Microbiologic and Radiographic Analysis of Ligature-induced Peri-implantitis with Different Dental Implant Surfaces

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Purpose: *The goal of this study was to evaluate microbiota and radiographic peri-implant bone loss associated with ligature-induced peri-implantitis.* ***Materials and Methods:*** *Thirty-six dental implants with 4 different surfaces (9 commercially pure titanium, 9 titanium plasma-sprayed, 9 hydroxyapatite, and 9 acid-etched) were placed in the edentulous mandibles of 6 dogs. After 3 months with optimal plaque control, abutment connection was performed. On days 0, 20, 40, and 60 after placement of cotton ligatures, both microbiologic samples and periapical radiographs were obtained. The presence of* Actinobacillus actinomycetemcomitans*,* Porphyromonas gingivalis*,* Prevotella intermedia/nigrescens*,* Campylobacter *spp,* Capnocytophaga *spp,* Fusobacterium *spp, beta-hemolytic* Streptococcus*, and* Candida *spp were evaluated culturally.* ***Results:*** P intermedia/nigrescens *was detected in 13.89% of implants at baseline and 100% of implants at other periods.* P gingivalis *was not detected at baseline, but after 20 and 40 days it was detected in 33.34% of implants and at 60 days it was detected in 29.03% of dental implants.* Fusobacterium *spp was detected in all periods. Streptococci were detected in 16.67% of implants at baseline and in 83.34%, 72.22%, and 77.42% of implants at 20, 40, and 60 days, respectively.* Campylobacter *spp and* Candida *spp were detected in low proportions. The total viable count analysis showed no significant differences among surfaces (P = .831), although a significant difference was observed after ligature placement (P < .0014). However, there was no significant qualitative difference, in spite of the difference among the periods. The peri-implant bone loss was not significantly different between all the dental implant surfaces (P = .908).* ***Discussion and Conclusions:*** *These data suggest that with ligature-induced peri-implantitis, both time and periodontal pathogens affect all surfaces equally after 60 days.* (Int J Oral Maxillofac Implants 2003;18:383–390)

Key words: *animal study, dental implants, peri-implantitis, periodontal diseases, surface characteristics*

Healthy soft and hard peri-implant tissue around dental implants is essential for long-term success.[1,2] The relationship between different dental implant surfaces and bacterial biofilm in peri-implantitis development has not been studied thoroughly. Cross-sectional microbiologic studies of dental implants with clinically healthy marginal peri-implant tissues in humans[3–8] and animals[9,10] have demonstrated a scattered submucosal microbiota dominated by facultative Gram-positive cocci and rods. In contrast, failing dental implants have been associated with periodontal pathogens, such as *Fusobacterium*, spirochetes, *Actinobacillus actinomycetemcomitans*, the black-pigmented species *Porphyromonas gingivalis* and *Prevotella intermedia*, and *Campylobacter rectus*.[3,11–13] These bacterial shifts have been reported to be caused by peri-implant bone loss, resulting in osseointegration failure.[10,14]

The importance of microbiologic factors in the development and progression of pathologic conditions in the tissues supporting dental implants is controversial. In addition, studies seeking to determine which implant surface (microstructure) or coating is more favorable for the progression of peri-implantitis are scarce.

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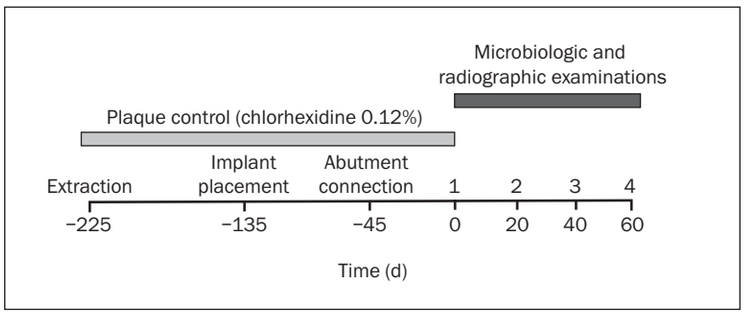

Fig 1 Outline of the experiment. Ligatures were placed around the implants on day 0 and were changed every 20 days, when microbiologic and radiographic procedures were performed. Animals: n = 6; implants: n = 36.

