The Role of Chlorhexidine on Endotoxin Penetration to the Implant-Abutment Interface (IAI)

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ABSTRACT

Purpose: The aim of this study is to assess the risk of endotoxin penetration to the implant-abutment interface (IAI) of implants with Morse-taper connection and the effect of chlorhexidine in the prevention of such penetration.

Materials and Methods: Thirty implants with Morse-taper connection were divided into three groups ($n = 10$/group) based on type of inoculation of the internal aspect of the implant. Implants in Group 1 were inoculated with 1 µl *Escherichia coli* for 24 hours; supernatant was removed and 0.5 µl of sterile saline was added. Implants in Group 2 were inoculated with 1 µl *E. coli* for 24 hours; supernatant was removed and 0.5 µl 0.2% chlorhexidine solution was added. Implants in Group 3 were inoculated with 0.5 µl of sterile saline and served as controls. Following inoculation procedures, implants were connected to standard abutments, immersed in sterile culture media, and loaded with 200,000 cycles of 160 N in a wear simulator. Samples were collected from the supernatant solution of each implant for endotoxin identification at the beginning of the loading cycle (T0) and following 9 hours (T9), 18 hours (T18), 27 hours (T27), 36 hours (T36), 45 hours (T45), and 54 hours (T54).

Results: For Group 1 and Group 2, there were statistically significant differences between the endotoxin concentration at T0 and the endotoxin concentration at the subsequent sampling points ($p < .05$ Kruskal–Wallis with Bonferoni corrections for intragroup comparisons). There were no statistically significant differences between Group 1 and Group 2 at all sampling points.

Conclusions: This study indicates that bacterial endotoxin can penetrate the IAI of implants with Morse-taper connection, and 0.2% chlorhexidine solution had no significant effect on that penetration.

KEY WORDS: chlorhexidine, dental implant, endotoxin, implant abutment, in vitro

INTRODUCTION

One of the major challenges for the construction of two-piece implant systems is the prevention of microbial infiltration into the internal implant parts through the implant-abutment interface (IAI) microgap. Microorganisms may grow into the IAI microgap[1–3] and set up a bacterial reservoir resulting in an area of inflamed soft tissue facing the fixture/abutment junction[4] and marginal peri-implant bone loss.[5–9] The size of the IAI microgap can be further increased under loading conditions,[10] introducing greater number of microorganisms in the internal aspects of the implant[11] and creating a "pumping" effect in close proximity to the peri-implant bone.[4]

Several in vitro studies have been performed evaluating the impact of IAI design on the amount of microbial penetration into the internal part of the dental implants[12] The design of the IAI can have an impact on the amount of microbial penetration into the internal

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part of a dental implant.[2,13,14] For instance, Quirynen and colleagues[2] in an in vitro study demonstrated microbial penetration of the fixture-abutment interface microgap of fixtures with an external hex design. Jansen and colleagues[13] reported microbial leakage in 13 different implant-abutment combinations using *Escherichia coli* as indicator bacteria. Among the different implant-abutment combinations, an implant with an internal connection and a silicon washer demonstrated the fewest cases of leakage. In an in vitro experiment utilizing loading forces, Steinebrunner and colleagues[14] evaluated bacterial leakage along the fixture-abutment interface microgap and discovered statistical significant differences between five implant systems with respect to number of chewing cycles and bacterial colonization. Specifically, implants with a trichannel internal connection showed bacterial leakage at significantly higher numbers of chewing cycles compared with implants with external hex, implants with internal connection and a silicon washer, and implants with internal hex with friction fit connection.

In recent in vitro studies under nonloading[15] and dynamic loading conditions,[16] it has been demonstrated that implants with Morse-taper connection had minimal contamination of the IAI microgap. These studies investigated the contamination of the IAI with identification of living bacteria (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *E. coli*). However, biologically small molecules, such as toxins and molecular constituents of the bacterial wall, can be responsible for inflammatory reactions. Endotoxin, a small molecule complex of lipopolysaccharides and proteins, is one of the most important toxins of gram-negative bacteria and plays a major role in bone destruction processes.[17] In a recent study,[18] an implant's ability to resist endotoxin infiltration was investigated for two implant systems with conical internal connections under static conditions. It was reported that both of the investigated implant systems failed to prevent endotoxin leakage.

