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ORIGINAL ARTICLE

Molecular leakage at implant-abutment connection—in vitro investigation of tightness of internal conical implant-abutment connections against endotoxin penetration

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Abstract Microleakage has been discussed as a major contributing factor for inflammatory reactions at the implant-abutment connection. In previous studies, the tightness against corpuscular bodies (viable bacteria) has been successfully investigated under static and dynamic conditions. The aim of this study was to investigate the tightness against endotoxins of two implant systems (AstraTech and Ankylos) with conical internal connections under static conditions. The inner parts of eight implants of each system were inoculated with endotoxin. Implants were screwed together with the respective abutments and stored under isostatic conditions in a supernatant of pyrogen-free water for 168 h. Supernatant samples were taken after 5 min, 24 h, 72 h, and 168 h, and endotoxin contamination was determined by the amebocyte-lysate test. Only one implant in the AstraTech group showed no sign of endotoxin contamination after 168 h, while the other implants showed contamination after varying storage times, respectively. The implants in the Ankylos group showed endotoxin contamination after only 5 min of storage in the supernatant solution. The tested internal conical implant-abutment connections appear to be unable to prevent endotoxin leakage. In average, Astra implants showed a higher tightness than Ankylos implants.

Keywords Microleakage · Endotoxin · Dental implants · In vitro investigation · Implant-abutment interface

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Introduction

Leakage at the implant-abutment connection is a major contributing factor for peri-implant inflammatory reactions. Prevention of microbial leakage at the implant-abutment connection is a major challenge for the construction of modern two-stage implant systems in order to minimize inflammatory reactions and to maximize bone stability at the implant neck. Gaps and cavities inside the implant, between implant, and the abutment are still present, even in modern implant systems (Fig. 1). The internal conical implant-abutment connection is considered to be mechanically more stable [1] and more tight than flat-to-flat connections or tube-in-tube connections.

Microbial leakage is an important factor for chronic inflammatory infiltration and marginal bone resorption [2, 3]. Implant manufacturers aim to reduce the leakage by increasing the stability of the implant-abutment connection. Therefore, reducing the mobility of this connection by

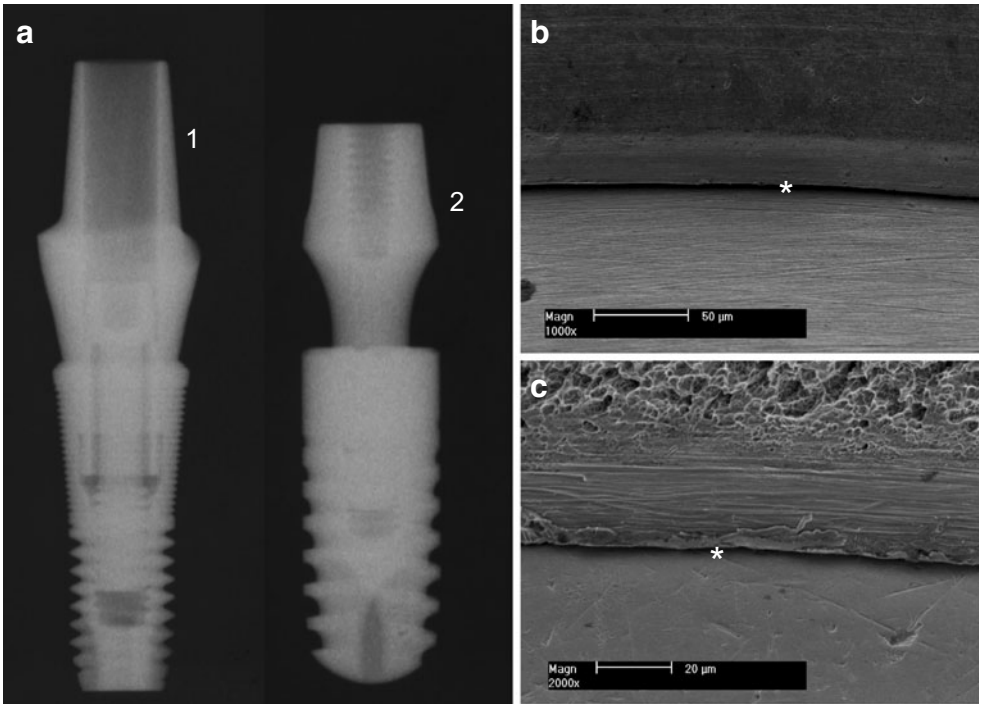

Fig. 1 **a** X-ray pictures of MicroThread-OsseoSpeed implant bolted with TiDesign Abutment (AstraTech) (*1*) and Ankylos Plus implant bolted with straight standard abutment (*2*). **b** Scanning electron microscope picture of the implant-abutment connection (AstraTech). **c** Scanning electron microscopy photo of the implant-abutment connection (Ankylos). The marginal gap between implant and abutment is marked with a *star*

