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Cetylpyridinium chloride suppresses gene expression associated with halitosis

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ABSTRACT

Objective: Halitosis is a common complaint affecting the majority of the population. Mouthrinses containing cetylpyridinium chloride (CPC) have been used as oral hygiene aids to suppress oral malodor. Although the clinical efficacy of these mouthrinses has been well-documented, the mechanism whereby CPC reduces malodor is less-well-understood. We hypothesized that CPC suppresses expression of the genes (*mgl* and *cdl*) and enzymes responsible for methyl mercaptan (CH_3SH) and hydrogen sulfide (H_2S) production by oral anaerobes associated with halitosis. In this study, the *mgl* and *cdl* expression of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in the presence of CPC was investigated.

Materials and methods: We used a microdilution method to determine the growth and production of volatile sulfur compounds (VSCs) by *P. gingivalis* W83 and *F. nucleatum* ATCC 10953 in respective media containing CPC (0.5 μg/mL to 1.5 μg/mL). For metabolic activity, we used an XTT {2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide} reduction assay. We used real-time RT-PCR and Western blotting to evaluate the effect of CPC at sub-MIC levels on mgl and cdl expression at the transcriptional and enzymatic levels.

Results: CPC inhibited the growth of *P. gingivalis* and *F. nucleatum* at MICs of 3 μg/mL and 2 μg/mL, and at MBCs of 6 μg/mL and 3 μg/mL, respectively. Compared with untreated controls, CPC at 1.5 μg/mL suppressed CH_3SH production of *P. gingivalis* by 69.84% ± 2.88% and H_2S production of *F. nucleatum* by 82.55% ± 8.36% ($p < 0.05$) without affecting metabolic activity. Inhibition of *mgl* mRNA (81.58% ± 20.33%) and protein (39.15% ± 6.65%) expression in *P. gingivalis* and inhibition of *cdl* mRNA (61.76% ± 13.75%) and protein (64.34% ± 1.62%) expression in *F. nucleatum* were also noted ($p < 0.05$).

Conclusion: CPC represents an effective agent for halitosis reduction by inhibiting the growth and suppressing the expression of specific genes related to VSC production in anaerobic periodontal pathogens.

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1. Introduction

Oral malodor is a common complaint among dental patients, often causing embarrassment and affecting interpersonal social communication.[1] The intensity of clinical oral malodor has been shown to be significantly associated with the levels of intraoral volatile sulfur compounds (VSCs), of which hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) are the major components.[2] It is now widely accepted that

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bacterial accumulation and food residues at the posterior, in the furrows and the papillary structure of the tongue contribute to VSC production.[3] Halitosis-associated bacteria are primarily Gram-negative periodontal pathogens, such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Solobacterium moorei* which can break down salivary and oral proteins into amino acids, some of which, *e.g.* methionine and cysteine, are further metabolized, yielding malodorous VSCs.[4]

It has been reported that H_2S is produced from L-cysteine by the enzymatic action of L-cysteine desulfhydrase (CD), which degrades the L-cysteine to produce pyruvate, ammonia and H_2S.[5] CH_3SH is produced from L-methionine by the enzymatic action of L-methionine-α-deamino-γ-mercaptomethane-lyase (METase), which produces α-ketobutyrate, methyl mercaptan and ammonia.[6] Previous studies have shown that CD and METase are detected in *P. gingivalis*, *F. nucleatum* and *Aggregatibacter actinomycetemcomitans*.[7] *F. nucleatum* has been reported to produce the highest amounts of H_2S through CD encoded by the *cdl* gene,[8,9] while *P. gingivalis* produces CH_3SH through METase encoded by the *mgl* gene.[10] In addition to contributing to halitosis, the VSCs have also been thought to play an important etiologic role in periodontal disease. Their penetration into the gingival pocket epithelium may lead to loss of collagen and bone, and delay wound healing.[11] Exposure to CH_3SH can also alter the protein synthesis of human gingival fibroblasts and inhibit cell migration in periodontal ligament cells.[12] *In vitro* studies have shown that H_2S is cytotoxic to gingival fibroblasts and epithelial cells.[13] Some investigations have reported that the production of H_2S by oral bacteria in periodontal sites is associated with the progression of periodontitis.[14]