Fig 2 Dental implant surfaces used in this experiment. *(Left to right)* Titanium plasma-sprayed, hydroxyapatite, machined surface on first 3 threads and acid-etched surface on the other threads, and commercially pure titanium.

Therefore, the aims of this study were *(1)* to identify, by culture tests, the presence of periodontal pathogens, and *(2)* to evaluate peri-implant bone loss by standardized radiography in ligature-induced peri-implantitis in dogs with endosseous implants having different surfaces.

MATERIALS AND METHODS

Animals and Anesthesia

Six adult, systemically healthy, male mongrel dogs were used. Dogs were 2 years old with an average weight of 18 kg. Animal selection, management, and surgical protocol routines were approved by the Institutional Animal Care and Use Committee at the Dental School at Araraquara. All surgical and clinical procedures, as well as the removal of microbial samples, were performed under general anesthesia accomplished by 0.05 mg/kg of subcutaneous preanesthesia sedation (atropine sulfate) and intravenous injection of chlorpromazine and thiopental.

Oral prophylaxis was performed for 2 weeks before teeth extraction. All mandibular premolars were then extracted, creating an edentulous ridge, and both the mandibular quadrants and the alveoli were allowed to heal for a period of 3 months. The maxillary premolars were extracted to avoid occlusal trauma interference. Plaque control was instituted during the healing period by scrubbing daily with 0.12% chlorhexidine and scaling and root planing once a month, until ligature placement (Fig 1).

Implant Design

Thirty-six dental implants with 4 different surfaces involving 3 different implant systems were used in this study. Nine commercially pure titanium (cpTi) implants (3i/Implant Innovations, Palm Beach Gardens, FL); 9 titanium plasma-sprayed (TPS) implants (Esthetic Plus; ITI/Straumann, Waldenburg, Switzerland), 9 hydroxyapatite (HA) -coated implants (Calcitek; Sulzer Medica, Carlsbad, CA); and 9 hybrid-surface implants (machined titanium in the first 3 threads and acid-etched in the other threads, Osseotite; 3i/Implant Innovations) were used. All implants had lengths of 10 mm and diameters of 3.75 mm (except the TPS, which had a diameter of 4.1 mm) (Fig 2).

Surgical Procedures

Under aseptic surgical conditions, the dental implants were placed after preparation of a full-thickness flap. The recipient sites were prepared using original instruments for each dental implant surface, according to the surgical techniques indicated by each implant manufacturer. The implants were randomly distributed among the dogs, so that each dental implant surface was represented at least once in each animal (Table 1). The implants were placed at the bone level, and a cover screw was attached to the implant, including the TPS dental implant, which had been modified in placement technique as indicated by the manufacturer. The flaps were sutured with single interrupted sutures to submerge all implants. Antibiotic coverage with potassium and sodium benzyl penicillin was given once a week for 2 weeks to prevent postsurgical infection. Paracetamol was given for pain control,

Table 1 Distribution of Dental Implants with Different Surfaces in 6 Dogs

	Right side			Left side		
Animal	PM2	PM3	PM4	PM2	PM3	PM4
1	cpTi	Acid	TPS	TPS	HA	Acid
2	cpTi	TPS	HA	HA	Acid	cpTi
3	HA	Acid	cpTi	cpTi	TPS	HA
4	TPS	HA	Acid	Acid	cpTi	TPS
5	HA	Acid	cpTi	cpTi	TPS	HA
6	TPS	HA	Acid	Acid	cpTi	TPS

PM2, PM3, PM4 = Mandibular premolars; cpTi = commercially pure titanium; TPS = titanium plasma-sprayed; HA = hydroxyapatite-coated; Acid = acid-etched surface.

and the sutures were removed after 10 days. A soft diet was instituted postsurgically.