constructing physically tight connections with a high level of precision in the sub-micrometer range is considered to be an important precondition for microleakage prevention [1]. Several investigators aimed to quantify microbial leakage of dental implants [4–8]. These studies investigated corpuscular bacterial leakage (size 1–10 µm, living bacteria). However, biologically small molecules like toxins and molecular constituents of the bacterial wall are responsible for inflammatory reactions. These small molecules can penetrate much smaller gaps than whole bacteria. It is well known that 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 [9].

To the authors' knowledge, only few data exist concerning microleakage phenomena of small molecules at implant-abutment connections. In an in vitro investigation, the sealing capability of three different implant-abutment connections was tested by placing toluidine blue increments solved in distilled water in the internal implant parts [10]. All three tested implant-abutment systems presented microleakage. This is in agreement with another study showing that two of the implant systems tested in the former study showed microgaps and micromovement between the abutment and its implant [1]. The third implant system was not included in the micromovement study, but it was shown that two other systems with conical implant-abutment connections did not show any microgaps or micromovements.

As endotoxin penetration might have more clinical relevance than dye penetration, it was the purpose of this investigation to evaluate molecular leakage of endotoxin in the two-implant systems with an internal conical implant-abutment connection, which did not show any microgaps or micromovements in the former study. Therefore, the hypothesis of the present study was that conical implant-abutment connections are tight enough to prevent endotoxin penetration.

Materials and methods

Implants and abutments

The following implants and corresponding abutments were used in this study:

1. MicroThread-OsseoSpeed 4.5×11 mm and TiDesign abutments 4.5/5.0–5.5, 3 mm (AstraTech Corp., Elz, Germany)
2. Ankylos Plus B11 4.5×11 mm and straight standard abutments b/3.0/4.0 (Dentsply Friadent, Mannheim, Germany).

The tested implants have internal conical implant-abutment connections. The Ankylos abutments were manufactured as one-piece abutments; whereas, the AstraTech abutments were multiple-part abutments, which were connected to the implant with a separate screw. All parts were standardized, prefabricated, and used as delivered by the manufacturers.

Endotoxin stimulus

Lipopolysaccharides from *Salmonella enterica* serotype Minnesota (source strain ATCC 9700; Sigma-Aldrich

Chemie GmbH, Taufkirchen, Germany), purified by phenol extraction, served as the standard endotoxin in this study. According to the manufacturer's information, this lipopolysaccharide contains endotoxin levels of 500,000 EU/mg. One nanogram of this endotoxin is equivalent to 5 EU (Limulus lysate assay) and 10 EU (chromogenic assay). One EU of endotoxin is equal to 1 IU.

The endotoxin test solution was prepared according to the manufacturer's instructions in order to achieve the highest concentration of endotoxin by diluting 100 mg purified endotoxin with 50 ml aqua ad injectabilia followed by mixing and warming to 70–80°C. A concentrated, though still hazy, solution (20 mg/ml) resulted.

Handling of the implants and abutments

To avoid endotoxin contamination, all implants, abutments, vials, forceps, and other instruments used in contact with the test materials were heat-treated prior to test procedure for at least 4 hours at 250°C in a dry oven, a well-accepted and highly effective depyrogenization procedure as recommended by the European Pharmacopoeia [11]. All procedures concerning the handling of implants, abutments, and the collection of samples during the experimental series were performed under sterile conditions in a microbiological cabinet under vertical laminar airflow.

Test setup

Specimens consisted of the tested implants, abutments, and a specimen holder made of V2A-steel with a central drill hole with an inner diameter equivalent to the respective implant diameter. Implants were screwed into the specimen holders to achieve an upright position. During the test phase, specimens were put in pyrogen-free glass dishes (diameter 50 mm and height 30 mm) made of Duran-glass (Duran Group Inc., Mainz, Germany). A supernatant of 10 ml aqua ad injectabilia (DeltaSelect Corp., Munich, Germany) was filled in to ensure that the microgap between implant and abutment was covered by the liquid level. To avoid contamination of the specimen, dishes were covered with Duran-glass upper shells (Duran Group Inc., Mainz, Germany). Dishes were then placed on a swing plate (HS250 basic, KIKA Labortechnik, Cologne, Germany) with constant horizontal alternate movement (20 motions/min).

