Influence of preformed bone defects on key pathogens and bone loss during experimental peri-implantitis formation in a canine model

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Abstract

Purpose: To determine the impact of experimentally preformed peri-implant crater-shaped bone defects on the evolution of in situ microbiota and development of bone defects compared to those induced over time by ligature placement only.

Methods: Implants were installed in the mandibles of eight dogs. Standardized bone defects were preformed in four test animals but not in the other four control animals, prior to implant (3.3 mm × 8 mm) installation. After 2 months of healing, peri-implantitis was induced with silk ligatures in both groups for 2 months. Microbial samples were obtained from implants and teeth for analysis at three time points (qPCR), and the average depths of the bone defects were measured.

Results: At the baseline, the total marker load of periodontal-pathogenic bacteria (TML) for teeth accounted for 5.2% (0-17.4%). After implant healing, TMLs for implants and teeth were comparable (7.1% [0.3-17.4%]). The TML of both groups was 3.5%, 2 months after ligature placement. Bone defects had a mean depth of 1.84 mm at preformed defects and 1.64 mm at control sites (P > 0.05).

Conclusion: Preformed defects in the test group showed comparable results to the control group in terms of TML, the incidence of periodontal-pathogenic bacteria, and bone defect depth.

Keywords: animal experiment, bacteria, bone defect, dental implant, polymerase chain reaction (PCR)

Introduction

Dental implants have become a valid treatment option for replacement of missing teeth, and high success rates of 97% and 75% over periods of 10 [1] and 20 [2] years, respectively, have been reported. However, biological complications can occur, and these may lead to peri-implant inflammation with concomitant inflammation of the mucosa and progressive marginal bone loss [3], also known as peri-implantitis, representing the main cause of late implant failure [4,5]. Derks et al. [6] conducted a meta-analysis showing that 22% of all implants in patients are affected by peri-implantitis to varying degrees. The clinical symptoms of peri-implantitis, such as bleeding on probing of the adjacent gingiva/mucosa, pain, suppuration, increased probing depth, radiographic bone loss, and the presence of abundant peri-pathogen bacteria, which colonize non-shedding surfaces in biofilms, are similar to those of periodontitis affecting natural teeth [7,8]. Peri-implantitis—like periodontitis—is induced by biofilm and associated with similar microbiota [7]. Dental implants affected by peri-implantitis can be treated using different approaches. However, no specific treatment protocol has been shown to be particularly advantageous, suggesting the need for further research on the pathogenesis and treatment of peri-implantitis.

[8]. In 1966, Rovin et al. [9] described the use of animal models to study the pathogenesis and possible treatment of periodontitis. This research model was adopted for peri-implantitis research in the early 1990s [10,11], and still remains in common use [12]. In these models, ligatures made of cotton are placed submarginally around healthy implants, allowing biofilm to accumulate and induce inflammation-related tissue breakdown [10]. Schwarz et al. [13] reported that the most commonly encountered defect was a saucer-shaped circumferential defect (Class -Ie), with incidences of 55% and 85% in humans and animals, respectively.

Regarding the microbiome associated with peri-implantitis, an experimental microbiological study conducted in dogs in 1992 linked the shift from aerobic to anaerobic flora around implants to pathologic conditions [14]. Porphyromonas gingivalis, Prevotella intermedia, and Tannella forsythia have higher populations in both peri-implant inflammation and periodontal defects [12]. More recently, ligatures have been used in these models to promote tissue breakdown within shorter periods than was the case in earlier models, which were prepared over periods of years [15]. A recently published study has described a canine model in which circumferential defects are preformed around implants, followed by induction of peri-implantitis with ligatures [16]. In the present canine study, a similar peri-implant model was adopted and crater-shaped defects were preformed with saucer-shaped burs before ligature placement. The aim was to determine the impact of pre-existing peri-implant crater-shaped bone defects on the evolution of the in situ microbiome and the progression of defects, relative to those induced over time by ligature placement only.

Materials and Methods

The study was approved by the Ethics Committee for Animal Research at the University of Murcia (Spain). The study protocol followed guidelines established by the European Union Council Directive of February 1, 2013 (R.D. 53/2013), and was registered with the Ministry of Animal Health (Murcia Regional Government; Reg. no. A1320141102). Eight Beagle dogs were purchased, aged approximately 1.5 years, from a licensed vendor in Murcia (Spain) for the study. Upon clinical assessment, the dogs were in good general health and showed no signs of oral viral or fungal lesions. No dog presented with any occlusal trauma. Animals were quarantined for vaccination with anti-rabies vaccine and administration of vitamins. Pre- and postoperatively, the animals were kept in individual cages. After three days, they received appropriate veterinary care with unrestricted access to water and standard laboratory nutritional support throughout the treatment period. All animals presented with intact dental arches without any oral viral or fungal lesions. This study followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines [17]. The timeline of the study is outlined in Fig. 1.

