representing the bacterial growth. Statistically significant p values are shown (*) on the upper side of the columns ($p < 0.05$, Kruskal Wallis, Dunn).

(Kruskal-Wallis, $p > 0.05$). However, ADR and NA did increase the H_2S concentration in *Fn* cultures after 24 h of exposure ($p < 0.05$). Only *Fn* showed a reduction in CH_3SH production by CORT after 24 h ($p < 0.05$). No other conditions changed the CH_3SH levels.

H_2S concentrations were significantly increased only in *Pe* cultures exposed to NA for 12 h ($p < 0.05$). No difference in H_2S production was observed for the groups exposed to ADR and CORT ($p > 0.05$). In addition, CH_3SH levels were not altered by exposure of *Pe* to catecholamines and CORT ($p > 0.05$).

No statistically significant differences ($p > 0.05$) were found in H_2S and CH_3SH levels among groups and their respective controls in *Pg* cultured for 12 or 24 h.

Pi was more sensitive to the effects of catecholamines and CORT than the other bacterial cultures. In this case, H_2S production was up-regulated in the groups exposed to ADR, CORT (12 and 24 h) and NA

(24 h) ($p < 0.05$). None of the experimental groups showed differences in CH_3SH production compared to their controls ($p > 0.05$).

Discussion

Clinical evidence has shown that stress can increase VSC concentrations, thus contributing to halitosis.⁶ The present study provides additional support for the role of stress substances on halitosis, as it was shown that ADR, NOR and CORT can up-regulate the production of VSC by microorganisms commonly found in sub-gingival biofilms.

This study reveals a variation in the growth rates of four periodontopathogenic microorganisms in response to the addition of stress hormones to their culture media. The growth of *Fn* and *Pe* was reduced by stress hormones, primarily after 24 h of exposure. In contrast, no significant effects on growth

