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ORIGINAL ARTICLE

An *in vitro* microbial-based model for studying caries-preventive agents

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Abstract

Objective. The aim of the study was to develop an *in vitro* microbial-based caries model to test potentially caries-preventive agents. ***Material and methods***. In this model, cariogenic *Streptococcus mutans* biofilms are grown on tooth samples within a reaction chamber hermetically surrounded by a bacteria-tight glove box allowing the manipulation of specimens during operation. The specimens were mounted in two rows on the inner and outer rims of a specimen turntable passing several inlet pipes transporting all necessary media. Using 64 lower incisors in 4 experiments, a 10 ppm NaF solution and an experimental potentially caries-preventive glycan solution were tested compared to a water control. The mean lesion depth was determined by confocal laser scanning microscopy. ***Results***. The depths of the caries-like lesions showed no statistically significant difference irrespective of whether the specimens were mounted on the inner or outer rim of the specimen turntable. As expected, the NaF solution inhibited the development of caries-like lesions almost completely. The experimental glycan solution revealed a statistically significantly lower demineralization depth compared to the control and a significantly higher depth compared to the NaF group. The system could be operated over a period of more than 9 weeks without unintentional contamination and the manipulation of the tooth specimens could be accomplished. ***Conclusions***. In conclusion, our *in vitro* system is suitable for testing potential caries-preventive agents in a reproducible way by using whole tooth samples and offers full access together with the possibility of manipulating the specimens during operation.

Key Words: *Biofilm, dental caries model, fluoride, glycan, Streptococcus mutans*

Introduction

In vitro models are suitable for testing the potential caries-preventive effect of new agents because they provide good control of experimental variables and they avoid the ethical conflicts of *in situ* experiments, animal or extensive clinical studies. Very simple and cost-effective chemical *in vitro* models allow the production of caries-like lesions, but their applicability is limited to factors which directly influence the de- and remineralization process [1–5].

Biofilm models have been developed to study the influence of potentially caries-preventive agents on pathogenic bacteria because it has been demonstrated that bacteria in biofilms are more resistant to antimicrobials than bacteria in suspension [6]. In order to be able to study the process of de- and remineralization, antimicrobial effects as well as possible interactions [7] simultaneously, artificial mouth models have been established (for review see [8]). Suitable models should include the development of (i) a dental biofilm and (ii) caries-like lesions in dental hard tissues as a consequence of a de- and remineralization process. *Streptococcus mutans*, which it is accepted plays a major role in dental caries, is widely used in these models [9–15]. We recently introduced a microbial-based caries model using whole tooth samples rather than small tooth segments, making it suitable to produce primary and secondary caries-like lesions around restorations and to investigate the caries-preventive effect of different filling materials and techniques [16,17]. So far, the model is not applicable for testing the caries-preventive effect of different treatment regimes and substances.

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The purpose of the present study was therefore to modify our *in vitro* microbial-based caries model to test potentially caries-preventive agents. The advanced model had to fulfil the following requirements: (i) operation with whole tooth samples including two treatment groups per turn (test and control), (ii) operation for 9 weeks without unintentional infection, and (iii) the possibility to manipulate the specimens during operation to prove its stability for complex experimental setups.

Material and methods

Principle of the microbial-based in vitro caries model

The principle of the bacteria-based *in vitro* caries model used in this study has been described in detail elsewhere [16]. Briefly, it consists of a custom-made reaction chamber (transparent poly methyl methacrylate box; L × H × W; 25 cm × 25 cm × 15 cm) hermetically surrounded by a bacteria-tight glove box (Model 90; Metall und Plastik, Randolfzell, Germany) that allows specimens to be manipulated and exchanged without necessarily terminating the experiment. Within the reaction chamber, a *S. mutans* (ATCC 25175) biofilm is cultivated on tooth specimens mounted on a turntable (poly-oxy-methylene, Ø 20 cm) rotated by a computer-controlled step motor (RS 440-436; RS Components, Mörfelden Walldorf, Germany). The specimens periodically pass several inlet pipes that provide them with all necessary media for growth of the *S. mutans* biofilm. For the development of caries-like lesions, the specimens receive an hourly repeating treatment regime of trypticase soy broth (TSB) (Becton Dickinson, Sparks, USA) artificial saliva [18], and a 10% sucrose solution.

The temperature in the reaction chamber is adjusted to 37°C by water bath heating. In order to use the system for the comparable testing of different potentially caries-preventive regimes or agents, the turntable carries two rows of specimens and additional inlet pipes to provide different media independently (Figure 1).

