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A simplified *in vitro* model for investigation of the antimicrobial efficacy of various antiseptic agents to prevent peri-implantitis

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ORIGINAL ARTICLE



ABSTRACT

The biofilm formation by oral bacteria on the implant surface is one of the most remarkable factors of peri-implant infections, which may eventually lead to bone resorption and loss of the dental implant. Therefore, the elimination of biofilm is an essential step for the successful therapy of implant-related infections. In this work we created a basic *in vitro* model to evaluate the antibacterial effect of three widely used antiseptics.

Commercially pure (CP4) titanium sample discs with sand blasted, acid etched, and polished surface were used. The discs were incubated with mono-cultures of *Streptococcus mitis* and *Streptococcus salivarius*. The adhered bacterial biofilms were treated with different antiseptics: chlorhexidine-digluconate (CHX), povidone-iodine (PI), and chlorine dioxide (CD) for 5 min and the control discs with ultrapure water. The antibacterial effect of the antiseptics was tested by colorimetric assay.

According to the results, the PI and the CD were statistically the most effective in the elimination of the two test bacteria on both titanium surfaces after 5 min treatment time. The CD showed significant effect only against *S. salivarius*.

Based on our results we conclude that PI and CD may be promising antibacterial agents to disinfecting the peri-implant site in the dental practice.

KEYWORDS

titanium, antimicrobial agents, peri-implantitis, *Streptococcus mitis*, *Streptococcus salivarius*

INTRODUCTION

Implantation is one of the most widely applied treatment options for tooth replacement. Titanium (Ti) and Ti alloys are the most common choices for dental implant materials because these materials are well tolerated by human tissues and they integrate easily with the bone to allow successful osseointegration [1]. Good clinical implantation intervention depends on various factors. Bacterial colonization, biofilm formation, and consequent peri-implantitis play major roles among the complications [2].

The oral microbiota is a diverse community, consisting of over 700 different bacterial and fungal species, which can form biofilm on soft and hard surfaces, including the implant surfaces [3]. Formation of a microbial biofilm is a complex and multi-step process. Most of the pioneer colonizers, belongs to *Streptococcus* genus and plays key role in the formation of multilayered dental plaque in oral cavity [4]. The formation and maturation of bacterial biofilm on the surfaces of dental implant have been associated with the etiology of peri-

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implant mucositis and peri-implantitis. Peri-implantitis is one of the major causes of unsuccessful implantation, since the periodontopathogenic bacteria can penetrate from the colonized neck part of an implant along the implant body in the gingiva and deeper hard tissue region, triggering inflammation around osseointegrated implants which may result in the loss of bone around an implant [5, 6].

Esposito et al. [7], published in their earlier study that 8–50% of implant removal processes are due to peri-implantitis. Because of the growing number of concerned patients every year, dentists need to address the proper treatment of peri-implant infections.

Currently, the treatment of peri-implantitis is done by mechanical debridement with or without adjunctive antiseptic agents. The most suitable chemical agent for disinfecting the peri-implant region has not yet been found because of the lack of comprehensive *in vitro* and *in vivo* experiments [8]. Several disinfectants have been tested with varying success.

In our earlier study we investigated the cytotoxic effect of three different disinfectant solutions (3% H₂O₂ solution, saturated citric acid (pH = 1) and CHX gel) on human epithelial cells attached to Ti surfaces [9]. Connecting to our previous study in this current work we done a basic research to observe the antimicrobial effects of decontaminant agents e.g., chlorhexidine-digluconate (CHX), chlorine dioxide (CD), and povidone-iodine (PI).

CHX has been the most frequently used agent in the adjuvant treatment of peri-implantitis for years as it has a broad-spectrum antimicrobial effect, both bactericidal and bacteriostatic, depending on the applied concentration [10]. CHX can also penetrate into the biofilm to damage the incorporated bacteria [11].

CD is used in various fields due to the excellent bactericidal and antiviral properties [12]. It can also diffuse easily into the biofilm and destroys the microbes forming the film [13].

PI has been used as a topical antiseptic in oral surgery and periodontal practice. PI has a wide spectrum of antibacterial and antiviral effects. The information regarding its effect on biofilms is limited [14]. Furthermore, the comparisons of antibacterial effect of CHX, CD, and PI have not yet been investigated.

The oral microbiota is a very complex ecological community that hard to model exactly, since it contains a huge number of culturable and unculturable bacteria which need special demands to their viability and proliferation [15, 16]. Our research group previously used *Streptococcus* strain as a model organism for dental research [17]. Therefore, in this study we also applied a simplified *in vitro* model and chose pioneer colonizers (*Streptococcus mitis*, *Streptococcus salivarius*) to our investigations.