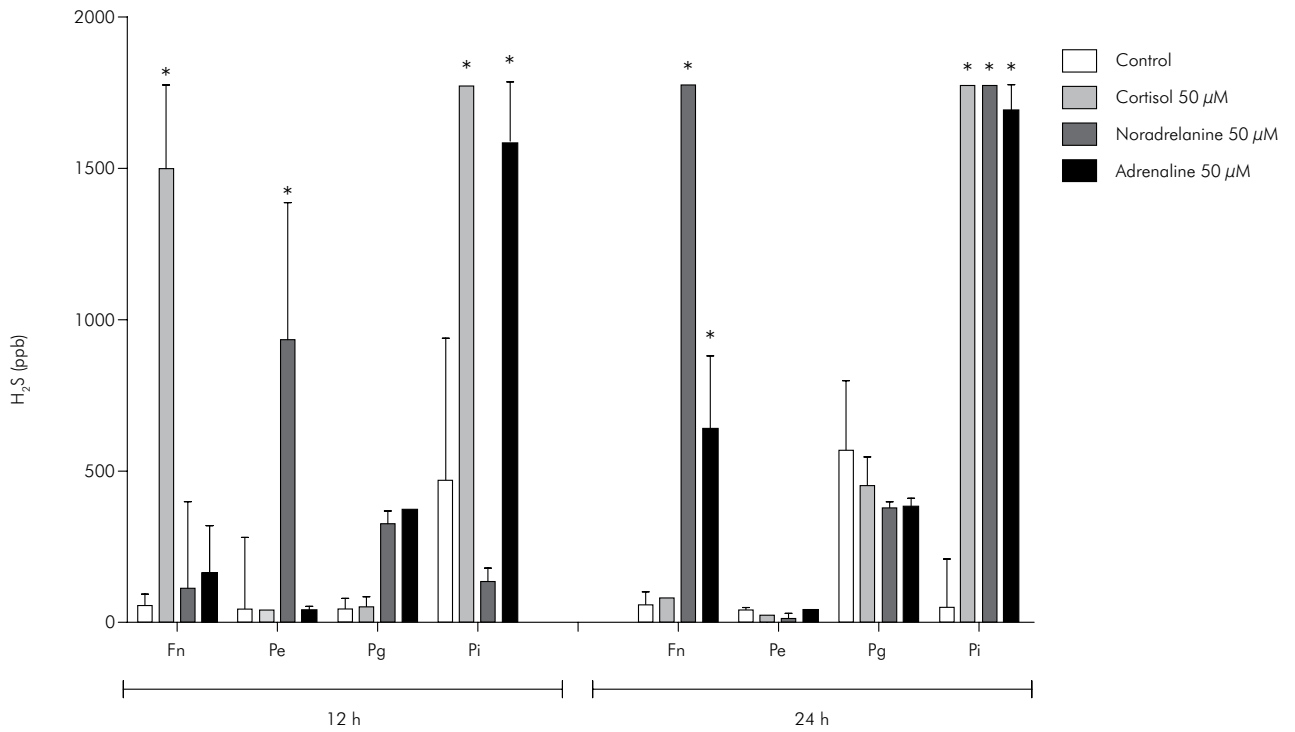


Figure 3. Median and interquartile deviation of H₂S concentrations (ppb) representing the VSCs production. Statistically significant *p* values are shown (*) on the upper side of the columns (*p* < 0.05, Kruskal Wallis, Dunn).

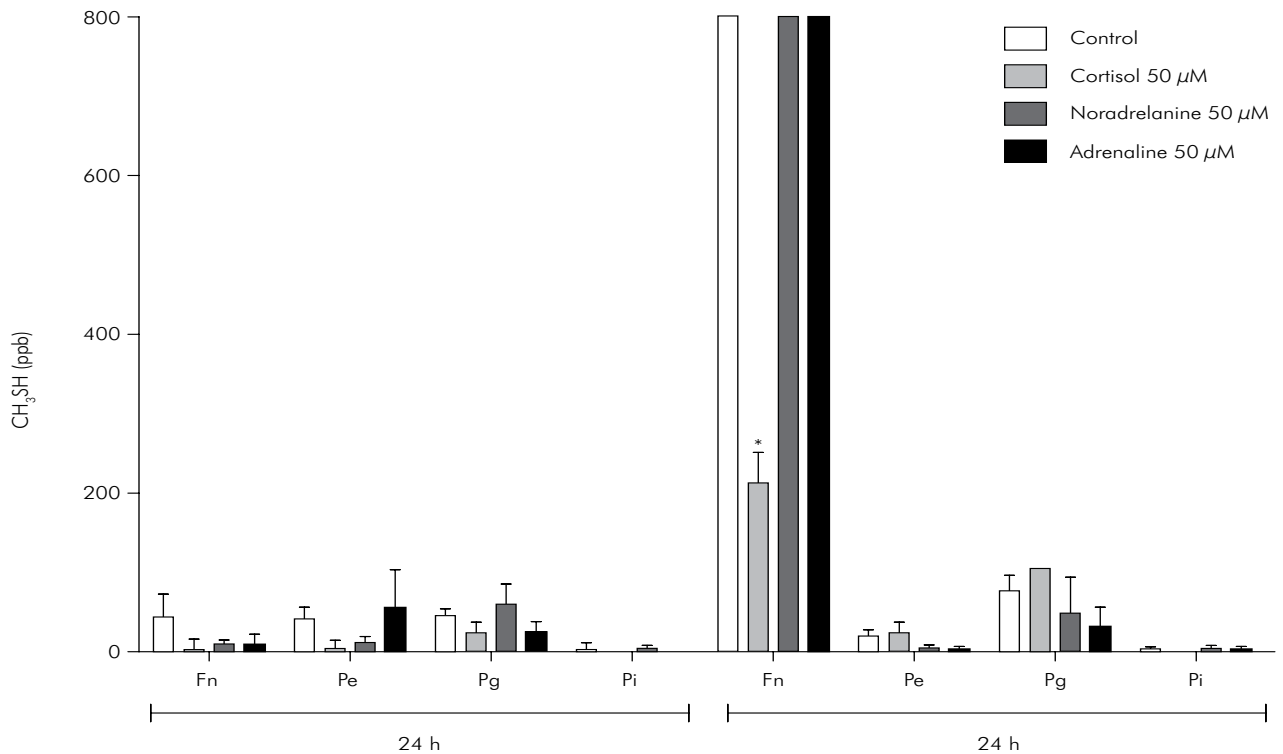


Figure 4. Median and interquartile deviation of CH₃SH concentrations (ppb) representing the VSCs production. Statistically significant *p* values are shown (*) on the upper side of the columns (*p* < 0.05, Kruskal Wallis, Dunn).

were observed in *Pg* and *Pi* for any of the substances tested. Most of these results are in agreement with the literature regarding the effects of catecholamines on bacterial growth. Roberts *et al.*¹³ demonstrated an inhibitory effect of catecholamines on *Fn* growth and no effect on the growth of *Pi* cultures, while Belay *et al.*¹² and Jentsch *et al.*¹⁷ showed no change in *Pg* growth patterns. However, some of the present findings are contrary to previous reports. It was reported that the growth rate of *Pg* was reduced when exposed to catecholamines.^{13,15} Additionally, cortisol was demonstrated to stimulate the growth of *Pg* cultures.²⁴ Our results showed no effects of NA, ADR or CORT on the growth rate of this pathogen. There were several differences in the experimental factors used in these studies, including the period of hormone exposure, bacterial strains and culture media used, catecholamine concentration and the method of growth assessment, which may have contributed to the divergent findings.