Anesthesia protocol

Acetompazine 0.12-0.25 mg/kg, buprenorphine 0.01 mg/kg, medetomidine 35 mg/kg with 10% zolazepam at 0.10 mg/kg, acepromazine maleate 0.12-0.25 mg/kg (Calmo-Neosan, Pfizer, Madrid, Spain), and medetomidine 35 mg/kg (Medetor 1 mg, Virbac, CP-Pharma GmbH, Burgdorf, Germany) were administered to pre-anesthetize the animals. The mixture was injected intramuscularly in the femoral quadriceps. General anesthesia was administered through an intravenous catheter (diameter: 22/20 G) in...
The cephalic vein with propofol at a slow constant infusion rate of 0.4 mg/kg/min. Conventional dental infiltration anesthesia ([articaine 40/0.0005 mg/mL; 1% epinephrine] [Articaine, Inibsa, Barcelona, Spain]) was administered at the surgical sites. All procedures were performed under the supervision of a veterinary physician.

Surgical procedure

Sixty-four teeth from eight dogs were removed. Following two implantation protocols after tooth extraction and healing, six implants (three per side) were installed in the mandibles of these dogs. The dogs were divided into two treatment groups of four dogs each: a “non-preformed” group (no preformed defects [n = 24]) and a “preformed” group (standardized marginal bone defects in the implantation bed [n = 24]). Bone defects were formed using a specially designed 2.8 mm-deep and 7-mm-wide bur with a saucer-shaped profile (Straumann, Switzerland). In both groups, identical implants (3.3 mm × 8 mm, BLT, Straumann, Switzerland), which healed in 2 months, were installed. Next, experimental peri-implantitis was induced with silk ligatures in both groups for another 2 months (Fig. 2). Ligatures were placed in the marginal part of the implants in both groups in order to facilitate plaque accumulation, without forced advancement of the ligature.

Data acquisition

The data were collected during the three sampling sessions: March (Baseline-BL), May (T1), and July 2018 (T2) (Fig. 3). The time differences between the baseline and T1 and T2 were 84 and 54 days, respectively. During the first session, two teeth (one from each side) were selected from a selection of four dogs (n = 8) were sampled at the baseline. The sampling procedure was performed using a sterile paper point inserted between the tooth and the gum. At the baseline, eight teeth from each dog (anterior, middle, and posterior teeth from each side) were extracted for implant installation. In general, six implants and two control teeth (one from each side) from all dogs were sampled with a sterile paper point at T1 and T2. As an exception, dogs 1034 and 9581 could not be sampled respectively for one and two implants.

Microbiological analysis

The sterile paper points were analyzed using a standard microbiological kit, iai PadoTest (Institut für Angewandte Immunologie IAI AG, Zuchwil, Switzerland). This kit relies on quantitative PCR (qPCR) to determine the total population of bacteria and the quantities of six key bacteria involved in periodontitis and peri-implantitis: Aggregatibacter actinomycetemcomitans, Filifactor alocis, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, and Treponema denticola. The microbiological test provided information about the total quantity of bacteria (total bacterial load, TBL) and quantities of each of the six key bacteria and their sum (total marker load, TML).

Bone defect analysis

Bone defect depths were assessed radiologically after implant installation and 2 months of ligature-induced peri-implantitis formation (Fig. 4). Defect depths were measured on periapical radiographs (Fiji, Image J) from the apical point of the defect to the implant shoulder by a calibrated and blinded examiner.

The average defect depth for each implant (mesial and distal measurements) and each dog was calculated to obtain separate data for each group (the “preformed” and “non-preformed” defect groups). The threads on each radiograph were used for calibration of the individual radiographs.

Data analysis

Means, standard deviations, medians, and interquartile ranges (IQR) were calculated for defect depths, and means for TBL and TML. TBL, TML, and bacterial load for each of the five key bacteria identified in this study were compared between the “preformed” and “non-preformed” groups at baseline (BL), T1, and T2. To determine whether extreme values were outliers, the Cook’s D was calculated. Values with a Cook’s D > 4/n were used as the cut-off.