MATERIALS AND METHODS

Preparation of disc surfaces for the experiment

In our experiments we used two different surface modified Ti discs. The discs (1.5 mm thick and 9 mm diameter) were

cut from commercially pure (CP4) Ti rods (Denti System®, Hungary). One type of the discs surfaces were modified by sand blasting, acid etching technique and the surfaces of the other discs were polished by the manufacturer. Before the experiments the samples were cleaned with acetone, then with 70% ethanol for 15 min, and rinsed with ultrapure water three times. Finally, the samples were sterilized at 160 °C for 45 min.

Investigation of the antibacterial activity of the three different antiseptic agents on mono-species biofilms

S. mitis and *S. salivarius* isolates from patients who had clinical symptoms of peri-implantitis were used in our experiments. The isolates were previously identified by matrix assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany).

After the isolation and identification, the bacteria were stored at –80 °C in Brain Heart Infusion (BHI) broth (Oxoid, UK) supplemented with 12% (v/v) glycerol. The strains were incubated at 37 °C for 24 h in 5% CO₂ atmosphere on blood agar plate containing 5% cattle blood (BioMérieux, S. A. Marcy l'Etoile, France) for experiments.

The mono-bacterial suspension was prepared in 1% glucose bouillon from the overnight blood agar plate of each strain. After incubation at 37 °C for 3 h under aerobic atmosphere, enriched with 5% CO₂, the optical density (OD) of the cultures reached the 0.5 McFarland density. The bacterial suspension was pipetted on the surfaces of discs in 24-well hydrophilic surface plate (Tissue Culture 24 well plate, Sarstedt, Nümbrecht, Germany).

We used different incubation time for the two test bacteria since our goal was to investigate the response of pioneer colonizer streptococci to antiseptic treatment in distinct laboratory conditions and create basic *in vitro* models for our further investigations. Therefore, we used a shorter incubation time for *S. mitis* (4.5 h) similarly to our previous experiment [17] and other researchers work [18]. However, we extended the incubation time to 48 h in case of *S. salivarius* where after 24 h we changed the culture medium for fresh 1% glucose bouillon. This was performed based on Rath et al. *in vitro* biofilm model who established in their work that already 24 h cultivation time of *S. salivarius* is enough for biofilm formation on titanium implant [19].

After incubation at 37 °C in CO₂ the developed biofilms were washed with 1× phosphate-buffered saline (PBS) (1×, pH: 7.2) to remove the less adherent cells. Then the attached bacterial cells were treated with 2 ml of three different oral antiseptics: CHX (Curasept ADS 220, 0.2%, Switzerland), PI (Betadine, 10%, Switzerland), and CD (Solumium dental, 0.12%, Hungary) for 5 min, since it can be optimal in the usual dental practice according our previous results [9] and other researchers [20]. The antiseptics were washed out from the implant surfaces by rinsed them with 1 ml 1× PBS.

In order to follow the metabolic activity of the bacterial biofilm 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium



bromide (MTT) (Sigma–Aldrich, Darmstadt, Germany) assay was used. This method was previously tested on epithelial cell culture by our research group [11]. After the antiseptic treatment and washing steps 50 μ L MTT solution (1 mg/ml final concentration) was added to 0.5 ml 1 \times PBS on the samples and incubated at 37 °C for 4 h. Then the solution was removed from each well and the remaining formazan crystals, which indicates the level of cell metabolic activity, were solubilized with 200 μ L of 0.04 mM HCl (Scharlab, Spain) in absolute isopropanol (Molar Chemicals, Hungary) and with 40 μ L of 10% sodium dodecyl sulfate (Sigma–Aldrich GmbH, Germany). The OD of solubilized formazan crystals were measured at 550 nm with an ELISA reader (Anthos Labtech Elisa Reader, Hungary).

The antibacterial effect of the antiseptics was compared with untreated control Ti discs with the developed biofilm which were only rinsed with sterile 1 \times PBS.

Statistical analysis

Statistical analysis was carried out using Statistica 13 (Dell Inc. USA). After test of normality (Shapiro–Wilk test) the comparisons within group were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test and T-test was used for comparison of independent samples. The means \pm SEM (standard error of the mean) were calculated for OD_{550nm} values measured by plate reader based on five parallel experiments (three measures in each group) carried out in different time points. Statistical significance was set at $P < 0.05$.