Numerous attempts have been made to reduce the inner bacterial colonization.[19–22] Chlorhexidine has been used effectively as an antiseptic for treatment of periodontal disease.[23] This is justified by its efficient antimicrobial and antifungal function.[24] Chlorhexidine has been used to prevent internal implant contamination as a 0.2% solution[20] or as a varnish.[21]

To our knowledge, there are no reports investigating the risk for endotoxin penetration to the IAI under loading conditions and the effect of chlorhexidine in the prevention of such penetration. Thus, the aim of the study is to evaluate the role of chlorhexidine on endotoxin penetration to the IAI of implants with Morse-taper connection under in vitro dynamic loading conditions.

MATERIALS AND METHODS

Implant Experiment Groups

For this study, three groups of implants with Morse-taper connection (ANKYLOS CX, B14, DENTSPLY Implants Manufacturing GmbH, Mannheim, Germany) were compared based on the type of inoculation of the internal part of the implant. Implants in Group 1 ($n = 10$) were inoculated with 1 µl *E. coli* (concentration = 1×10^8 CFU/ml) and incubated for 24 hours at 37°C; the supernatant was removed, and 0.5 µl of sterile saline was added. Implants in Group 2 ($n = 10$) were inoculated with 1 µl *E. coli* (concentration = 1×10^8 CFU/ml) and incubated for 24 hours at 37°C; the supernatant was removed, and 0.5 µl of sterile 0.2% chlorhexidine gluconate solution was added. Implants in Group 3 ($n = 10$) were inoculated with 0.5 µl of sterile saline and served as controls. Prior to the inoculation, implants were screwed into specimen holders made of V2A steel. All inoculations were performed with a single-channel pipette and endotoxin-free pipette tips under sterile conditions. The inoculants were pipetted into the deepest point of the internal lumen of each implant.

Following the final inoculations, abutments were connected (Standard C/ Abutment b/3.0/6.0 straight, DENTSPLY Implants Manufacturing GmbH) to the implants with a torque of 25 Ncm. The abutment connections were performed under sterile condition with an implant torque controller ensuring proper abutment insertion torque without touching the internal or the external surfaces of the implants. A different set of sterile instruments was utilized for each implant. All specimens were examined carefully for inoculant outflow from the IAI. All procedures concerning implant inoculation and handling of the implants were performed in a microbiological cabinet under vertical laminar airflow.

In Vitro Dynamic Loading

Subsequent to securing the implants into the specimen holders and connecting the abutments, a custom made chamber was secured to the specimen holder with a

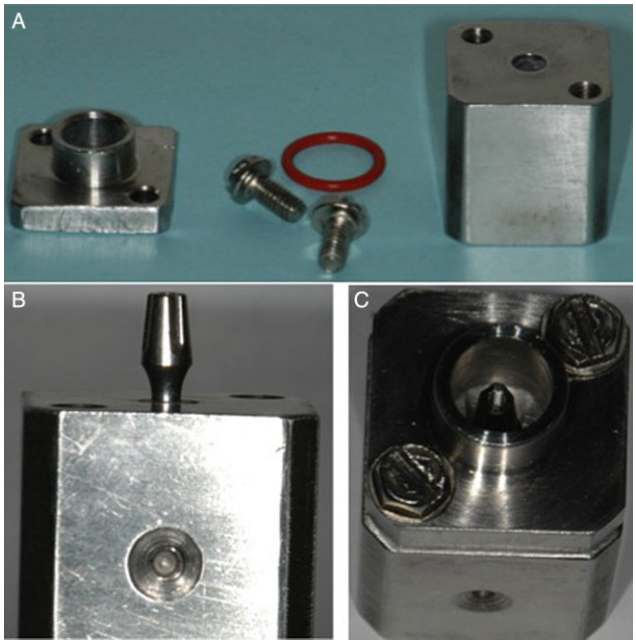

Figure 1 *A*, Custom chamber, silicon O-ring, and specimen holder. *B*, Implant with abutment secured at the specimen holder. *C*, Specimen holder assembled with custom chamber and silicon O-ring.

silicon O-ring positioned between the chamber and the specimen (Figure 1). The chamber was subsequently filled with a sterile Luria broth (Fisher Scientific, Hampton, NH, USA) covering the IAI and covered with a wax foil. Dynamic loading was applied using a chewing simulator (CS-4.2 chewing simulator, Mechatronic, Feldkirchen-Westerham, Germany). The cyclic fatigue load was applied to each abutment with a round stainless steel stylus at an angle of 30° through a hole in the wax foil. A force of 160 N was applied for a total of 200,000 cycles at 1 Hz. The chewing simulator was operating in a microbiological cabinet under vertical laminar airflow at all times.