Preparation of the implants

Under sterile conditions, eight implants of each system were inoculated with 0.5 µl of endotoxin test solution: AS1-AS8 (AstraTech) and AN1-AN8 (Ankylos) using a single-channel pipette (Research Pippette, Eppendorf Corp., Hamburg, Germany) and endotoxin-free pipette tips (epTips, Eppendorf Corp). The endotoxin was then carefully pipetted into the deepest point of the internal lumen of each implant. Then, the abutments were connected to the implants according to the manufacturers' protocols using a calibrated torque controller of the respective implant manufacturer, without touching the outer or inner surfaces of the implants. After pulling tight the abutment screw, all specimens were examined carefully with regard to overpressing of instilled endotoxin solution.

Sample collection

During the observation period, tests were performed at 0 min (control setup), 5 min, 24 h, 72 h, and 168 h (AS1–AS8 5 min to AS1–AS8 168 h and AN1–AN8 5 min to AN1–AN8 168 h). Samples were collected using a single-channel pipette (Research Pippette, Eppendorf Corp.) and endotoxin-free pipette tips (epTips, Eppendorf Corp.). Samples were collected from the supernatant solution of each implant and the controls. The sample size was 200 µl. Aqua ad injectabilia and implants of each group without contamination served as control (control). After pipetting, each sample was put into a pyrogen-free test tube (Safe-lock micro test tubes, Eppendorf Corp.) and stored frozen until analysis.

Test procedure

The QCL-1000® Chromogenic limulus amebocyte lysate (LAL) test (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) was used for endotoxin detection. LAL is a quantitative test for gram-negative bacterial endotoxin. The test is based on the findings of Bang who found that endotoxin caused a fatal intravascular coagulation in the horseshoe crab species (*Limulus polyphemus*) [12]. It was found that this coagulation was the result of an endotoxin-initiated cascade reaction. This reaction is caused by the enzymatic conversion of a clottable protein (Coagulogen), which is derived from the circulating blood cell (amebocyte) of *L. polyphemus* [13, 14] (Fig. 1). In the chromogenic LAL assay, the coagulogen is completely or partially removed and replaced by a chromogenic substrate [15]. The chromogenic LAL assay usually proceeds in two stages: Firstly, a proenzyme is activated by gram-negative bacterial endotoxin. The initial rate of activation is determined by the endotoxin concentration. Secondly, following the addition of the chromogenic substrate, a chromophore releasing stage follows. The released chromophore turns the solution a yellow color. The altered optical density is measured in a spectrophotometer at 405–410 nm [16].

After defrosting, samples were tested according to manufacturer's recommendations for the microplate method.

The microplate was pre-equilibrated at 37±1.0°C in a heating block. After dispensing the standard controls and supernatant samples, pipetting of 50 µl LAL in every sample was performed at time (T)=0 using a multichannel pipettor. After each step, mixing of the solutions was performed by repeatedly tabbing at the site of the microplate. At T=10 min, 100 µl chromogenic substrate solution, pre-warmed to 37±1.0°C, was added. At T=16 min, 100 µl of a stopping reagent was added, and the absorbance was measured at 405 nm in a spectrophotometer. An overall Friedman test was used to evaluate differences of endotoxin contamination over the period of 168 h in each tested implant system. Multiple comparisons of all sampling points with one-sided Wilcoxon signed-rank tests were performed to evaluate differences in endotoxin contamination between the sampling points of each implant system. Differences of endotoxin contamination after 168 h between the two tested implant systems were tested by rank sum test. For all statistical tests, the level of significance was adjusted for multiple comparisons and set at 5%.

Results

Standard endotoxin

Figure 1 shows the absorbance at 405 nm of the ten times diluted standard endotoxin solution used for inoculation of the tested implants versus endotoxin concentration. The coefficient of correlation (R) for the individual mean Δ absorbance at 405 nm of the standards versus their respective endotoxin concentration was 0.989 and therefore in the range of the manufacturer's recommendation (≥0.980).