There are some reports in the literature demonstrating VSC production by periodontopathogenic bacteria. Previous studies revealed that saliva and dental plaque from patients with periodontal disease produces large quantities of VSC, thus contributing to malodor.^{25,26,27} Our study shows that periodontal bacteria produce significant VSC levels *in vitro* and that this production is altered by stress hormones. We observed that the major effects of catecholamines and CORT were on H₂S levels, mainly in the case of *Fn*, *Pe* and *Pi*. For these bacteria, exposure to these substances caused an increase in H₂S concentration. Only CORT changed the production of CH₃SH, and this effect was inhibitory in *Fn* cultures treated with this glucocorticoid. Given these results, it is possible to assume that the main roles of ADR, NA and CORT are in H₂S production. To better understand the effects of stress hormones, it will be necessary to utilize enzymatic, genomic and proteomic approaches.

Interestingly, the growth and VSC production of *Pg* was not affected by any of the stress hormones tested. It is possible that *Pg* is less sensitive to hormonal influence in the favorable environment of our experiment, which offered a rich-nutrient culture media along with controlled atmosphere and temperature. Previous studies have observed that

adrenaline and noradrenaline have strong effects on bacterial growth mainly when the bacteria are cultivated in poor nutrient media.^{18,28} For this reason, those authors designed a special medium, serum-SAPI minimal medium, to mimic the challenging *in vivo* environment. However, this medium was not used in our study. This could be the reason for our observations of slight effects of hormones on growth and VSC production, especially in the case of *Pg*. It is possible, for example, that these organisms (and others showing negative growth responses) utilize catecholamines for up-regulation of virulence expression rather than for growth¹³ and VSC production.

When taking into consideration the times of exposure (12 and 24 h) in our study, differing effects were found. In some experiments, significant effects of the hormones were noted at both times, while the hormone effects were observed at only one time period (12 or 24 h) in other experiments. We expected to observe that a substance that showed an effect at 12 h of exposure would have the same effect at 24 h. As demonstrated, however, this was not always the case. These stress-related substances have short half-lives and could be degraded during the time-period of our experiments. Kennedy *et al.*²⁹ demonstrated that catecholamines are stable in saliva for up to 2 h at 4 °C. In our study, however, catecholamine concentrations in the culture medium after 12 and 24 h were not determined, and one can imagine that degradation was faster due to the higher temperature. We hypothesized that the bacteria might be able to use this degradation by-product to stimulate VSC production; however, further studies are necessary to elucidate this hypothesis. With regard to the degradation of CORT, it is known that this hormone remains stable in saliva at ambient temperature for up to one week or more³⁰ and, therefore, would not be degraded during our experimental period.

By comparing the VSC production and growth patterns of bacteria exposed to NA, ADR and CORT, it was possible to observe differing results. These findings suggest a species-specific interaction of ADR, NA and CORT, which leads to differing effects in terms of growth and gas production. It is worth highlighting that, even in some situations where growth was reduced, VSC levels were stimulated. Because of this,

it is possible that these stress-related substances have different cellular mechanisms whereby they affect growth and VSC production. Additionally, considering that fewer bacteria could produce higher levels of VSC in the presence of these hormones, it is important to evaluate the clinical importance of this observation. It is possible that these results could be the beginning of an explanation for why patients without advanced periodontal disease or other clinical signs of inflammation have increased complaints of halitosis in stressful situations and have higher VSC levels than non-stressed patients.⁶ Investigations of the exposure of catecholamines and cortisol in multi-species biofilm models, animal models for halitosis and clinical studies should be carried

out to better understand the relationship of stress and VSC production.

Conclusions

In conclusion, our results indicate that the hormones adrenaline, noradrenaline and cortisol can modulate the growth of periodontopathogenic microorganisms and the production of VSC, thus elucidating another possible mechanism of action for the relationship between stress and the development of halitosis.

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