Archives of Oral Biology 84 (2017) 133–138

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Research Paper

Inhibition of malodorous gas formation by oral bacteria with cetylpyridinium and zinc chloride

Jeong Hyun Kang[a], Dae Jeong Kim[b], Bong Kyu Choi[c], Ji Woon Park[b,*]

[a] Department of Oral medicine and Orofacial pain, Institute of Oral Health Science, Ajou University School of Medicine, 164, World cup-ro, Yeongtong-gu, Suwon, Gyeonggi-do, 16499, Republic of Korea

[b] Department of Oral Medicine and Oral Diagnosis, School of Dentistry and Dental Research Institute, Seoul National University, 101, Daehak-ro, Jongno-gu, Seoul, 03080, Republic of Korea

[c] Department of Oral Microbiology and Immunology, School of Dentistry, Seoul National University, 101, Daehak-ro, Jongno-gu, Seoul, 03080, Republic of Korea

ARTICLE INFO

Keyword:
Bacteria
Gas chromatography
Halitosis
Microbiology
Periodontitis
Sulfur

ABSTRACT

Objective: The antimicrobial efficacy of zinc- ($ZnCl_2$) and cetylpyridinium-chloride (CPC) and their inhibition capacity on volatile sulfur compound (VSC) production by oral bacterial strains were investigated.

Design: Minimum inhibitory concentrations (MIC) and growth curves were determined for $ZnCl_2$, CPC, and CPC with $ZnCl_2$ solutions against eight oral microorganisms (*Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Tannerella forsythia*, *Staphylococcus aureus* and *Streptococcus mutans*) known to be involved in the pathophysiology of both halitosis and periodontal disease. Gas chromatography was applied to measure VSCs (H_2S, CH_3SH, $(CH_3)_2S$) production levels of each strains following exposure to the solutions.

Results: $ZnCl_2$ and CPC effectively inhibited growth of all eight strains. $ZnCl_2$ was generally more effective than CPC in suppressing bacterial growth excluding *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia*. Synergism between CPC and $ZnCl_2$ was shown in *A. actinomycetemcomitans*. The MIC for CPC was significantly lower than $ZnCl_2$. VSC production was detected in five bacterial strains (*A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *T. denticola*, and *T. forsythia*). Each bacterial strain showed unique VSCs production profiles. H_2S was produced by *F. nucleatum*, *P. gingivalis*, and *T. denticola*, CH_3SH by all five strains and $(CH_3)_2S$ by *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *T. denticola*. Production of CH_3SH, the most malodorous component among the three major VSCs from mouth air was evident in *F. nucleatum* and *T. forsythia*.

Conclusion: Both $ZnCl_2$ and CPC effectively inhibit bacterial growth causative of halitosis and periodontal disease, resulting in a direct decrease of bacterial VSCs production.

1. Introduction

Halitosis, an unpleasant odor emanating from the mouth, causes personal distress and negatively influences social relationships resulting in a drastic reduction in quality of life (Rayman & Almas, 2008). However the exact pathophysiology of halitosis is still to be fully elucidated. Although controversy still exists, microbiota of the subgingival areas and tongue dorsum have been regarded as primary causative factors (Hughes & McNab, 2008). Gram negative anaerobic bacteria have been known to produce volatile sulfur compounds (VSCs) which are major constituents of halitosis gas through proteolytic decomposition of organic material accumulated in periodontal pockets and on the tongue (Tonzetich, 1977; Wåler, 1997b). Positive correlation was detected between the progression of periodontal pockets and the production of VSCs by periodontal pathogens (Tonzetich & McBride, 1981; Yaegaki & Sanada,1992a, 1992b) and the posterior dorsal tongue has been regarded as one of the main VSCs producing sites because of its large surface area and high bacterial load within a densely populated biofilms (Hess, Greenman, & Duffield, 2008).