RESULTS

Disinfectant efficacy of oral antiseptics on *S. mitis* biofilm determined by MTT colorimetric assay

The results of antimicrobial activity of antiseptics against *S. mitis* are shown in Fig. 1. Evaluation of the three disinfectants antibacterial activity against the pioneer colonizer indicated that among antiseptics the PI and the CD showed significant difference both on the polished (ANOVA $P = 0.0005$) and the sand blasted, acid etched (ANOVA $P = 0.0004$) Ti

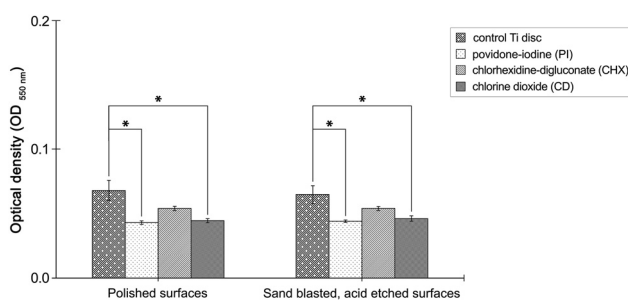


Figure 1. Determination of the antibacterial efficiency of different antiseptic agents by MTT colorimetric assay for *S. mitis*. The values were calculated from five independent experiments and are shown as mean \pm SEM. Asterisks denote significant differences ($*P < 0.05$)

surfaces compared with the untreated control Ti discs after the 5 min treatment time. We presented the measured OD values on Fig. 1 however, we converted these data to percent values and mentioned them in this way in the text (data are not shown here). The attachment to the control Ti surface was considered 100% (highest OD value) and the number of metabolically active cells on the surfaces was expressed in relative percentages in the results section.

Based on our MTT results all antiseptic decreased the cell metabolic activity in biofilm on sand blasted, acid etched and polished surfaces. However, the PI and CD showed significant cell reduction on both surfaces ($P < 0.05$).

The PI was the most effective antiseptic against the *S. mitis* cells incubated for 4.5 h, since it decreased the number of active cells with 37% (OD₅₅₀ = 0.043 \pm 0.001) on polished surface compared with the control disc (OD₅₅₀ = 0.068 \pm 0.008) after 5 min treatment time ($P = 0.0012$). We observed completely similar tendency with regard the sand blasted, acid etched surfaces. The decrease of the metabolically active cells was 33% (OD₅₅₀ = 0.044 \pm 0.001) after rinsing with PI compared with the untreated control Ti discs (OD₅₅₀ = 0.065 \pm 0.007) ($P = 0.0007$).

Disinfectant efficacy of oral antiseptics on *S. salivarius* biofilm determined by colorimetric MTT assay

The disinfectants dissolving effects on *S. salivarius* biofilm developed for a prolonged incubation time (48 h) are detailed in Fig. 2. According to our results all tested agents significantly decreased the amount of metabolically active cells in *S. salivarius* biofilm on polished surfaces compared with the untreated Ti surfaces *in vitro* (ANOVA $P < 0.0001$). The most pronounced antibacterial activity was attributed to PI, which is eliminated 65% (OD = 0.048 \pm 0.003) of biofilm forming cells on polished surface after 5 min treatment time ($P = 0.0002$). However, the CD also eliminated a remarkable percent of the biofilm (60%) (OD = 0.056 \pm 0.001) compared with the control polished discs (OD = 0.139 \pm 0.01, $P = 0.0002$). Considering the three agents significant differences could be observed between the PI and CHX ($P = 0.0002$) and in this respect between the CD and CHX ($P = 0.0006$) as well.

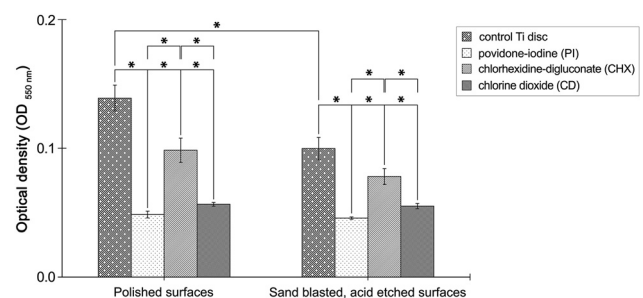


Figure 2. Effect of antiseptics on the *S. salivarius* cells using MTT colorimetric assay. The values were calculated from five independent experiments and are shown as mean \pm SEM. Asterisks denote significant differences ($*P < 0.05$)

On the sand blasted, acid etched surface the metabolic activity of *S. salivarius* cells decreased in the biofilm when treated with all three antiseptics compared with the control discs (ANOVA $P < 0.0001$). Furthermore, the PI and CD showed significantly higher antibacterial activity against *S. salivarius* compared with the CHX treatment (PI vs. CHX $P = 0.0007$, CD vs. CHX $P = 0.0212$).