Various approaches have been undertaken in attempts to control oral malodor, including the use of masking products, the mechanical reduction of microorganisms and their substrates and the chemical neutralization of odorous compounds.[2,3,14] Toothpastes and mouthrinses containing antimicrobial compounds such as chlorhexidine (CHX), zinc chloride, stannous fluoride, essential oils and cetylpyridinium chloride (CPC) are the products commonly used to reduce oral malodor.[5] CHX-containing mouthrinses have been shown to be successful in reducing supragingival plaque as well as bacteria on the tongue.[15] Stannous-containing sodium fluoride dentifrices, such as that containing 0.454% stabilized stannous fluoride sodium hexameta-phosphate, have been reported to demonstrate significant anti-malodor activity.[16] Certain metal ions, particularly zinc, are well-known to reduce or inhibit the formation of VSCs.[17] Zinc chloride may render VSCs non-volatile and may also inhibit bacterial cysteine proteases, which are partly responsible for the production of VSCs.[17,18] In addition to antimicrobial chemicals, natural antimicrobial compounds from plant sources have been incorporated into oral hygiene products to reduce oral malodor.[19] Daily use of a mouthrinse containing plant essential oils (eucalyptol, menthol and methylsalicylate) can provide clinically significant benefit in this respect.[19,20] These natural flavouring agents in chewing gums have also been shown to have a short-term germ-killing effect against halitosis-associated oral bacteria in human saliva.[21–23] Recently, epigallocatechin gallate (EGCg), a polyphenolic catechin from tea (*Camellia sinensis*), has been suggested as an alternative agent for halitosis management, due to its ability to inhibit the growth and halitosis-associated *mgl* gene expression of *P. gingivalis*.[10]

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound that has demonstrated plaque-reducing benefit and has a long history as a safe and effective component of oral hygiene products.[24] CPC exhibits a broad spectrum of antimicrobial activity against plaque bacteria and fungi in the oral cavity.[15,25] As a surface-active agent, CPC binds non-specifically to charged protein and modifies surface tension of the bacterial cell wall, thus leading to cell wall leakage and affecting cell metabolism.[26] Daily use of mouthrinses containing CPC was found to reduce VSCs and oral malodor.[27] However, limited studies have explored the mechanism whereby CPC contributes to this effect. We hypothesized that CPC reduces halitosis by its antimicrobial activity against halitosis-associated oral bacteria, and by suppressing the expression of genes associated with VSC production. In this study, the effect of CPC on VSC production and *mgl* and *cdl* gene expression of *P. gingivalis* and *F. nucleatum* was investigated.

2. Materials and methods

2.1. Test bacteria and growth conditions

P. gingivalis W83 and *F. nucleatum* ATCC 10953, purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), were used in this study. *P. gingivalis* was grown in Todd-Hewitt Broth (THB) with 0.001% hemin and 0.0001% vitamin K in an anaerobic chamber (37 °C, 10% H_2, 5% CO_2 and 85% N_2; Forma Scientific, Inc., Marietta, OH, USA). *F. nucleatum* was grown anaerobically at 37 °C in Schaedler broth (OXOID LTD., Basingstoke, Hampshire, England).

2.2. Bacterial susceptibility assay

The minimal inhibitory concentrations (MICs) and minimum bactericidal concentration (MBC) of CPC were determined according to the microdilution method in 96-well microtiter plates.[28] All plates were incubated in an anaerobic incubator at 37 °C for 48 h, and growth was determined spectrophotometrically at 600 nm by means of a microplate reader (PowerWave 200, Bio-Tek Instruments, Winooski, VT, USA).

2.3. CH_3SH and H_2S production assay

P. gingivalis and *F. nucleatum* were grown in the presence of serially diluted CPC in 96-well microtiter plates for 48 h. Quantities of L-methionine (10 μL, 0.6%, w/v) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (10 μL, 0.06%, w/v) were then added to each well for determination of CH_3SH production. For H_2S production, 10 μL of L-cysteine (0.3%) and 10 μL of lead acetate (1.2%) were added instead.[30] All plates were further incubated anaerobically for 12 h at 37 °C. The production of CH_3SH and H_2S was determined by measuring absorbance of cell-free supernatant at 430 nm and 550 nm and was expressed as a percentage of the non-treated control.