There have been attempts to clarify specific microorganisms involved in the production of oral malodor. Analysis of tongue and subgingival plaque from halitosis patients using polymerase chain reaction detected six periodontopathogenic bacteria including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola,* and *Tannerella forsythia* (Yasukawa, Ohmori, & Sato, 2010). Especially gram negative and

* Corresponding author at: Orofacial Pain Clinic, Department of Oral Medicine and Oral Diagnosis, School of Dentistry and Dental Research Institute, Seoul National University, 101, Daehak－ro, Jongno－gu, Seoul, 03080, Republic of Korea.
E-mail address: ankara01@snu.ac.kr (J.W. Park).

http://dx.doi.org/10.1016/j.archoralbio.2017.09.023
Received 21 April 2017; Received in revised form 21 June 2017; Accepted 24 September 2017

benzoyl-DL-arginine-2-napthylamide positive periodontal pathogens such as *F. nucleatum*, *Prevotella loescheii, Porphyromonas endodontalis*, *P. gingivalis*, *P. intermedia*, *T. denticola,* and *T. forsythia* have been regarded as principal pathogens in the formation of halitosis (De Boever & Loeschem, 1995; Kato, Yoshida, Awano, Ansai, & Takehara, 2005; McNamara, Alexander, & Lee, 1972; Persson, Edlund, Clasesson, & Carlsson, 1990; Tonzetich & McBride 1981; Yasukawa et al., 2010). Although *Staphylococcus aureus* and *Streptococcus mutans* are not well known VSCs producing bacteria, several studies have evaluated their involvement in early stages of biofilm formation and also the pathogenesis of periodontitis and peri-implantitis (Colombo et al., 2013; Lee & Wang, 2010; Nishihara & Koseki, 2004). However reports which investigated the direct role of oral bacteria in VSCs production are rare and primary gas measurements of VSCs produced by oral bacteria with gas chromatography (GC) are sparse.

To manage halitosis, the main strategy would be the reduction of already existing VSCs and eradication of VSCs producing microorganisms in the oral cavity. Currently available methods work by mechanically and/or chemically reducing microorganisms and substrates or by neutralizing odoriferous compounds (van den Broek, Feenstra, & de Baat, 2008). Compared to mechanical removal, chemical mouthrinses have less limitation in reaching all acknowledged oral areas and removing both bacteria and VSCs. Zinc- and cetylpyridinium-chloride (CPC) have been known as one of the most widely used chemotherapeutic agents in commercial mouthrinses (Fedorowicz, Aljufairi, Nasser, Outhouse, & Pedrazzi, 2008; Rioboo et al., 2012; Roldan, Winkel, Herrera, Sanz, & Van Winkelhoff, 2003). Zinc's affinity for sulphydryl groups allowed its interaction with sulfur in the substrate or precursors of VSCs to form insoluble sulfides through oxidization (Ng & Tonzetich, 1984). CPC is an amphiphilic quaternary compound which known as destructing the bacterial cell wall structure (Pitten & Kramer, 2001).

To assess the effect of two clinically applied chemotherapeutic agents, zinc and CPC, on microbial growth and VSCs production we tested their impact on eight bacterial strains including *A. actinomycetemcomitans, F. nucleatum, P. gingivalis, P. intermedia, T. denticola, T. forsythia, S. aureus,* and *S. mutans* which have well known roles in both halitosis and periodontal disease. Minimum inhibitory concentrations (MIC) and growth curves were determined for zinc chloride, CPC, and zinc chloride with CPC solutions against 8 micro-organisms. Gas chromatography (GC) was applied to measure VSCs (H_2S, CH_3SH, $(CH_3)_2S$) production levels following exposure to each bacterial cultures.

2. Materials and methods

2.1. Chemicals and microbiological media

Chemicals were all analytical grade or better and were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated. Microbiological media including supplements were obtained from Becton Dickinson (B-D, Sparks, MD, USA) and prepared according to the manufacturer's instructions.

