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Efficacy of a mouthrinse based on hydroxyapatite to reduce initial bacterial colonisation *in situ*

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ABSTRACT

Objective: The present *in situ* - investigation aimed to specify the impact of pure hydroxyapatite microclusters on initial bioadhesion and bacterial colonization at the tooth surface.
Design: Pellicle formation was carried out *in situ* on bovine enamel slabs (9 subjects). After 1 min of pellicle formation rinses with 8 ml of hydroxyapatite (HA) microclusters (5%) in bidestilled water or chlorhexidine 0.2% were performed. As negative control no rinse was adopted. *In situ* biofilm formation was promoted by the intraoral slab exposure for 8 h overnight. Afterwards initial bacterial adhesion was quantified by DAPI staining and bacterial viability was determined *in vivo*/*in vitro* by live/dead-staining (BacLight). SEM analysis evaluated the efficacy of the mouthrinse to accumulate hydroxyapatite microclusters at the specimens' surface and spit-out samples of the testsolution were investigated by TEM.
Results: Compared to the control ($2.36 \times 10^6 \pm 2.01 \times 10^6$ bacteria/cm^2), significantly reduced amounts of adherent bacteria were detected on specimens rinsed with chlorhexidine 0.2% ($8.73 \times 10^4 \pm 1.37 \times 10^5$ bacteria/cm^2) and likewise after rinses with the hydroxyapatite testsolution ($2.08 \times 10^5 \pm 2.85 \times 10^5$ bacteria/cm^2, $p < 0.001$). No demonstrable effect of HA-particles on *Streptococcus mutans* viability could be shown. SEM analysis confirmed the temporary adsorption of hydroxyapatite microclusters at the tooth surface. Adhesive interactions of HA-particles with oral bacteria were shown by TEM.
Conclusion: Hydroxyapatite microclusters reduced initial bacterial adhesion to enamel *in situ* considerably and could therefore sensibly supplement current approaches in dental prophylaxis.

1. Introduction

Hydroxyapatite particles and clusters of different size may have the potential to develop into a valuable agent in preventive dentistry (Hannig, Basche, et al., 2013; Lelli et al., 2014). Although the authority of fluoride application as premier choice of standard oral prophylaxis measures is still undisputed, so far, neither bacterial adhesion nor acid-associated demineralization of dental hard tissue can be prevented completely. Alternative preventive treatment strategies aim to affect the physical, electrostatic and thermodynamic interactions that promote initial microbial adhesion and biomineralisation at the tooth surface (Besinis, De Peralta, Tredwin, & Handy, 2015; Hannig & Hannig, 2010b; Kensche et al., 2016; Wessel et al., 2014). In this context, the acquired pellicle qualifies as the key structure to be modified by oral health care products (Hannig, Basche, et al., 2013; Hannig & Hannig, 2009; Weber, Hannig, Poetschke, Höhne, & Hannig, 2015). However, the wide variety of individual test preparations as well as the complexity of their potential active compounds has to be considered critically when evaluating the validity of studies testing oral health care preparations. This sometimes makes determination of effective antibacterial agents difficult, as most commercially available products contain a mixture of several active compounds (Hannig, Basche, et al., 2013). The pure components have rarely been tested *in situ*.

In recent years, growing attention has been drawn to nanoparticles, especially nanoparticulate metals and metal oxides (Allaker, 2010; Besinis, De Peralta, & Handy, 2014; Hannig & Hannig, 2010b). Their possible benefits in biofilm management are suggested to be due to their shape, size-dependent active surface area, chemical reactivity and bacterial interactions (Allaker & Memarzadeh, 2014; Beyth, Houri-Haddad, Domb, Khan, & Hazan, 2015; Hannig & Hannig, 2010b). In preventive dentistry,

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biomimetic micro- or nanocrystallite hydroxyapatite or other calcium-phosphate based systems such as CPP-ACP are promoted for their caries-preventive effect (Grychtol, Basche, Hannig, & Hannig, 2014; Hannig, Basche, et al., 2013; Hannig & Hannig, 2010b; Najibfard, Ramalingam, Chedjieu, & Amaechi, 2011). Unfortunately, most studies rather focus on their potential to repair demineralized surface lesions instead of investigating their ability to decrease bacterial adhesion or -growth (Lelli et al., 2014; Lu, Meng, Li, & ZM, 2007; Tschoppe & Kielbassa, 2011; Zhang et al., 2015).

With the aim to clarify the antibacterial potential of hydroxyapatite at the tooth surface, an *in situ* study performed by our group investigated the effect of different subfractions of a commercially available preparation (*Biorepair*) on initial bacterial colonization (Hannig, Basche, et al., 2013). The results indicated no noticeable antibacterial but a strong antiadhesive effect of zinc-carbonate hydroxyapatite (Hannig, Basche, et al., 2013). In line with our findings, Palmieri et al. observed an inhibitory effect of a comparable preparation on *Streptococcus mutans*- and *Mitis Group Streptococci* colonization of saliva-conditioned microtiter plates *in vitro* (Palmieri, Magi, Orsini, Putignano, & Facinelli, 2013). The inhibition of biofilm formation by zinc oxide has repeatedly been shown, wherefore zinc-ions are added to some oral health care products (Grenho, Salgado, Fernandes, Monteiro, & Ferraz, 2015; Gu et al., 2013; Lynch, 2011). In comparison, the antibacterial or anti-adhesive potential of pure hydroxyapatite has so far not been sufficiently investigated and is rather based on hypothesis. In theory, hydroxyapatite particles could impair bacterial adhesion due to their pH-dependent surface charge influencing electrostatic interactions (Harding, Rashid, & Hing, 2005; Venegas, Palacios, Apella, Morando, & Blesa, 2006). Furthermore, blocking of specific bacterial adhesins has been suggested based on *in vitro* investigations (Venegas et al., 2006).