Besides these results the quantitative evaluation of bacterial adhesion to different control Ti surfaces revealed that the density of metabolically active cell was significantly lower on the polished surface than on the sand blasted, acid etched groups, respectively ($P = 0.0063$). We did not find such difference at *S. mitis* between non treated control polished and sand blasted acid etched surfaces (Fig. 1).

DISCUSSION

In dentistry the dental implants are widely used for tooth replacement however, the implant surfaces similarly to the tooth surfaces provides possibility for attachment of bacteria and for formation of a complex biofilm which can cause inflammation around peri-implant tissues and influence the long-term success of osseointegration of implants [21].

Generally in dentistry a smooth Ti surface is developed to provide epithelial attachment and prevent plaque formation. The rough part of Ti implant responsible for proper connective tissue attachment, and grant the anchorage of implant in the bone. The peri-implant inflammation caused by pathogens can spread apically from the colonized neck part of Ti implant toward the deeper bone tissue region therefore, we used two different surface roughness of dental implant (polished and sand blasted, acid etched) to model this complex process on two test bacteria (*S. mitis*, *S. salivarius*) in our *in vitro* model [22].

According to McDonnell and Russell [10] CHX is one of the most well-known and widely used antiseptic agent in dentistry, but based on our results it is showed significant cell reduction only in case of *S. salivarius* after 5 min exposure time. The CHX reduced 29% of metabolically active cells while the other two agents destroyed double amount of proliferating *S. salivarius*.

Based on MTT data the PI and CD were the two most effective antiseptic agents against *S. mitis* and *S. salivarius*. Moreover, both agents are proved to be significantly better compared with CHX in the elimination of the *S. salivarius* biofilm developed for 48 h. Our results are in concordance with Herczegh et al. [23] who established in their work that the CD was more effective compared with CHX after 5 min treatment time.

Our model demonstrated that the PI was the most effective in the *in vitro* elimination of both bacterial biofilms. However, in case of *S. mitis* we could not observe significant difference between PI and CD. Hosaka et al. [24] reported that even 0.5 min application of PI was effective in the *in vitro* killing both of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* and according to other studies the CD

was also effective in reducing the plaque of *F. nucleatum*. [25] Currently there are no data in the literature about the comparison of the effectiveness of PI and CD in the same *in vitro* experimental setting.

Besides these three antiseptics there are several other agents to control biofilm associated peri-implantitis. [10] However, choosing an ideal antiseptic to the therapy of peri-implantitis is influenced not only by effectiveness but also by the lack of side effects of agent. CHX is the most widely used gold standard antiseptic for plaque control [26] but CHX have some adverse properties. It stains teeth or it can cause oral mucosa desquamation or the burning sensation of the oral mucosa [27, 28]. However, the anti discoloration system (ADS) presented in Curasept used by our experiment can reduce the risk of discoloration and eliminates the unpleasant taste-disturbance [29]. PI has also adverse effects including allergy or hypersensitivity to the solution, and it can cause a reversible yellowish discoloration as well, however the short durations of using PI in low concentration could decrease these effects [30, 31]. In our experiment we used a short 5 min exposure time, furthermore, Kanagalingam et al. reported in their review that there have been no clinical reports on development of microbial resistance after the PI treatment while, in contrast bacterial resistance to CHX has been observed [32]. Based on our MTT assay data, we determined that PI could be a promising oral antiseptic in the prevention of peri-implantitis.

According to other researchers [33] CD can react with four amino acids (cysteine, methionine, tyrosine, tryptophan) which have vital role in living organism and microbes can not develop resistance against CD, it can not cause real harm to humans as it is not able to penetrate into the deep tissues [33]. CD is effective in relatively low concentrations and the new membrane technology developed by Noszticzsiusz et al. [33] allows the production of high purity CD solution without any by-products. These properties could make it an ideal antiseptic beside PI in dental practice to treat inflammation caused by microorganisms.

CONCLUSION

In our study we compared the antimicrobial effect of three antiseptics (CHX, PI, CD) on *S. mitis* and *S. salivarius* in mono-species biofilm models adhering to Ti surfaces using MTT colorimetric assay. The antimicrobial property of all tested disinfectants have been known, however there are limited information in the literature about their comparison.

Our data indicated, that PI and CD had remarkable eliminating property against *S. mitis* and *S. salivarius* cells in biofilm after 5 min of treatment time. Considering the conflicts in available literature regarding the cytotoxic properties of agents we established that the PI and CD means advantageous disinfectants, since they are effective *in vitro* against the biofilms of the pioneer colonizers under aerobic conditions.



In the light of our results we plan the further optimization of our *in vitro* model by using other pathogenic anaerobic bacteria or pathogenic fungal species and changing the treatment time or concentration of antiseptics for their most effective clinical application.

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