Archives of Oral Biology 85 (2018) 10–15

Contents lists available at ScienceDirect

Archives of Oral Biology

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

Research paper

A dual energy micro-CT methodology for visualization and quantification of biofilm formation and dentin demineralization

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ARTICLE INFO

Keywords:
Dental biofilm
Microcosm model
Micro-CT
Dual-energy

ABSTRACT

Objective: The aim of this study was to induce artificial caries in human sound dentin by means of a microcosm model using human saliva as source of bacteria and to apply a novel dual-energy micro-CT technique to quantify biofilm formation and evaluate its demineralization potential.
Design: Eight sound third molars had the occlusal enamel removed by cutting with a diamond disk and five cylindrical cavities (± 2 mm diameter; ± 1.5 mm depth) were prepared over the dentin surface in each specimen (n = 40 cavities). After sterilization, each specimen received the bacterial salivary inoculum obtained from individuals without any systemic diseases presenting dentin caries lesions and were incubated in BHI added of with 5% sucrose for 96 h to allow biofilm formation. After that, two consecutive micro-CT scans were acquired from each specimen (40kv and 70kv). Reconstruction of the images was performed using standardized parameters. After alignment, registration, filtering and image calculations, a final stack of images containing the biofilm volume was obtained from each prepared cavity. Dentin demineralization degree was quantified by comparison with sound dentin areas. All data were analyzed using Shapiro-Wilk test and Spearman correlation using $\alpha = 5\%$.
Results: Dual-energy micro-CT technique disclosed biofilm formation in all cavities. Biofilm volume inside each cavity varied from 0.30 to 1.57 mm^3. A positive correlation between cavity volume and volume of formed biofilm was obtained (0.77, $p < 0.01$). The mineral decrease obtained in dentin was high (± 90%) for all cavities and all demineralized areas showed mineral density values lower than a defined threshold for dentin caries (1.2 g/cm^3).
Conclusion: Dual-energy micro-CT technique was successful in the quantification of a microcosm human bacterial biofilm formation and to quantify its demineralization potential *in vitro*.

1. Introduction

The oral cavity is inhabited by more than seven hundred microbial species (Marsh & Martin, 1999) and dental plaque has nowadays been recognized as a microbial biofilm. Biofilms are specifically defined as a *"matrix-enclosed bacterial population adherent to each other and/or to surfaces or interfaces"* (Costerton, Stewart, & Greenberg, 1999). Many intrinsic and extrinsic factors impact the composition, metabolic activity, and pathogenesis of these highly diversified oral microenvironments (Zaura & ten Cate, 2004). However, organic acid production due to bacterial metabolism on the tooth surface is considered to be the major cause of dental caries, leading to mineral loss and cavitation of the teeth.

Some experimental studies have demonstrated formation of *in vitro* enamel and dentin caries-like lesions using various demineralizing agents or organic acids (Marquezan et al., 2009; Moron et al., 2013; Pacheco et al., 2013; ten Cate, 2015). In general, *in vitro* artificial caries models produced by acid solutions or gels are dealing only with physico-chemical aspects of demineralization, whereas the *in vivo* caries progression is mediated by a metabolic active microbial biofilm. Moreover, demineralization in genuine caries-affected dentin is often manifested as sporadic islands of demineralization, instead of a continuous demineralization gradient from the lesion surface to the inner region, ranging in lesion depth and degree of tubular occlusion (Qi

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http://dx.doi.org/10.1016/j.archoralbio.2017.09.034
Received 27 December 2016; Received in revised form 30 September 2017; Accepted 30 September 2017

et al., 2012). For this reason, a biological *in vitro* model which mimics the oral conditions seems to be more reliable than experiments based on subsurface acid demineralization (Arnold et al., 2001).

Microbiological models of artificial caries formation include single-species or a microcosm approach. In the single-species artificial caries challenge, a pure strain of a known cariogenic bacteria is used to grow into an organized biofilm. In a microcosm model, saliva samples from patients presenting carious lesions are pooled and incubated (Antonio et al., 2011).

Micro-CT is becoming increasingly popular in dental research, as it allows non-destructive morphological, volumetric and mineral density measurements of hard tissues. Dual-energy micro-CT is a variation of the technique capable to detect subtle variation in the attenuation coefficient of one object using two image stacks obtained after data acquisition in two distinct input energies (Hsieh, 2009). In fact, this technique can be especially used to detect low x-ray absorption materials and has been recently applied to detect an "artificial" dental biofilm created with hydrogel around tooth surfaces (Vyas et al., 2014). For this reason, a dual-energy acquisition could be of aid in the characterization of microbial induced caries models, since the volume of produced biofilm after the microbiological assay could possibly be estimated.

Thus, the aim of this study was to apply a dual-energy micro-CT technique to visualize and quantify biofilm formation over sound human dentin submitted to a microcosm model for artificial caries formation using saliva as a source of cariogenic bacteria. Moreover, this biofilm model was also characterized regarding demineralization potential in sound dentin using a micro-CT and digital image processing approach.