After a healing period of 3 months, healing abutments were connected, according to the indication of each dental implant system. After 45 days and healing of the soft tissue, cotton floss ligatures were placed in a submarginal position around the dental implants and sutured in the peri-implant mucosa to hold the ligatures in position. The positions of the ligatures were checked twice a week. Peri-implant bone loss was accelerated by tying further ligatures at 20-day intervals for a period of 60 days, or until the implants had a loss of about 40% of radiographic bone height.[15]

Microbial Samples

Peri-implant microbial samples were obtained with paper points immediately before ligature placement and 20, 40, and 60 days after ligature placement from the mesiodistal sites of all dental implants, as described by Slots and Listgarten.[16]

Supramucosal debridement at the sample site was initially performed with a sterile plastic curette and dry gauze after isolation from saliva using cotton tips/wool and suction. Four sterile paper points were subsequently inserted into the peri-implant sulci, as far apical as possible, for a period of 20 seconds, at baseline and immediately after removal of the ligatures at 20, 40, and 60 days. The paper points and cotton floss ligatures were removed and placed into 3-mL vials containing viability-medium Göteborg anaerobically (VMGA) III.[17] All samples were collected by the same operator and coded by an assistant for blind identification. The microbiologic procedures were initiated within 24 hours.

The samples were centrifuged for 60 seconds and serially diluted tenfold in peptonated water to between 10^{-1} and 10^{-6} for quantitative evaluation of colony-forming units/milliliter (CFU/mL) and to obtain isolated colonies for qualitative identification. Aliquots of 0.1 mL of the dilutions were plated onto enriched tryptic soy agar (ETSA)[18] and tryptic soy-serum-bacitracin-vancomycin (TSBV) agar[19] in a standardized manner. ETSA plates were incubated in anaerobic jars containing an atmosphere with mixed gas (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at 37°C for 7 to 10 days. TSBV agar plates were incubated in a 5% carbon dioxide atmosphere for 5 days at 37°C. The bacterial species were identified from anaerobic cultures based on gram-stain, aerotolerance, colony morphology, esculin hydrolysis,[20] [alpha]-glucosidase and N-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolysis,[21] oxidase, and catalase activities. Total viable count (TVC) and cultivable microbiota, including *P gingivalis*, *P intermedia/nigrescens*, *Fusobacterium* spp, *Capnocytophaga* spp, *beta-hemolytic Streptococcus*, *Campylobacter* spp, and *A actinomycetemcomitans* detection, were performed based on colony morphology and positive catalase tests.[19] *Candida* spp identification was also performed.

Radiographs

Baseline periapical radiographs were taken at the time of ligature placement and at 20, 40, and 60 days after ligature-induced peri-implantitis to evaluate changes in bone levels. The standardized radiographs were obtained with a customized occlusal index fabricated from a film holder by affixing a silicone bite block made of polyvinyl siloxane putty impression material.

A dental x-ray machine equipped with a 35-cm-long cone was used to expose the periapical intraoral film (Agfa Dentus, Size 0; Agfa Gevaert, Mortsel, Belgium). Exposure parameters were 70 kV (peak), 15 mA, and 0.25 seconds at a focus-to-sensor distance of 37 cm. The linear distance between a fixed point in the abutment and the first visible bone-to-implant contact was determined at the mesial and distal of each implant digital image. The