Bacterial Culture Conditions

E. coli DH5α (Invitrogen, Carlsbad, CA, USA) was grown in Luria broth and cultured at 37°C to midlogarithmic phase. The optical density, which corresponded with a concentration of 1×10^8 CFU/ml, was determined. Briefly, serial dilutions of bacteria collected at 1-hour intervals were plated on Luria agar (Fisher Scientific) plates, and the optical density was noted at time of collection. After which, plates were allowed to incubate overnight at 37°C and the resulting colonies counted. The optical density that corresponded to 1×10^8 CFU/ml was used as a reference for preparing the bacterial solution for all future experiments.

Sample Collection

At the beginning of the experiment (prior to implant loading) (T0), following 9 hours (T9), 18 hours (T18), 27 hours (T27), 36 hours (T36), 45 hours (T45), and 54 hours (T54) at the end of the loading cycles, samples were collected using a single-channel pipette and endotoxin-free pipette tips from the supernatant solution of each implant. After pipetting, each sample was placed into a pyrogen-free test tube and stored frozen until analysis.

Endotoxin Quantification

Endotoxin levels were detected and semiquantified using a QCL-1000® Chromogenic limulus amebocyte lysates (LAL) assay (Lonza, Walkersville, MD, USA) according to the manufacturer's instruction. Briefly, 50 µl of endotoxin standards (0.1, 0.25 and 1.0 EU/ml) and samples were added in duplicate to a 96-well microplate. After which, 50 µl of LAL was added to each well and allowed to incubate for 10 minutes. A 100 µl of prewarmed (37°C) substrate solution was added and allowed to incubate at 37°C for 6 minutes followed by the addition of 100 µl of acetic acid 25% v/v to stop the reaction. The absorbance at 405 nm was read on a spectrophotometer (Epoch, BioTek Laboratories, Seattle, WA, USA). Endotoxin units/ml was calculated by a standard curve and best-fit linear trend line.

Statistical Analysis

For description of the data, mean values and standard deviations were calculated. The Kruskal–Wallis test was applied to evaluate differences between the three groups regarding endotoxin concentration for each sampling point. The same test was applied to evaluate differences within groups at different sampling points. A p value of <0.05 was considered as statistically significant.

RESULTS

The mean endotoxin concentrations and standard deviations for each group at all sampling points are illustrated in Figure 2. For Group 1, Group 2, and Group 3, the mean (SD) endotoxin concentration at T0 was 0.05 (0.01) EU/ml, 0.01 (0.03) EU/ml, and 0.06 (0.03) EU/ml, respectively (data not shown in Figure 1). For Group 1 and Group 2, there were statistically significant differences between the endotoxin concentration at T0 and the endotoxin concentration at the subsequent

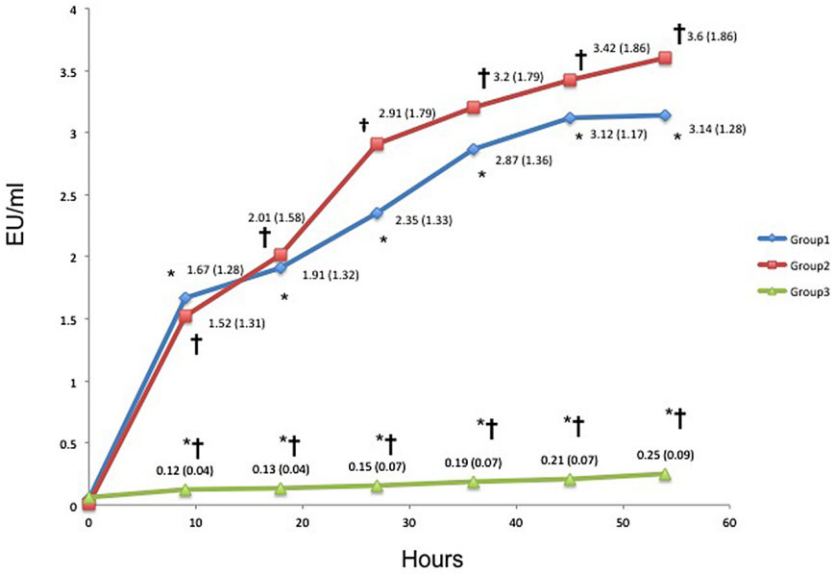

Figure 2 Mean endotoxin concentrations (SD) at different sampling points according to different implant groups. *$p < .05$ Group 1 versus Group 3 Kruskal–Wallis with Bonferoni corrections. †$p < .05$ Group 2 versus Group 3 Kruskal–Wallis with Bonferoni corrections.

sampling points ($p < .05$ Kruskal–Wallis with Bonferoni corrections for intragroup comparisons). There were no statistically significant differences for the remaining intragroup comparisons.