2. Materials and methods

2.1. Tooth selection and sample preparation

Eight sound human third molars were obtained from patients living in the city of Rio de Janeiro, Brazil. The experimental protocol of the present study was approved by the Ethical Committee of the host institution (CEP-HUCFF) and was registered at government databases (Plataforma Brazil) under the number 54941416.9.0000.5257. All methods were performed in accordance with the National Commission for Research Ethics (from portuguese, Comissão Nacional de Ética em Pesquisa − CONEP) guidelines and regulations. They were randomly numbered (1 to 8) and stored in a 0.1% thymol solution at 4 °C until used. Complete removal of occlusal enamel and two apical thirds of the tooth root was performed using a diamond disk monted on a cutting machine (Isomet, Buehler, Lake Bluff, IL, USA). Five regular cavities in dentin were prepared with a spherical diamond drill (1013, KG Sorensen) using a high speed dental handpiece without exposing the pulp. In total, 40 cavities were prepared by inserting at least half of the bur diameter (± 1 mm) into the dentin. The tooth fragments were fixed in 24-well polystyrene tissue-culture plates (TPP, Zellkultur Testplatte 24F) with hard wax which was also applied on the outer surface of the teeth to ensure that the formed biofilm would contact only the occlusal surface of the specimen. The plates were then sterilized under UV light for 40 min before receiving the microbial inoculum (Bertolini et al., 2014).

2.2. Biofilm formation by a microcosm model

The inoculum comprised unstimulated whole mixed saliva collected from three volunteers aged 7–13 years (mean 9.6 years) into a graduated collection tube. The subjects were in good general health and not taking any medication. Inclusion criteria included having at least one first permanent molar presenting a dentinal caries lesion. The volunteers and their caregivers gave their informed consent for participation. Subjects were instructed not to consume food or beverages except for water during 1 h before saliva collection, which occurred in the morning. Unstimulated whole saliva produced in the first 30 s was discarded and after that, the volunteers were instructed to spit out every 30 s for exactly 5 min with the aid of a sterile funnel and a graduated tube. The mean dmft, DMFT and mean whole saliva flow rate of each volunteer were registered (4.3, 2.0, and 0.58 mL/min, respectively).

One milliliter of saliva from each volunteer was placed into a tube and mixed using a vortex. Serial dilutions (10^{-1} to 10^{-8}) were performed, and inoculated onto BHI-S agar, Mitis Salivarius agar added sorbitol, kanamycin sulfate and bacitracin (Kimmel & Tinanoff, 1991), Rogosa agar and Chromagar, on short plates in duplicate. Plates were incubated anaerobically for 48 h at 37 °C and then, CFU/mL was counted. From this suspension, 8.1×10^7 CFU/mL of all oral microorganisms were counted. From those, 1.6×10^3 CFU/mL of *Streptococcus mutans*, 4.9×10^5 CFU/mL of *Lactobacilus ssp*. and 3.6×10^2 CFU/mL of *Candida ssp*. were identified. Each well containing one tooth specimen received 20 µL of the inoculum (1.5×10^8/well) and was completed with Brain-Heart infusion growth media added 5% sucrose. The system was incubated in microaerofilia for 7 days at 37 °C to allow biofilm growth. Every 24 h, growth media with sucrose in each well was renewed (1000 µL/well) by aspiration with a pipette and the pH of the system was around 4. All procedures were performed inside a laminar air-flow chamber, under aseptic environment. Fig. 1 illustrates a baseline specimen, the same specimen after biofilm formation and after removing the microbial accumulation.

2.3. Micro-CT acquisition and reconstruction

After the period of biofilm formation, BHI culture medium was removed from each well with a pipette and the specimens were scanned in a micro-CT (Skyscan 1173, Bruker, Kontich, Belgium). The first

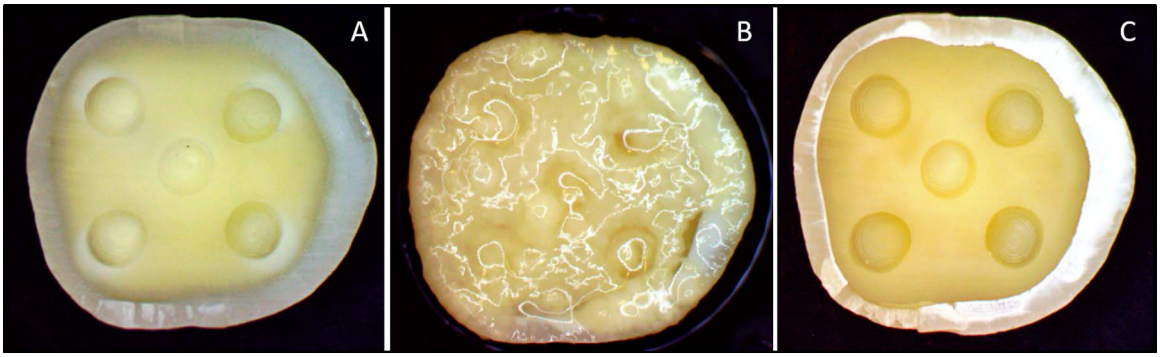

Fig. 1. Sample 5. A) Baseline tooth specimen showing sound dentin with prepared cavities. B) Same specimen after biofilm formation during 96 h. C) Same specimen after cleaning of microbial accumulation, showing artificial caries formation. Dentinal lesion is evidenced by the darker yellow color of dentin, compared to the sound specimen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)