Using the Mann-Whitney U-test for independent samples, the bacterial loads and defect depths of the treatment groups were compared. When comparing outcomes at the different time points, the Wilcoxon signed-rank test for dependent samples was used. Mixed linear regression models were used to examine the influence of different factors on the outcomes, with the advantage that the effect of the animal could be taken into account. The model included a point in time, tooth/implant position of the implant in the mandible, side of the mandible, and bacterial load (when considered as a
factor) as fixed effects, as well as the dog as a random effect. The Dunnett-Hsu method was used to adjust for multiple comparisons. All statistical analyses were performed using the statistical software, R version 3.4.3 (R Core Team 2018, Vienna). The significance level was set to an alpha level of $P = 0.05$.

**Results**

In general, the surgical procedures and healing were uneventful, except for one tooth and two implants. Microbiological sampling could not be performed for one control tooth in one dog at T2; i.e., two implants could not be measured at T2, because the radiographic data were unreadable. Further data for one implant in the test group were omitted from statistical analysis of bone defect depths due to complete bone loss.

**Total Bacterial Load**

TBL values ranged widely between 0.9 million and 212.8 million bacteria (Fig. 5). Authors observed a decrease in TBL at T1 and T2 for both “preformed” (test) and “non-preformed” (control) from the TBL measured at the baseline in all eight animals. Before therapy, the mean TBL for teeth was 82 million bacteria (range = 31-175.8 million). After implant installation and 2 months of healing, decreased but comparable mean values of TBL were found for the teeth ($\bar{x}$ for the control group = 23.3 million; $\bar{x}$ for the test group = 13.3 million) and the implants ($\bar{x}$ for the control group = 18.4 million; $\bar{x}$ for the test group = 16.3 million). Mean values of TBL 2 months after ligature placement remained relatively consistent for the teeth ($\bar{x}$ for the control group = 23 million; $\bar{x}$ for the test group = 6.6 million) and implants ($\bar{x}$ for the control group = 13.3 million; $\bar{x}$ for the control group = 19.1 million). The increase in mean TBL at T2 for implants in the “preformed” group was largely due to the presence of an outlier (TBL = 212.8 million).

**Total Marker Load**

TML was based on five bacterial species because *A. actinomycetemcomitans* was not detected. The TML showed values ranging between 0 and 7.3 million (Fig. 6). There was a slow but continuous decrease in the TML over the three sampling sessions. Before therapy, the mean TML for the teeth was 2.7 million bacteria (range = 0.7-7.3 million). After implant installation and 2 months of healing, decreased mean values of TML for the teeth ($\bar{x}$ for the control group = 1.9 million; $\bar{x}$ for the test group = 0.9 million) and the implants ($\bar{x}$ for the control group = 1.4 million; $\bar{x}$ for the test group = 1.3 million) were recorded. Mean values of TML 2 months after ligature placement remained relatively consistent around teeth ($\bar{x}$ for the control group = 0.7 million; $\bar{x}$ for the test group = 0.1 million) and implants ($\bar{x}$ for control group = 0.6 million; $\bar{x}$ for the test group = 0.4 million). The difference between TBL and TML for all samples showed that bacteria, with an average of 19.6 million bacteria (range = 0.9-212.1 million), were present other than the five key species.

**Specific periopathogens**

Two of the six bacterial species investigated—*P. gingivalis* and *T. denticola*—were detected in 100% of the teeth and implants sampled. Three other bacterial species were detected only in a fraction of the samples: 99.2% for samples for *T. forsythia*, 95.5% for *F. alocis*, and 35.3% for *P. intermedia*. The sixth bacterial species—*A. actinomycetemcomitans*—remained systematically undetected. Follow-up of the control teeth (Table 1) revealed that *P. gingivalis* had the highest mean bacterial count, followed in order by *T. forsythia*, *T. denticola*, *F. alocis*, and *P. intermedia*. Only at the baseline did *P. intermedia* have a larger population than *F. alocis*, which remained undetected. Furthermore, the control group showed quasi-systematically higher mean bacterial counts than those from the test group. The follow-up of implants (Table 2) revealed a similar pattern as that for the control teeth, with *P. gingivalis* showing the highest mean bacterial count, followed in order by *T. forsythia*, *T. denticola*, *F. alocis*, and *P. intermedia*. The mean counts of the five bacteria were comparable between the control and test groups at both sampling points (T1 and T2).

**Comparison of defect depths between the preformed and non-preformed groups**

At the time of implant installation, the defects had a depth of 2.8 mm. After 2 months of peri-implantitis induction, the depth in preformed defects was 1.84 mm, compared with 1.64 mm in the control group (Table 3). Figure 3 shows the median (IQR) defect depths for mesial and distal sites, as well as for the test and control groups. A two-sample $t$-test comparing the two treatments yielded no statistically significant difference ($P = 0.34$). Figure 7 shows the change in peri-implant bone height in the test and control groups during ligature placement; a positive bone height change in the test group was observed relative to the control group.

