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Hydrogen sulfide production from subgingival plaque samples

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ABSTRACT

Periodontitis is a polymicrobial anaerobe infection. Little is known about the dysbiotic microbiota and the role of bacterial metabolites in the disease process. It is suggested that the production of certain waste products in the proteolytic metabolism may work as markers for disease severity. Hydrogen sulfide (H_2S) is a gas produced by degradation of proteins in the subgingival pocket. It is highly toxic and believed to have pro-inflammatory properties. We aimed to study H_2S production from subgingival plaque samples in relation to disease severity in subjects with natural development of the disease, using a colorimetric method based on bismuth precipitation. In remote areas of northern Thailand, adults with poor oral hygiene habits and a natural development of periodontal disease were examined for their oral health status. H_2S production was measured with the bismuth method and subgingival plaque samples were analyzed for the presence of 20 bacterial species with the checkerboard DNA–DNA hybridization technique. In total, 43 subjects were examined (age 40–60 years, mean PI 95 ± 6.6%). Fifty-six percent had moderate periodontal breakdown (CAL > 3 < 7 mm) and 35% had severe periodontal breakdown (CAL > 7 mm) on at least one site. *Parvimonas micra*, *Filifactor alocis*, *Porphyromonas endodontalis* and *Fusobacterium nucleatum* were frequently detected. H_2S production could not be correlated to periodontal disease severity (PPD or CAL at sampled sites) or to a specific bacterial composition. Site 21 had statistically lower production of H_2S ($p = 0.02$) compared to 16 and 46. Betel nut chewers had statistically significant lower H_2S production ($p = 0.01$) than non-chewers. Rapid detection and estimation of subgingival H_2S production capacity was easily and reliably tested by the colorimetric bismuth sulfide precipitation method. H_2S may be a valuable clinical marker for degradation of proteins in the subgingival pocket.

1. Introduction

Periodontitis is, along with caries, the most common oral disease worldwide. In severe cases it may lead to tooth loss and edentulousness. Under healthy conditions, the microorganisms in the richly colonized oral cavity live in balance (homeostasis) with the host, forming biofilms on mucosal surfaces and teeth [1]. When the bacterial load, by poor oral hygiene, is increased the balance can be disturbed and dysbiosis is formed [2]. The plaque (biofilm) on teeth surfaces accumulates and changes in composition over time, consequently leading to a soft tissue inflammation in the gingiva – gingivitis, diagnosed based on characteristic signs such as swelling and redness. Gingivitis may over time, further result in periodontitis where the host response degrades the periodontal ligament and supporting alveolar bone, clinically registered as loss of clinical attachment (CAL) and increasing probing pocket depth (PPD). The disease progression and severity is highly subject dependent and leads to tooth loss in some individuals while others do not experience periodontal destruction despite persistent gingival inflammation for many years (long-standing gingivitis). Gingivitis is a reversible process where the inflammatory host response can be eliminated if the bacterial overload is reduced [3,4]. The lost supporting alveolar bone in periodontitis is, however, irreversible. Periodontal disease is induced by dysbiosis in the plaque but the exact mechanisms and role of the microorganism are still unclear, however, it is likely that bacterial activity, growth and production of toxic metabolites trigger the inflammatory response.

According to the ecological plaque hypothesis [5], there is a change in the environment of the subgingival pocket, implying increased flow of gingival crevicular fluid, providing serum constitutes such as proteins, hormones and vitamins, favoring

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fastidious proteolytic bacterial species. This results in a biofilm of a high diversity of mainly Gram negative and anaerobe bacteria [6,7]. The diseased subgingival pocket is slightly alkaline with a negative redox potential, as compared to positive mV recordings at healthy sites [8]. The dysbiotic bacterial activity results in an increased production of enzymes and a cascade of metabolic end products such as nitrogen compounds (e.g., ammonia), volatile sulfur compounds (e.g., hydrogen sulfide, methyl mercaptan, dimethyl disulfide) and short carboxylic acids (butyric acid, valeric acid, propionic acid, caproic acid, phenyl acetic acid etc.) in addition to other bacterial products such as endotoxins and phenyl compounds (e.g., indole) [9].

The presence and activity of microorganisms in the subgingival pocket can be studied at different levels depending on the question addressed. New techniques are constantly developing in the search to "map" the entire plaque and identify the genome of all the species present. Another approach of interest is the expression of proteins, proteomics, associated to mixed bacterial communities rather than single species alone. The enzymatic activity concerning the hydrolysis of benzoyl-DL-arginine-naphthylamide is an example of a protein studied and a test developed, the so-called BANA, identifying a specific activity only found for a small number of bacterial species [10,11]. Yet another level in studying the plaque is the net effect of the entire biofilm, the result of the proteins – the metabolites, metabolomics.