Table 2 Microorganisms Detected During the Experiment

	0 days				20 days				40 days				60 days			
Microorganism	cpTi	TPS	HA	Acid	cpTi	TPS	HA	Acid	cpTi	TPS	HA	Acid	cpTI*	TPS	HA†	Acid‡
P gingivalis	0	0	0	0	4	2	4	2	4	2	4	2	2	2	3	2
P intermedia/ nigrescens	2	0	2	1	9	9	9	9	9	9	9	9	7	9	8	7
Campylobacter spp	0	0	0	0	2	1	0	1	4	1	0	1	1	1	0	1
Fusobacterium spp	2	0	3	0	6	7	6	5	6	8	7	6	5	7	7	6
Beta-hemolitic *streptococcus*	3	1	3	0	7	8	7	8	6	6	6	8	6	6	6	6
Candida spp	0	0	0	0	2	1	2	1	0	0	0	0	0	0	0	0

*n = 7; †n = 8; ‡n = 7: Dental implants excluded because of 40% radiographic bone loss at 40 days.

mesial and distal values were averaged to obtain a mean implant value. Relative peri-implant bone loss was measured to avoid interference by the different dental implant macrostructures used in this study.

All measurements were made independently by 2 of the authors. If discrepancies were 0.5 mm or less, the mean value of the 2 measurements was used. In situations with greater discrepancies, the images were analyzed again and discussed until consensus was reached.

Data Analysis

The TVC were transformed into CFU/mL using predetermined conversion factors to account for dilution and the size of the evaluated surface on the plate. Data were then analyzed with respect to dental implant surface, time of ligature placement, and relative bone loss via nonparametric analysis of variance (Kruskal-Wallis test) with alpha equal to .05. Differences between groups were assessed by the Dunn test. Microorganism analysis was performed after logarithmic transformation. All tests were stratified according to the dog (unit of analysis), ie, n = 6.

RESULTS

Microbiologic Analysis

Microbiologic data were available for analysis from 36 sites/implants in 6 dogs (6 sites per animal). Five implants (2 cpTi, 1 HA, and 2 acid-etched) did not receive additional ligatures after 40 days of ligature induction since they already demonstrated 40% bone loss; therefore at 60 days, just 31 implants were analyzed. Therefore, 139 microbiologic samples were analyzed over the experimental period.

Table 2 summarizes the positive samples for each dental implant surface at all times for each microorganism. *A actinomycetemcomitans* and *Capnocytophaga* spp could not be detected in any of the samples examined.

In the TVC, there were no statistically significant differences between the dental implant surfaces ($P = .813$). However, after ligature placement, statistical significance was observed among the different time periods ($P \leq .0024$) (Fig 3a). The numbers of pathogens found taken following ligature breakdown increased. The TPS and acid-etched surfaces were observed, on average, to be less colonized. With respect to time, the baseline measurement was statistically significantly different from measurements taken at the other times.

P gingivalis was not detected at baseline. At other times, low colonization was detected: 12 dental implants (4 cpTi, 2 TPS, 4 HA, and 2 acid-etched) were colonized at days 20 and 40. At day 60, the number of implants testing positive decreased; 2 cpTi implants and 1 HA implant did not receive ligatures because there was already 40% peri-implant bone loss. There were no significant quantitative differences among the dental implant surfaces ($P = .704$) between days 0 and 20, 40, and 60 ($P > .05$) (Fig 3b).

P intermedia/nigrescens was detected at baseline on 5 dental implants (2 cpTi, 2 HA, and 1 acid-etched). At days 20, 40, and 60, all implants were colonized. The quantitative difference was not significant among implant surfaces ($P = .877$); significance was observed between day 0 and days 20, 40, and 60 ($P \leq .0033$) (Fig 3c).

Fusobacterium spp was identified on 4 dental implants (2 cpTi and 2 HA) at baseline. At days 20, 40, and 60, respectively, 24 implants (6 cpTi, 7 TPS, 6 HA, and 5 acid-etched); 27 implants (6 cpTi, 8 TPS, 7 HA, and 6 acid-etched); and 25 implants (5 cpTi, 7 TPS, 7 HA, and 6 acid-etched) implants were colonized by *Fusobacterium* spp. Significant differences were observed between day 0 and days 20, 40, and 60 ($P \leq .047$), except for the cpTi surface ($P = .143$). There was no significant difference between the different dental implant surfaces ($P = .375$) (Fig 3d).