2.2. Preparation of bacteria

The eight laboratory strains tested including *A. actinomycetemcomitans* (ATCC 43718), *F. nucleatum* (ATCC 25586), *P. gingivalis* (ATCC 33277), *P. intermedia* (ATCC 25611), *S. aureus* (ATCC 29213), *S. mutans* (ATCC 25175), *T. denticola (*ATCC 33521), and *T. forsythia* (ATCC 43037) were obtained from the Department of Microbiology of the School of Dentistry at Seoul National University. All bacteria were stored in a sterile *vial* at −70 °C. Growth of *F. nucleatum* and *P. gingivalis* was carried out in brain heart infusion broth (Difco Laboratories, MI, USA) containing hemin (10 μL mL^{-1}) and vitamin K (1.0 μL mL^{-1}) (Sigma-Aldrich, MO, USA). *P. intermedia* was also cultured in brain heart infusion without any supplement. *A. actinomycetemcomitans* was grown in brain heart infusion broth containing only vitamin K (1.0 μL mL^{-1}). *T. denticola* and *T. forthysia* were grown in modified new oral spirochete (NOS) medium (12.5 g L^{-1} of brain heart infusion broth; 10 g L^{-1} of trypticase; 2.5 g L^{-1} of yeast extract; 0.5 g L^{-1} of sodium thioglycolate; 1 g L^{-1} of L-cystein; 0.25 g L^{-1} of L-asparagine; 0.2% sodium bicarbonate; 50 mL of heat-inactivated fetal bovine serum; 0.0006% thiamine pyrophosphate; pH 7.4) (Sigma-Aldrich, MO, USA) containing vitamin K and N-acetylmuramic acid, but in the case of *T. forsythia*, additional hemin (10 μL mL^{-1}) was added to the same broth. *S. aureus* and *S. mutans* were grown in tryptic soy broth (TSB) (Sigma-Aldrich, MO, USA). All bacterial suspensions except *S. aureus and S. mutans* were incubated at 37 °C under anaerobic conditions for 24 h before adding solutions containing zinc chloride ($ZnCl_2$) and/or CPC. *S. aureus* and *S. mutans* were incubated under aerobic condition at 37 °C prior to adding the solutions.

2.3. Antimicrobial agents

The solutions tested included $ZnCl_2$ (Hambakwooseum Bio®, Seoul, Korea), CPC (Hambakwooseum Bio®, Seoul, Korea) and their combination. All solutions were made with deionized water only without any other additives. The MICs of $ZnCl_2$ were in the range of 0.0625–0.50% and those of CPC were 0.015625% in seven bacterial strains except for *F. nucelatum* (0.00195313%). After determination of the MICs of each agent for every bacterial strain, the direct exposure test and VSC measurements were done under the MIC of each agent.

2.4. Broth microdilution method for MIC

The MIC was determined as the lowest concentration of each agent inhibiting the growth of a particular bacterium as determined by the optical density (OD) of the broth. Broth dilution was carried out in 96-well culture plates. Bacterial suspensions were prepared in appropriate liquid media and their initial concentrations were adjusted to OD 600 nm. Twofold dilutions of $ZnCl_2$ and CPC solutions were prepared in appropriate broth culture media from stock solution. Aliquots (150 μL) of each dilution of $ZnCl_2$ and CPC were dispensed in the 96-well culture plate. Each bacterial suspension (150 μL) was added to each well and incubated under aerobic/anaerobic conditions at 37 °C for 4–30 h. The absorbance was measured at 600 nm. The highest dilution of $ZnCl_2$ and CPC at which a drastic decrease in bacterial growth occurred was determined by OD and defined as the MIC.

2.5. Direct exposure test

To evaluate the antimicrobial effects of the solutions, time-response growth curves were acquired. MICs of each agent, $ZnCl_2$, CPC, and $ZnCl_2$ with CPC was added to the broth containing each bacterial strains then incubated under aerobic/anaerobic conditions at 37 °C. The growth phase was determined by measuring ODs of the cultures at 600 nm at 1–6 h intervals using a spectrophotometer (SoftMax®, CA, USA). The initial OD was set to approximate 0.2. The experiments were performed in triplicate. Media without any antimicrobial agent served as a negative control in each experimental condition. The entire procedure of the experiment was performed three times.

2.6. Direct VSCs measurement with gas chromatography

The bacterial strains were cultured in a 100 mL Pyrex flask containing appropriate media with or without $ZnCl_2$ and/or CPC solutions at their MICs and were incubated under aerobic/anaerobic conditions at 37 °C without shaking for 4–36 h according to each strain. The headspace air above each bacterial culture was passively collected in a gas-tight syringe (SampleLock, 10 mL Vol, Hamilton Co, Reno, NV) with negative pressure and then injected directly into the injector port of a GC system (6890N Network GC System, Agilent Technologies,