Study design

Two different potentially caries-preventive agents were tested in four experiments for their ability to inhibit the development of caries-like lesions compared to a water control (Table I). These agents were (i) a 10 ppm NaF solution and (ii) an experimental glycan solution prepared by hydrolysis of commercially available porcine gastric mucine (saliva medac, Hamburg, Germany) which had shown a caries-preventive effect in rat experiments [19].

We used 64 lower human incisors that had been carefully examined for the absence of enamel developmental defects or irregularities. After removal of the roots, a stainless steel pin was fixed with composite resin into the pulp chamber. The diameter of the pin corresponded to drill-holes on the specimen turntable. A custom-made positioning device ensured that the labial surface of the teeth was positioned at an angle of 25° to the horizontal. Since all fluids were applied by dripping, the gentle slope that had been empirically determined in previous experiments ensures a flow of fluid on the specimen surface and better reproducibility of biofilm development.

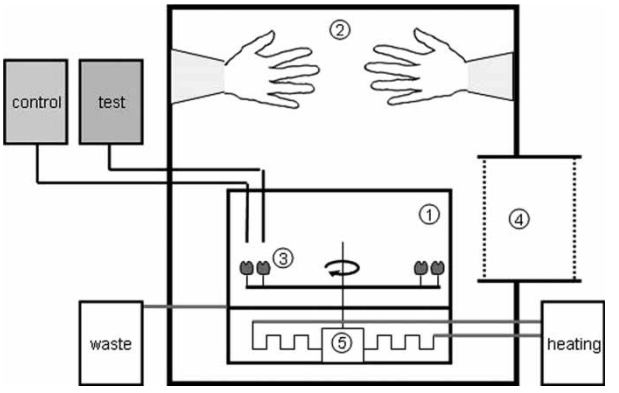

Figure 1. *In vitro* bacteria-based caries model set-up: (1) reaction chamber, (2) sterile environment within glove box, (3) two rows of specimens on rotating turntable passing different media inlet pipes, (4) air lock for transfer of all material, and (5) step motor.

Before entering the artificial mouth, all specimens were transferred to a Tupperware box equipped with moistened plastic foam. The box was wrapped in a paper/plastic peel pouch and sterilized using ethylene oxide gas sterilization [16].

Before each of the four experiments (Table I), the reaction chamber was aseptically loaded with new specimens within the protected environment of the glove box. All experiments (Table I) were carried out using the same treatment parameters. After final setup and installation of the specimens, the angular velocity of the specimen turntable was adjusted to 24°/s and the fluid flow for each medium (see below) was adjusted so that continuous dripping could be observed at the tip of the inlet pipes (0.4 ml/min). After 2 days of operation without *S. mutans* infection, in order to check all physical parameters of the system, the specimens were inoculated on the morning of day 3 by dripping a *S. mutans* suspension through separate inlet pipes (0.4 ml/min, approximately 1 ml/tooth). To prepare the inoculum, *S. mutans* was first grown on blood agar plates (Sifin GmbH, Berlin, Germany) for 3 days. Single colonies were transferred to 30-ml of trypticase soy broth (TSB) and were incubated anaerobically at 37°C overnight. During the inoculation procedure, all other media were stopped. Over a period of 14 days the specimens received a continuously repeating treatment regime of TSB solution for 5 min,

Table I. Set of experiments, each experiment lasted for 16 days

	Experiment I	Experiment II	Experiment III	Experiment IV
Day	1–16	17–32	33–48	49–64
Inner circle of turntable	NaF[1] ($n=8$)	Control ($n=8$)	Glycan ($n=8$)	Control ($n=8$)
Outer circle of turntable	Control ($n=8$)	NaF[1] ($n=8$)	Control ($n=8$)	Glycan ($n=8$)

[1]NaF = Sodium fluoride.

followed by 15 min of artificial saliva. A 10% sucrose solution was applied for 5 min every hour instead of the TSB solution. The test and the control solutions were applied parallel with the application of the sucrose solution.

During the experiment, samples of waste fluid were taken each week to control the mono-inoculation with *S. mutans* of the system. The samples were plated on Columbia blood agar (Sifin GmbH, Berlin, Germany) and Mitis salivarius bacitracin agar (20% sucrose and 200 IU/l bacitracin, BD Difco) and were incubated anaerobically at 37°C for 3 days.

At the end of each experiment, the teeth were taken from the reaction chamber and new specimens were installed. The plaque was removed with a soft bristled toothbrush and buffered saline. After cleaning, the specimens were stored in sterile buffered saline until caries examination.