Hydrogen sulfide (H_2S) is an end product of the proteolytic activity, proposed to be of interest in gingivitis and periodontitis [12] but the impact in disease development is largely unknown. It is a volatile, foul smelling gas produced by degradation of cysteine in the subgingival pocket [13–15]. It is a gas known for its toxic properties [16] with pro-inflammatory elements [17], recently proposed as a gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO) [18]. The bacterial production of H_2S *in vitro* has previously been reported for many species frequently encountered in subjects with periodontitis e.g., *Fusobacterium* spp., *Parvimonas micra*, and *Treponema denticola* by degradation of cysteine or glutathione [19–22]. The gas is of major interest in the research of halitosis (bad breath) and is believed to be one of the key factors for oral malodor [23,24]. Measurement of H_2S in gingival crevices has previously been conducted in several studies where the production has been measured with lead acetate impregnated paper strips [13,14], trapping device and gas chromatographic analyses [12] and a technologically advanced probe for sulfide detection [25]. All these studies have tried to elucidate the presence and role of H_2S in periodontal disease. Nevertheless, the methods used have been either time consuming and insensitive, such as the lead acetate method, or too advanced and expensive for use in the clinics or field studies, as the latter two. Therefore, the existing methods have not been used and tested in a greater extent at clinical settings. The fact that the gas is volatile and easily converts to polysulfides has further hampered this task. Thus, comprehensive knowledge of the role of H_2S in bacterial metabolism, the possible effect on local environment and the host, and its relation to clinical parameters in health and disease is still lacking.

H_2S may serve as a marker molecule for the increased proteolytic activity by the bacteria in the subgingival pocket in gingivitis and periodontitis. Thus, it is likely that deep pockets and severe periodontitis cases have higher H_2S production than shallow pockets and cases with no or little periodontal breakdown. Recently, our group modified a colorimetric method for H_2S estimation *in vitro* based on bismuth sulfide precipitation (unpublished data). The method was applied for subgingival *ex vivo* measurements and tested under field conditions among adults of the Karen Hill tribe of northern Thailand, a population with a natural progression of periodontal disease without disturbing intervention on the subgingival microbiota by regular dental treatment. The aim of this study was to investigate the H_2S producing ability of subgingival samples in relation to clinical and microbiological parameters on site and individual level.

2. Materials and methods

2.1. Subjects

The subjects participating in the study were members of the Karen Hill tribe selected from five villages in Omgoi district, Chiang Mai province in northern Thailand. During a week our group accompanied a mobile dental team organized by The Princess Mother Medical Voluntary Foundation, Bangkok, Thailand, that also ethically approved to the study. Inclusion criteria were subjects between 40 and 60 years of age. The participants were selected randomly, informed of the study and voluntarily consented to participate. The subjects were interviewed for their age, smoking habits, betel chewing habits, oral hygiene habits and sugar consumption. This population is living in a remote area with underdeveloped infrastructure, and has no access to dental care and therefore represent an inviolate population for a cross-sectional study on H_2S production in subgingival plaque in relation to clinical parameters and the composition of the microorganisms inhabiting the biofilm.

2.2. Clinical examination

The clinical examination was performed in day light with the help of a mouth mirror and a dental and periodontal probe prior to the examination and potential treatment performed by the mobile dental team. Following parameters were registered: Decayed Missed Filled Teeth (DMFT), Probing Pocket Depth (PPD) $\geq$ 4 mm at four sites per tooth, Bleeding on Probing (BoP), Clinical Attachment Level (CAL) $\geq$ 3 mm and Plaque Index (PI). The periodontal status of the participants was divided into three groups; gingivitis (BoP but $\leq$3 mm CAL), mild periodontitis (CAL > 3 mm but <7 mm), severe periodontitis (at least one site CAL $\geq$ 7 mm, suspected loss of teeth due to periodontitis).

2.3. The bismuth method

The bismuth method was performed as previously described [22] with some modifications. Shortly, a mini-sponge (3M ESPE, Forsbergs Dental, Gothenburg, Sweden), absorbing approximately 10 μl, was inserted with a forceps in the mesial subgingival pocket of 16, 21, 31 and 46 for approximately 10 s. The sponge was then moved to 100 μl bismuth solution (0.4 mol l^{-1} triethanolamine – HCl pH 8.0, 10 mmol l^{-1} bismuth(III)chloride, 20 μmol l^{-1} pyridoxal 5-phosphate monohydrate, 20 mmol l^{-1} EDTA and 40 mmol l^{-1} L-cysteine) in a well of microtiter plates (96 MicroWell Plates Nunc, Roskilde, Denmark) and the color of the sponge was registered after 2 h as no color change (−), small color change to black (+), medium color change (++) and maximal color change (+++).

2.4. Bacterial sampling

After sampling for the bismuth test, plaque samples for the checkerboard DNA–DNA hybridization analyses were made with a curette and inserted in Eppendorf tubes of 100 μl TE-buffer (10 mmol l^{-1} Tris HCl, 1 mmol l^{-1} EDTA, pH 7.6). To lyse the cells, 100 μl 0.5 mol l^{-1} NaOH was added and the samples were transported to the laboratory (Department of Oral Microbiology and Immunology, Institute of Odontology, University of