Evaluation of endotoxin contamination of supernatant samples

Endotoxin contamination of supernatant samples was measured at the time of inserting the inoculated and bolted implants (AS1–AS8 and AN1–AN8) in the supernatant solution (first sampling point) and after 5 min (second sampling point), 24 h (third sampling point), 72 h (fourth sampling point), and 168 h (fifth sampling point). In the AstraTech group (AS1–AS8), three implants showed no sign of contamination after 5 min of staying in the supernatant solution (AS2, AS4, and AS7). After 24 and 72 h, two implants showed no sign of contamination of the supernatant solution (AS2 and AS4), and only one implant showed no sign of endotoxin contamination after 168 h (Fig. 2). All implants in the Ankylos group (AN1–AN8) showed endotoxin contamination after 5 min of staying in the supernatant solution There was a significant increase in endotoxin contamination with longer storage times for both implant systems (p≤0.001). Differences were significant for all comparisons of sampling points (AstraTech, p≤0.022; Ankylos Plus, p≤0.018). Significantly, less endotoxin contamination was found in the AstraTech group at every sampling point (p≤0.05; Tables 1 and 2).

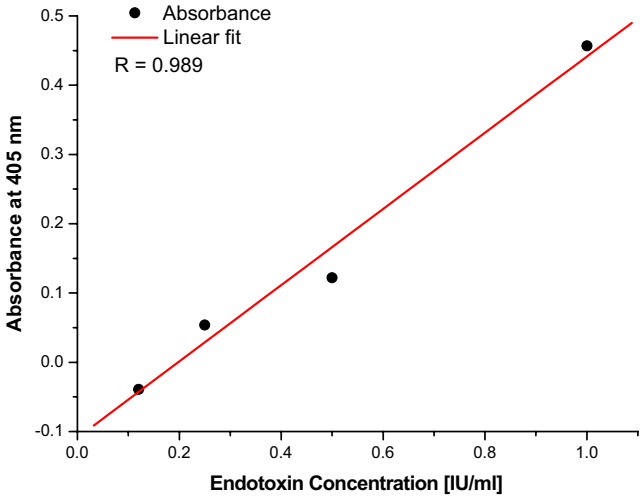

Fig. 2 Absorbance of the ten times diluted standard endotoxin solution used for inoculation versus endotoxin concentration. R=correlation coefficient

Discussion

The present investigation has demonstrated that even internal conical implant-abutment connections were not tight on the molecular level.

To ensure that all implant parts used in this study were free of endotoxin, all implants, abutments, and abutment screws were heat-treated for 4 h at 250°C in a dry heat oven. However, it remains unclear, whether this treatment might be responsible for a decline in tightness of the implant-abutment connection.

To avoid rarefaction of the inserted endotoxin solution, no washing procedures of the bolted implants were performed. Depyrogenization procedures with dry heat have been described as the most efficient way of depyrogenization [11]. However, inoculation would have led to an evaporation of the endotoxin solution and, therefore, was not performed. To avoid measurement errors due to a contamination of the outer surface of the implants with endotoxin, the first sample was taken immediately after putting the inoculated implants in the supernatant solution. If contamination occurred in the first sample, this implant was excluded from the study. By choosing a volume of 0.5 µl for inoculation, no signs of over-pressing of the inoculated endotoxin solution were observed in our study. Jansen et al. found an outside contamination rate of 56.8% (21 of 37) for Ankylos implants and of 60% (24 of 40) for AstraTech

Table 1 Endotoxin contamination (mean, medium, minimum, and maximum) of the supernatant samples at five sampling points during observation period of up to 168 h

There is a significant increase in endotoxin contamination for both implant systems ($p \leq 0.001$) with significant differences for all multiple comparisons (AstraTech, $p=0.022$; Ankylos Plus, $p=0.018$)

[a] International units

		Sampling points				
		0min (IU[a]/ml)	5min	24h	72h	168h
AstraTech ($N=8$)	Mean	0	1.3	2.0	2.1	2.4
	Median	0	0.7	3.0	3.0	3.1
	Minimum	0	0.0	0.0	0.0	0.0
	Maximum	0	3.2	3.2	3.2	3.3
Ankylos Plus ($N=8$)	Mean	0	3.1	3.2	3.2	3.4
	Median	0	3.2	3.2	3.2	3.3
	Minimum	0	2.4	3.2	3.2	3.2
	Maximum	0	3.2	3.3	33	4.2

implants [17]. In that study, inoculation was performed with the same volume (0.5 µl) as in the current study, but by inoculating the tip of the abutment screw, while in the current study, inoculation was performed by pipetting directly into the implant using a single-channel pipette. This seems to be a more efficient way, because the inoculated solution was placed directly in the hollow space beneath the abutment screw tip without contact with the threads. An outward pressing along the threads and the conical connection seems to have thereby been avoided.