Both Group 1 and Group 2 had statistically significant higher endotoxin concentration compared with Group 3 at sampling points T9, T18, T27, T36, T45, and T54 ($p < .05$ Kruskal–Wallis with Bonferoni corrections for intergroup comparisons). There were no statistically significant differences between Group 1 and Group 2 at all sampling points.

DISCUSSION

The present study showed that the placement of chlorhexidine 0.2% into the internal parts of the implant did not have a significant impact in preventing or decreasing the endotoxin penetration from the IAI. Bacterial endotoxin penetrated the IAI microgap of implants with Morse-taper connection under in vitro dynamic conditions. The increase in endotoxin concentration was significant following 9 hours of loading. The endotoxin concentration at the subsequent sampling points was not statistically significantly higher than the 9-hour sampling time point.

The bacterial penetration into the IAI microgap has been of interest because microorganisms can establish a bacterial reservoir resulting in a significant inflammatory cell infiltrate and bone loss at the marginal portion of the implant.[25,26] The dental implant we utilized in the present experiment has an internal Morse-taper connection between the implant and the abutment, with a 5.7° taper angle.[27] This specific type of implant has been evaluated in vitro for the risk of bacterial penetration into the IAI microgap in several studies under nonloading[13,15,28,29] and dynamic loading conditions.[16] The range of the percentage of implants with bacterial penetration to the IAI from those studies was 0 to 50%. In the current study, we reported that 100% of the specimens showed endotoxin leakage from the IAI. The main reason for this difference in the results from the previously mentioned studies can be related to differences in the size between whole live bacteria and bacterial endotoxin.[30] An additional reason that may explain the different findings from the previous mentioned studies is the different loading conditions that the implants were subjected. In the majority of the studies that evaluated the risk for bacterial leakage of the IAI for the specific type of implant, loading was not applied to the abutment.[13,15,28,29] It has been reported that load application under in vitro experimental conditions can significantly increase the risk for bacterial contamination of the IAI.[11] In comparison to the study[16] that dynamic loading conditions have been applied, there is a difference in both magnitude (15 N vs 160 N) and direction (90° vs 30°) of the applied load.

The size of the IAI microgap of the investigated implant has been evaluated with scanning electron

microscopy,[13] conventional radiography,[31] and monochromatic hard x-ray synchronton radiography[10] under nonloading and loading conditions. Variations in the size of IAI microgap have been reported depending of the method of evaluation and loading conditions. For instance, Jansen and colleagues,[13] using scanning electron microscopy under nonloading condition, reported 4 μm microgap between the implant and the abutment. This observation is in contrast with Zipprich and colleagues[31] that did not observed microgap formation even when the abutment was loaded with 200 N force at a 30° angle. Furthermore, Rack and colleagues[10] using monochromatic hard x-ray synchronton radiography reported a 22-μm microgap formation when applied a load of similar magnitude and direction as with the previously mentioned experiment. The size of the endotoxin molecules can be smaller than 1 to 2 μm[30] allowing the penetration through the IAI in all of the specimens of the present experiment.