**Discussion**

This study using a canine model was conducted to assess the influence of preformed saucer-shaped peri-implant defects on key pathogens and the development of bone defects in the early stage of peri-implantitis, compared to those induced solely by ligature placement.

After ligature placement, deep pocketing, suppuration, and loss of alveolar bone were observed. This animal model has been widely chosen in studies evaluating the pathogenesis and treatment of peri-implantitis [10]. Although the tissue breakdown induced by ligatures does not reflect the natural development and progression rate in humans, the defect sizes...
and shapes seem to resemble those occurring naturally in humans [18]. In 2006, Schwarz et al. [13] compared experimentally induced peri-implantitis defects in dogs with defects in humans. They concluded that the configuration and size of experimental ligature-induced defects seemed to follow those of naturally occurring defects. The authors further considered that circumferential crater-shaped bone loss without any dehiscence was the defect most often observed in humans (55.3%) and dogs (86.6%). That study, similar to the one published by Seong et al. [16] in 2019, involved a peri-implantitis model where direct acute trauma led to circumferential defects. This type of defect initiation doubtless does not represent the condition generally observed in a clinical setting. Nevertheless, upon comparison of microbiological data and defect depths, the authors found no significant difference between the “newly” proposed and conventional peri-implantitis models.

Some authors have expressed concerns about spontaneous regression in peri-implant defects caused by acute trauma [16]. The findings of the present study were consistent with these results. At the time of implant installation, preformed defects showed a standardized defect depth of 2.8 mm and a width of 7 mm. At the end of the study, preformed defects had an average depth of 1.84 mm, showing regression of bone defect depth after traumatic induction of peri-implant defects (Fig. 7). As no X-ray plates or measurements were obtained at T1, the extent to which preformed defects showed regression during 2 months of healing after implant installation or during active ligature-induced peri-implantitis (2 months) remained unclear. However, there is a need for caution when implementing this model in studies assessing regenerative processes in peri-implantitis defects, as the results could be biased by spontaneous regression

As the difference in depths between the control and treatment groups was not statistically significant (less depth for the control than for treatment), the statistical power of the study to confirm that the treatment was not inferior, considering a tolerance range of 0.4 mm as clinically insignificant, was post-calculated. The power of the study with four animals per group was 0.76, compared with 0.89 for six animals per group. This indicates that our study was under-powered.

Nevertheless, comparing the depths of non-preformed and preformed defects, the latter were observed to result in a more homogenous and predictable defect size (Fig. 6). Clarification of the predictability and uniformity of defect sizes would be helpful for future studies designed to investigate and compare preclinical peri-implantitis treatments.

With regard to study design, the present study was part of two other ongoing studies that used these two different defect models. Therefore, no split-mouth design was appropriate. The authors consider these circumstances to be an advantage, rather than a shortcoming, in terms of the 3Rs in animal research (Replacement, Reduction and Refinement). Furthermore, no specimens for further histological analysis could have been retrieved for this reason.

Microbiological studies revealed similar findings with respect to the TML for the “non-preformed” and “preformed” groups, showing an initial increase at T1, followed by a decrease at T2. A decrease in TBL was observed at T1 and T2 in both groups, from the TBL recorded at the baseline.

As the TBL represents all bacteria present, this observation might be due to a decrease in abundant symbiotic bacteria with a shift to dysbiosis [19]. Although a minimal decrease in TML was observed at different time points (baseline, T1, and T2), the mean TML of the different groups remained comparable.

In this study, the silk ligatures induced peri-implantitis in 2 months (8 weeks). Charalampakis et al. [20], in a similar canine study, described the application of silk ligatures for 10 weeks followed by a period of 25 weeks of plaque accumulation after ligature removal. During the 25-week period of plaque accumulation, the subgingival bacterial load increased significantly over time. A longer study period, as well as ligature removal, might have led to an increase in TBL/TML of the implants.

A. actinomycetemcomitans was never detected, which indicates that this bacterium at least did not play a major role in peri-implantitis in this study. P. intermedia was found only in a fraction of the samples, whereas P. gingivalis showed the highest counts, followed in order by T. forsythia, T. denticola, F. alocis, and P. intermedia. Thus, three Gram-negative bacterial species (P. gingivalis, T. forsythia, and T. denticola) were found in higher amounts than the Gram-positive F. alocis and Gram-negative P. intermedia.

