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Colonization of *Enterococcus faecalis* in a new SiO/SiO_2-microtube *in vitro* model system as a function of tubule diameter

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ABSTRACT

Objectives. Endodontic pathogens can penetrate deep into dentinal tubules and therefore survive the chemo-mechanical disinfection procedures. Bacterial penetration has been mainly studies using sliced infected human teeth which, besides creating artifacts, can hinder the observation of the inner tubules due to the dense and opaque dentin structure.

The aim of the present study was to develop a standardized dentin model by using artificial SiO/SiO_2-microtubes of different diameters and lengths to test the penetration ability of *Enterococcus faecalis*.

Methods. E. faecalis was grown in Schaedler fluid media for 24 h and thereafter cell density was settled to 10^3 cells/ml by addition of fresh media. The bacterial solution was then incubated for 2, 3, 5 and 10 days with the SiO/SiO_2-microtubes of different diameters (2–5.5 μm) and lengths (100–500 μm). The colonization of the tubes was evaluated by phase-contrast microscopy and the amount of colonization was determined by using a colonization index (CI; 0-none, 1-mild, 2-moderate, 3-heavy).

Results. The diameter of the tubes strongly influences the microbial colonization. After 2 days of cultivation the 5.5 μm tubes showed a moderate to heavy colonization (CI 2–3). In comparison, the 2 and 3 μm tubes were clearly less colonized at the same point in time. In detail: at day 3, only mild to moderate bacteria colonization (CI 1–2) were found in the 3 μm tubes and at day 10 penetration of the 2 μm tubes just started. The colonization of the 5.5 μm tubes was also influenced by their length. In case of the longer microtubes, though, a smaller share of heavily colonized tubes was observed.

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Significance. Our results show that *E. faecalis* was able to penetrate and reproduce within the standardized SiO/SiO_2-microtubes in a short time. To examine the mechanisms of bacterial adhesion and invasion into tubular structures the 2 μm tubes could serve as a model system because the diameters are similar to those of dentinal tubules.

1. Introduction

After an endodontic infection the removal of the necrotic and gangrenous pulp tissue as well as the eradication of the invading microbial species has a major influence on the long term treatment success. Basically this is accomplished by a meticulous chemo-mechanical cleaning of the root canal system [1–4]. Nevertheless, microbes hidden in dentinal tubules or in lateral canals can survive the disinfection procedure which often leads to reinfection [5–8].

In particular infections with the Gram-positive bacterial species *Enterococcus faecalis* are sometimes difficult to control. As it is shown, the prevalence of *E. faecalis* in persistent cases ranges between 22 and 70% [9–11]. The species is able to survive medical treatments with calcium hydroxide [12] and the application of clindamycine, tetracycline and Lugol's solution [13,14].

Furthermore, the species is able to penetrate directly into the dentinal tubules which have a very tight lumen that are 1–2 μm wide and up to 5 mm long [15–17]. *E. faecalis* can be sheltered within the dentinal tubules and eventually survive the disinfection procedure. Remaining bacteria are then able to recolonize the entire root canal system which leads to persistent infections [18–22].

Up to now, studies focusing on bacterial invasion of dentinal tubules commonly use infected and afterwards fractured or sliced human teeth [15–17,23,24]. In those *in vitro* studies it was found that colonization of the dentinal tubes by *E. faecalis* ranged between 10 and 200 μm after incubation for several weeks [23,24].

One of the major problems examiners often experience is the changing anatomical morphology of the tubules among the specimens. This implicates a non-standardized approach. Moreover, the forces applied during the breaking process often lead to stress-induced irregularities that have a negative impact on the overall results. If such a method is used, the fracture often occurs by incident, too. Because the fractures are different among the specimen, the recorded data often lack reproducibility. Likewise, the use of light microscopy is rather limited due to the dense dental material which only allows optical access to those tubules that are located at the very surface.

In this context, rolled-up nanotech [25–27] conveniently provides strain-engineered transparent microtubes with tunable length and diameter on substrate surfaces [28]. Previous work implies that rolled-up microtubes are biocompatible and useful for growth analysis and viability studies of complex eukaryotic and yeast cells in 3D confined space [25,29–32]. It can, therefore, be expected that the 3-dimensional architecture of the microtubes has a major influence on the migration and colonization of other living cells, too.

From the dental aspect, the question is whether it is possible to develop an *in vitro* model on the basis of rolled-up microtubes that can possibly be used in future to simulate the bacterial penetration and colonization of the dentins. For this reason, it was the aim of the present study to first develop a new *in vitro* model system for bacterial penetration of *E. faecalis* using transparent SiO/SiO_2 microtubes.

2. Materials and methods

2.1. Cultivation of the model organism

In the present *in vitro* study, we investigated the colonization of SiO/SiO_2-microtubes with facultatively anaerobic, Gram-positive bacterium *E. faecalis* (DSMZ 20376). Cell growth was realized in 10 ml of Schaedler culture medium (Oxoid Ltd., Hampshire, UK) under standard anaerobic conditions for 24 h. The optical density (OD_{546nm}) of bacterial suspension was set to a level of 0.1 (approx. 10^3 cells/ml) for incubation of the tubes.

2.2. Preparation of SiO/SiO_2-microtubes

Transparent silicon oxide rolled-up microtube arrays were fabricated on Si substrates by the deposition of a pre-stressed oxide layer on a patterned photoresist sacrificial layer and the subsequent removal of this sacrificial layer as reported previously [25,28,29]. Their dimensions (*e.g.* length, diameter) are controlled by tunable parameters of the fabrication process. The microtubes within the array have the same diameter but vary in length (Fig. 1). For analysis, we used microtubes that are 2 μm, 3 μm and 5.5 μm in diameter with lengths of 100 μm, 200 μm, 300 μm, 400 μm and 500 μm. The total number of microtubes was 3150, so that 210 tubes were studied in each group.

Chips with microtubes were glued on slides with transparent UV curing adhesive (Conloc® UV 685). Glue polymerization and sample sterilization were achieved simultaneously by irradiation with an UV lamp (Superlite, M+W Dental, wavelength 254 nm) for 15 min (Fig. 2a).

2.3. Investigation of bacterial invasion

Silicone cell culture chambers were applied to these slides and 3 ml of bacterial suspension prepared in advance was added (Fig. 2b). To eliminate remaining air within the microtubes, cell culture chambers were covered with Parafilm® M and evacuated by a vacuum pump (Fig. 2c). Subsequently, the bacterial