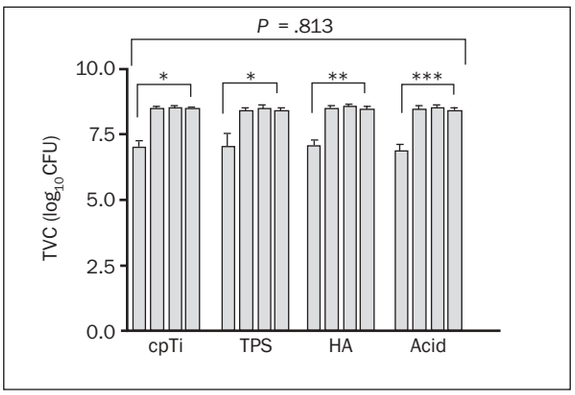

Fig 3a Means and standard deviations of total viable count (TVC) on different dental implant surfaces at baseline and 20, 40, and 60 days. *P = .0015; **P = .0014; ***P = .0024.

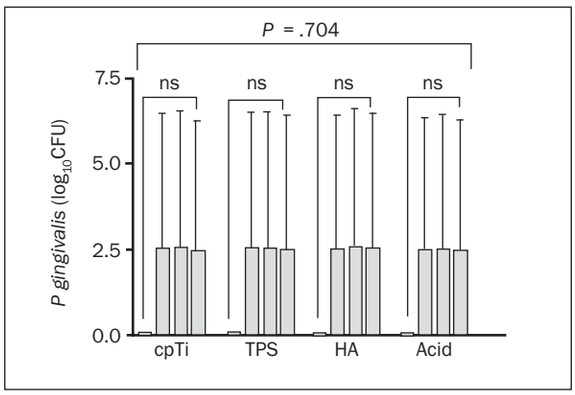

Fig 3b Means and standard deviations of *P gingivalis* on different dental implant surfaces at baseline and 20, 40, and 60 days. ns = non-significant ($P > .05$).

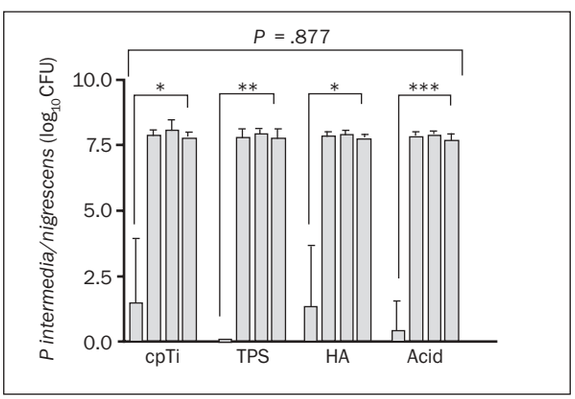

Fig 3c Means and standard deviations of *P intermedia/nigrescens* on different dental implant surfaces at baseline and 20, 40, and 60 days. *P = .002; **P = .003; ***P = .0008.

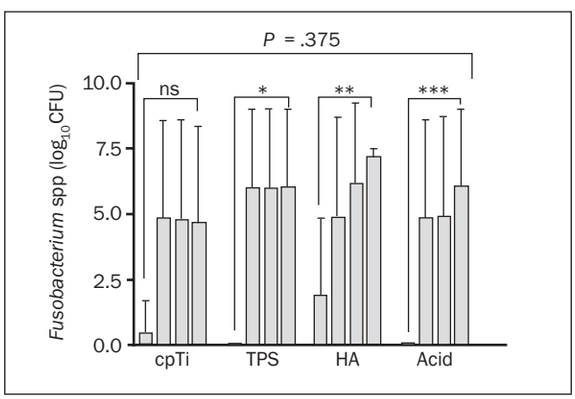

Fig 3d Means and standard deviations of *Fusobacterium* spp on different dental implant surfaces at baseline and 20, 40, and 60 days. ns = non-significant ($P > .05$); *P = .018; **P = .028; ***P = .047.