Data and opinions regarding peri-implantitis-associated bacteria have been mostly inconsistent. A systematic review from 2016 supported a direct association between peri-implantitis and the periopathogen red cluster bacteria, P. gingivalis, T. denticola, and T. forsythia, and the orange cluster bacteria, P. intermedia and Campylobacter rectus. P. gingivalis and T. forsythia were the most common bacterial species. Although less studied, C. rectus has also been reported to be associated with periodontitis

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### Table 1 Mean (standard deviation) populations (in millions) of the five key bacterial species at the different sampling points

| Time   | Group  | P. gingivalis | T. forsythia | T. denticola | F. alocis | P. intermedia |
|--------|--------|--------------|--------------|--------------|-----------|---------------|
| T1     | Control | 0.68 (0.63)  | 0.49 (0.47)  | 0.19 (0.13)  | 0.02 (0.02) | 0.01 (0.02)   |
| Test   | Control | 0.75 (1.01)  | 0.39 (0.45)  | 0.23 (0.24)  | 0 (0.01)   | 0 (0)         |
| T2     | Control | 0.21 (0.28)  | 0.19 (0.28)  | 0.15 (0.15)  | 0.08 (0.19) | 0.04 (0.10)   |

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### Table 2 Mean (standard deviation) populations (in millions) of the five key bacterial species at the different sampling points

| Group   | P. gingivalis | T. forsythia | T. denticola | F. alocis | P. intermedia |
|---------|--------------|--------------|--------------|-----------|---------------|
| No defect (control) | 1.91 | 0.71 | 1.45 | 2.48 | 1.64 (0.75) |
| Preformed defect (test) | 2.02 | 1.73 | 2.08 | 1.54 | 1.84 (0.22) |

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### Table 3 Mean defect depths (mm) of ligature-induced peri-implantitis in the preformed (test) and non-preformed (control) bone defects after 2 months

| Group   | Dog (no. of implants) | Mean defect depth (mm) |
|---------|-----------------------|------------------------|
| No defect (control) | 1 (5) | 0.91 |
| Preformed defect (test) | 2 (6) | 0.71 |

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### Fig. 7 Changes in defect depth and peri-implant bone height. A. Box-plots showing median (IQR) for mesial sites, distal sites, and total in the non-preformed and preformed groups; B. Box-plots showing the change in peri-implant bone height after ligature placement
Furthermore, in a recent canine study, an early increase in the T. forsythia population after ligature placement was associated with an increase in the pocket probing depth (PPD) [22]. These findings are consistent with the present observations.

In contrast, Dabdoub et al. [23], in 2013, found in a representative cohort in which peri-implantitis-infected implants showed peri-pathogenic bacteria at only 37% of the sites examined. These observations were partially corroborated by a systematic review in 2017, which – on the one hand – described periodontitis and peri-implantitis as different entities in terms of microbial composition, peri-implantitis showing a more heterogeneous and complex microbiome than periodontitis predominated by non-culturable Gram-negative species. On the other hand, red and orange complex bacteria, i.e. P. gingivalis and P. intermedia, are encountered more frequently in peri-implant inflammation than in healthy gums [24]. Consequently, the authors indicated the need for more research.

In the present study, the patterns of peri-pathogenic bacteria, in terms of distribution and amount, found in “preformed” defects mirrored those formed in “non-preformed” defects after ligature-induced inflammation and tissue breakdown. A comparison between the two groups sampled during the same experimental period revealed that most implants (85.2%) showed a general overlap of their bacterial compositions. The detection rate of P. intermedia was only 39.7% and only small numbers were present, suggesting a limited role of this bacterium in peri-implantitis. A comparison of TML values at the different sampling points revealed a general decrease around implants and apparently around control teeth. The TML values for the “non-preformed” and “preformed” groups at T1 were similar, suggesting that the tapered drill did not influence the control marker values. TML values were comparably decreased in the “non-preformed” and “preformed” groups, suggesting an immune reaction and a reduction in the extent of the peri-implantitis.

In conclusion, preformed peri-implant crater-shaped bone defects showed comparable results in terms of TML, the incidence of periodontal pathogenic bacteria, and defect depth after 2 months of ligature-induced peri-implantitis in this canine model. These results suggest that the protocol used in the present peri-implantitis model does not create any differences in defect size or microbial composition.

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Conflict of Interest

Yann Gager is an employee at Genolytic Co., which supervised the laboratory work and analysis using the microbiological kit iai Padotest. Benjamin Pippenger works at Institut Straumann AG, which provided the materials for this study.

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