In order to contribute to the assessment of pure hydroxyapatite microclusters as possible components of oral health care products, the present study investigated their potential to affect an *in situ* formed pellicle and to influence initial microbial adhesion and viability. Therefore a pure hydroxyapatite microclusters containing mouthrinse was prepared and investigations were based on *in situ* experiments. The hypothesis of this study claims that the purely hydroxyapatite particles based mouthrinse would not reduce bacterial adhesion and -viability *in situ* within the first 8 h of biofilm formation as effectively as the goldstandard chlorhexidine-digluconate.

2. Materials and methods

2.1. Subjects and specimens

The *in situ* experiments were realized with the participation of 9 volunteers, all healthy members of the laboratory staff or students aged 23–45. As prerequisite, all investigations corresponded to an ethics committee's review and approval (EK 147052013) and all volunteers had given informed written consent about participation in the study. The participants confirmed to be non-smokers. Initially, good oral health with no signs of gingivitis, caries or unphysiological salivary flow rate was ensured on the basis of an oral examination by an experienced dentist. In preparation of the *in situ* experiments, individual upper jaw splints were adjusted for all participants. In order to gain *in situ* pellicle samples, cylindrical enamel slabs measuring 5 mm in diameter and 1 mm in height were prepared as test specimens from bovine incisor teeth of 2-year old cattle (Hannig, Basche, et al., 2013; Kensche, Basche, Bowen, Hannig, & Hannig, 2013; Kensche et al., 2016). The surfaces were wet-ground and polished in a standardized grinding procedure with up to 4000 grid abrasive paper and the resulting smear layer was removed by steam jet and ultrasonication (US) with 3% NaOCl for 3 min. Afterwards, all slabs were washed twice for 5 min in distilled water activated by US, followed by disinfection in 70% ethanol for 10 min (US), before finally being washed and stored in distilled water for 24 h.

2.2. Tested preparations and application protocol

In order to enable the exposure of 4 test specimens to the oral environment at a time, cavities were prepared in the buccal aspects of the splints in region of the premolars and the first molar of every quadrant. The samples were placed on each splint with polyvinyl siloxane impression material (Provil novo light regular set, Heraeus Kulzer, Germany) so that only the specimens' surfaces were exposed to the saliva. Before insertion of the splints, participants were instructed to brush their teeth without toothpaste and to rinse thoroughly with tap water. After the splints have been carried intraorally for 1 min to allow initial pellicle formation on the surfaces, rinses with 8 ml of the test preparations described below were carried out for 1 min, followed by continuous intraoral exposure of the slabs for 8 h overnight (23 pm–7 am). In the morning at the lab, the slabs were removed from the splints and rinsed thoroughly with running tap water to remove any non-absorbed salivary remnants (Grychtol et al., 2014; Hannig, Basche, et al., 2013; Kensche et al., 2013). Further processing was performed *in vitro*.

The tested experimental rinsing solution was supplied by Dr. Kurt Wolff, Bielefeld, Germany. It contained only pure hydroxyapatite (HA) microparticles in distilled water (5 g HA dispersed in 100 ml bidestilled water). Referring to dynamic light scattering measurements performed by the company, the median particle size of the crystallites was 130 nm varying from 105 nm to 400 nm. A chlorhexidine-based mouthwash was used as reference (0.2% chlorhexidine-digluconate, meridol med CHX 0.2%, GABA, Lörrach, Germany) and samples carried intraorally without being subjected to any mouthrinse served as negative control. To avoid mutual interferences of the test solution and the control, experiments were carried out on different days.

2.3. Fluorescence microscopy

Fluorescence microscopic detection of bacteria as well as determination of their viability was performed on the basis of 10 randomized microscopic ocular grid fields per sample (Jung et al., 2010; Kensche et al., 2013). The particular size of the ocular grid fields (0.0156 mm^2) allowed calculating the number of cells per square centimeter. All epifluorescent analyses were performed at 1000-fold magnification (Axioskop II, ZEISS, Oberkochen, Germany).

2.4. DAPI staining and glucan visualization

As a standard method of fluorescent microscopic investigation, DAPI (4′,6-diamidino-2-phenylindole) was used to visualize bacteria present in the investigated samples. The dye is taken up into bacteria where it binds to adenine/thymidine-nucleic acids of double-stranded DNA, forming fluorescent units. DAPI is applicable for the detection and quantification of adherent microorganisms, however, without differentiation of viability. Additionally, glucans, as major structure molecules of the extracellular matrix were fluorescence microscopically visualized by Alexa Fluor 574 conjugated Concanavalin A. The staining was conducted as described in earlier studies (Hannig et al., 2007; Jung et al., 2010; Kensche et al., 2013). In brief, the enamel slabs were washed in saline solution and then covered with 1 ml DAPI-Methanol working solution (1 μg/ml) or, as simultaneous staining