The risk of the endotoxin penetration to the IAI for implants with the same IAI characteristics has been evaluated under nonloading conditions.[18] It was reported that 100% of the specimens exhibited endotoxin leakage from the IAI, with the higher endotoxin concentrations identified after 5 minutes of connecting the abutment to the implant. The observation of early endotoxin penetration into the IAI is in agreement with the present study, even though there are several differences in the methodology between the two studies. In the present study, we used bacterial cultured media in order to contaminate the internal part of the implant and not purified extracted endotoxin. The main reason that we used bacterial cultured media was the fact that we wanted to evaluate the effect of chlorhexidine application to the risk of endotoxin penetration to the IAI. Due to the bactericidal effect of chlorhexidine,[24] one may expect higher amounts of endotoxins to be released from the lysed bacteria at the internal part of the implant that have the potential to penetrate from the IAI. The results of the present study showed that higher concentrations of endotoxin were identified at the supernatant of implants of the Group 2 (0.2% chlorhexidine treated) following the 18th hour sampling point even though the differences were not statistically significant compared with implants of Group 1. The second reason for the use of bacterial cultured media is the evaluation of endotoxin production from bacteria that potentially penetrated the IAI and enter the sterile culture media that surrounded the specimens. Bacteria that potentially penetrated the IAI were provided with the nutrients and the space to grow in the custom chamber and follow the cycle of bacterial growth (lag phase, logarithmic phase, stationary phase, and decline phase). *E. coli* enter the decline phase and lyse following approximately 20 hours.[32] Thus, one may expect an increase of endotoxin concentration following lysed bacteria in the external culture media after 27 hours (T27). In the present study, we observed an increase in the amount of endotoxin concentration of the supernatant from T0 to T54, even though there was a statistically significant difference in concentration only between T0 and T9. Thus, we speculate that the increase of endotoxin concentration during the experiment was mainly because of endotoxin production from lysed bacteria at the internal part of the implant rather from bacteria that penetrated the IAI and lysed at the external culture media.

Early bacterial colonization of implant surfaces and peri-implant tissues can occur within minutes after implant installation.[33] The timing of the contamination of the internal parts of the implants through the IAI has not been studied extensively in clinical studies. It has been reported that after 25 days following the second-stage surgery and healing-abutment connection, moderate to high levels of eight different putative periodontal pathogens including *A. actinomycetemcomitans* and *P. gingivalis* could be identified in the internal aspects of the implant.[3] In the present study, we observed a significant increase on endotoxin penetration through the IAI 9 hours following the insertion and loading of the abutments. Clinically, this will correspond to the time of prosthetic rehabilitation of the patient. However, it seems that endotoxin penetration into the IAI can occur without any loading application to the implant abutment.[18] Thus, it is reasonable to expect that endotoxins will penetrate in the internal parts of the implant soon after an abutment is connected to an implant (one or two stage treatment).

In the present study, we failed to identify a statistically significant effect of chlorhexidine 0.2% on the endotoxin penetration to the IAI over time. The volume of chlorhexidine solution that we used was minimal (0.5 μl) because of the fact that we tried to avoid any solution overflow following placement of the abutment. The 0.5 μl volume of solution has been confirmed not to produce any overflow following abutment connection for the specific implant system in several in vitro

studies.[13,18,28] The utilization of larger volumes of chlorhexidine, in order to rinse the internal part of the implant, is more clinically relevant. However, such a protocol would have potentially removed all the cultured *E. coli* and made difficult to evaluate the effect of chlorhexidine on the endotoxin penetration to the IAI. In addition, repeated applications of chlorhexidine would require disconnection of the abutments, which can potentially affect the results of the study. Thus, the findings of the current study cannot be directly related to decontamination protocols utilizing larger amounts and/or repeated applications of chlorhexidine.

The use of endotoxin as a marker to identify molecular microleakage to the IAI is highly demanding on laboratory process and hygiene standards during the testing. Despite all efforts for sterile experimental conditions, we still observed minimal endotoxin concentration in the supernatant of implants of Group 3. This environmental endotoxin concentration should be taken into account when interpreting the results.

Few clinical studies[21,34] have focused on the decontamination of the inner-implant cavity of two-stage implants. In a recent study,[34] it was reported that the application of a 1% chlorhexidine gel in the internal part of the fixture before abutment placement and screw tightening could be an effective method to reduce bacterial colonization over a 6-month period. In addition, Groenendijk and colleagues[21] reported that the internal implant decontamination with 0.2% chlorhexidine solution led to a reduced gingival index and crevicular fluid flow compared with saline-treated controls.

The clinical significance of the present findings have not yet been evaluated. The implant system that we tested has been demonstrated both histologically[35] and clinically[36] minimal marginal bone loss over time, with mineralized tissue on the implant platform when the implant was placed with the IAI in subcrestal positions. Thus, the amount of endotoxin penetration from the IAI with or without placement of chlorhexidine in the internal parts of the implant might have a limited effect on marginal bone levels over time. However, the exclusion of bacteria and bacterial products from peri-implant regenerative procedures is considered of paramount importance to obtain clinical success.[37]

CONCLUSIONS

Within the limits of this study, it indicates that bacterial endotoxin can penetrate the IAI of implants with internal Morse-taper connection. The study failed to find a significant effect of 0.2% chlorhexidine solution on endotoxin penetration to the IAI over time.

CONFLICTS OF INTEREST

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