Our results reveal that endotoxin leakage occurs at internal conical implant-abutment connections within a short storage time. Endotoxin contamination was found in supernatant samples after inoculation of two-stage implants of two different manufacturers with bacterial endotoxin and incubation in a supernatant endotoxin-free solution within an overall observation period of 168 h. So, molecular microleakage containing endotoxin occurs in two-stage implants, even if they showed good results for bacterial leakage of corpuscular size [17]. Our findings are similar to those of Coelho et al. who found that three different types of two-stage implants showed a significant increase in microleakage phenomena after inoculating the internal implant parts with toluidine blue [10], but there might be some differences in penetration behavior between the endotoxin molecules and this color marker, so this comparison must be made with caution.

A significant increase ($p \leq 0.001$) in endotoxin contamination of supernatant samples was measured for both tested implant systems. Significantly, less endotoxin contamination was measured in the AstraTech group at all sampling points. Considering the short time period (5 min) before endotoxin concentration occurred in the supernatant solution of most specimens (five specimens of the Astra group and eight specimens of the Ankylos group), the tendency of better performance in the AstraTech group (one implant remained without contamination after 168 h) seems to be negligible.

Table 2 Significantly less endotoxin contamination was measured in the AstraTech group

Differences of endotoxin contamination at four sampling points were tested by rank sum test

Sampling points	*p* values
5 min	0.008
24 h	0.003
72 h	0.005
162 h	0.021

Since the discovery of microbial colonization of the internal parts of two-stage implants [6, 18, 19], there is very little data regarding the analysis of the microleakage at the implant-abutment connection. Different methods for verification of microleakage phenomena were described in these studies. One method was to analyze the migration of bacteria (*Escherichia coli*) and the growing of microbial cultures from supernatant samples [5, 7, 17]. Another study used colored tracing probes to analyze microleakage photometrically [20]. Our method is very unique and, to the best of the authors' knowledge, there are no other studies using endotoxin for microleakage detection. The only comparable study about the tightness of the two tested implant systems was published by Jansen et al. [17]. After 14 days of incubation, they detected contamination in 50% of the Ankylos implants (eight of 16) and in 69% of the AstraTech implants (11 of 16). The use of endotoxin in our study is advantageous because of the small molecular weight of endotoxin molecules. A molecular weight of 50–100 kDA was described by Jann et al. [21]. The gap size at the implant-abutment connection of conical abutments was measured by Jansen et al. A marginal gap size of 1–2 µm was found for the AstraTech system and of 4 µm for the Ankylos system. It appears that endotoxin molecules are able to pass through gaps in this dimension more quickly than bacteria with a diameter of 1.1 to 1.5 µm. These findings might explain why a higher rate of leakage and a faster increase in endotoxin contamination for these two implant systems was found in our study. Nevertheless, the use of endotoxin as a marker to verify molecular microleakage makes high demands on laboratory processes and hygiene standards during testing.

Based on the findings of Broggini et al., it was assumed that the creation of a microgap at the bone level in combination with microbial leakage and a persistent bacterial presence leads to accumulation of inflammatory cells, which, when combined with osteoclast formation/growth, results in alveolar bone loss [2]. The role of endotoxin in bacterial bone destruction has been well described in recent years. In a review of the international literature, Nair et al. stated that in the case of lipopolysaccharide, this gram-negative polymer binds to osteoblasts or other cells within and stimulates them to release cytokines and eicosanoids, which then induce the recruitment and activation of osteoclasts [9]. This underscores the importance of evaluating not only the tightness of implant-abutment connections regarding bacterial cells but also regarding endotoxins.

Conclusions

The experimental setup was considered valid and produced consistent data. The hypothesis that conical implant-abutment connections are tight enough to prevent endotoxin penetration must be rejected. For the Astra implants, significantly less endotoxin penetration was observed compared to the Ankylos system. Some Astra implants stayed tight over the entire trial period, and all investigated Ankylos implants showed penetration after 5 min.

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Conflict of interests The authors declare that they have no conflict of interest.

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