Caries evaluation

The caries evaluation procedure used in this study has been described in detail elsewhere [16,20]. Briefly, all teeth were embedded in PMMA resin (PalaXpress, clear; Heraeus Kulzer, Wehrheim, Germany). After setting, the specimens were cut in half along the tooth axis in an oro-labial direction using a 100-µm-thick diamond saw (Zeitz 1600; Ernst Zeitz, Wetzlar, Germany) and were polished with silicon carbide paper (4000 grit). Both halves were analysed using a confocal laser scanning microscope (Zeiss LSM 510; Carl Zeiss, Jena, Germany) to measure the auto fluorescence of the demineralization. Using the measuring tool of the Zeiss LSM 510 image browser software (Carl Zeiss, Jena, Germany), the depth of the demineralization was determined at 10 sites in 0.5 mm increments along the advancing front of the lesion starting at the incisal edge.

Statistics

The normal distribution was confirmed with the Kolmogorov-Smirnov test. The mean value and the standard deviation for each parameter were calculated; ANOVA was used for the statistical analyses. The level of statistical significance was set at $\alpha = 0.05$.

Results

The weekly bacteria control showed that manipulation of the reaction chamber could be accomplished without unintentional contamination. The depths of the caries-like lesions showed no statistically significant difference between the control groups irrespective of whether the specimens were mounted on the inner or outer rim of the specimen turntable (Table II). Also within the test groups, no significant difference could be found with regard to the position on the turntable.

Evaluation of the different experimental conditions confirmed that the NaF solution inhibited the development of caries-like lesions almost completely, showing a mean relative decrease in lesion depth of 91.8% compared to control. The experimental glycan solution revealed a statistically significantly lower demineralization depth compared to the control and a significantly higher depth compared to the NaF group (Table II). The mean relative decrease in lesion depth compared to the control was 43.5%.

Discussion

Compared to *in vivo* models, a major advantage of *in vitro* bacteria-induced caries models is the control of most experimental variables. A variety of so-called artificial mouth models has been developed using either a culture bath or a dripping technique to provide the bacteria with all necessary media [11–14,16,20]. The dripping technique offers the advantages that (i) the air-biofilm surface of the mouth is realized in the reaction chamber, (ii) the individual teeth receive a separate local environment, and (iii) the requirement of the media is smaller in relation to the number of specimens and results in a more realistic volume to sample ratio.

Table II. Mean lesion depth (µm) ± standard deviation

Experiment	NaF[1]	Glycan	Control
I	5 (±11)	–	82 (±19)
II	9 (±10)	–	94 (±21)
III	–	44±12	75 (±15)
IV	–	52 (±16)	89 (±19)
All	7[b](±10)	48[ac] (±13)	85[ab] (±19)

[1]NaF = Sodium fluoride.
[a]Significantly different ($p < 0.05$) to NaF group.
[b]Significantly different ($p < 0.05$) to glycan group.
[c]Significantly different ($p < 0.05$) to control group.

The dripping technique requires the installation of pipes which provide the fluids for the surface of the specimens. To avoid the necessity of one pipe for each fixed specimen [12], the use of specimen turntables has been described in which all specimens pass the same inlet pipes [11,13,16]. The evaluation of different caries modulating agents requires avoidance of cross-contamination of the teeth. Deng et al. [15] described a modification of a model (constant depth film fermentor) with a turntable carrying out 180° instead of 360° movements. This modification separates the turntable into two segments which can then be provided with different media [13,15]. Our treatment groups are placed on the inner or outer rim of the turntable providing each circle with corresponding inlet pipes. In this experimental setting the specimens on the inner circle pass the dripping inlet pipes slightly slower than specimens on the outer circle. The results indicate that this situation does not result in significant differences in the depth of caries-like lesions between the groups.

Most artificial mouth models use flattened and ground tooth sections to minimize the caries-inhibiting effect of surface irregularities and fluoride-rich layers. The present study was designed as a pilot study for further experiments investigating combinations of different treatment regimes and filling materials and therefore required the use of whole tooth samples rather than tooth segments. This might explain the degree of variability in terms of the lesion depth. However, our data revealed no significant difference in the lesion depth between the control groups of repeated experiments. The lower lesion depth in the NaF and the Glycan group reveals that the *in vitro* model is suitable for simultaneous testing of potentially caries-preventive agents.

In contrast to other artificial mouth models [8,13,20], our system offers the possibility to considerably manipulate test specimens under gnotobiotic conditions. Loading and unloading of the reaction chamber as well as manipulations of the specimens during operation could easily be performed by integrating the reaction chamber with a sterilized glove box. This reliably prevented the system from being inadvertently contaminated over a time period of 9 weeks.

Conclusions

In conclusion, our *in vitro* system is suitable for testing potentially caries-preventive agents in a reproducible way by using whole tooth samples, and offers full access along with the possibility of manipulating the specimens during operation.

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