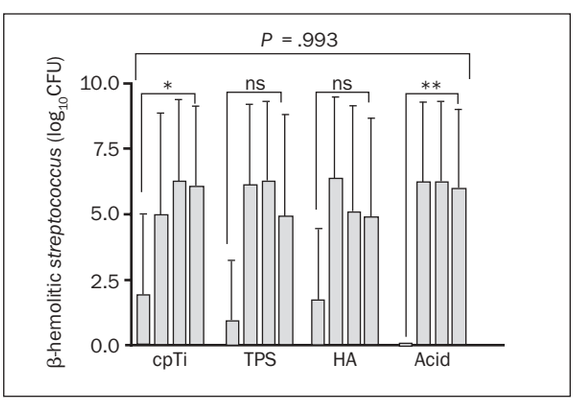

Fig 3e Means and standard deviations of beta-hemolytic *Streptococcus* on different dental implant surfaces at baseline and 20, 40, and 60 days. ns = non-significant ($P > .05$); *P = .028; **P = .013.

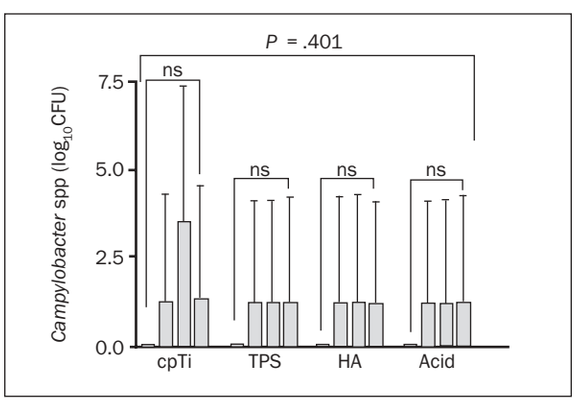

Fig 3f Means and standard deviations of *Campylobacter* spp on different dental implant surfaces at baseline and 20, 40, and 60 days. ns = non-significant ($P > .05$).

Seven dental implants (3 cpTi, 1 TPS, and 3 HA) tested positive for beta-hemolytic *Streptococcus* at baseline. At the other time points, 30 implants (7 cpTi, 8 TPS, 7 HA, and 8 acid-etched); 26 implants (6 cpTi, 6 TPS, 6 HA, and 8 acid-etched); and 24 implants (6 cpTi, 6 TPS, 6 HA, and 6 acid-etched) were colonized at days 20, 40, and 60, respectively. Differences among the dental implant surfaces were not observed (P = .993), although a significant difference between times was demonstrated for the cpTi and acid-etched surfaces ($P \leq .0284$) (Fig 3e).

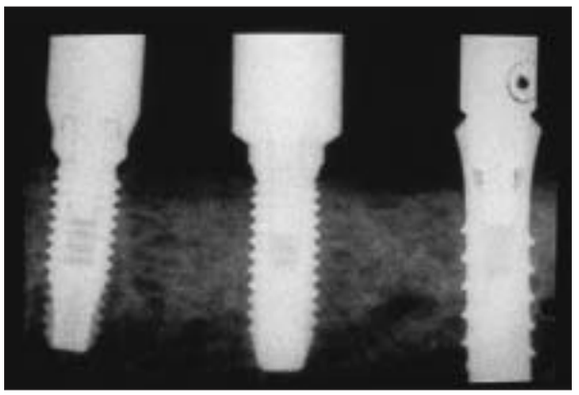

Fig 4a Periapical radiograph taken at baseline.

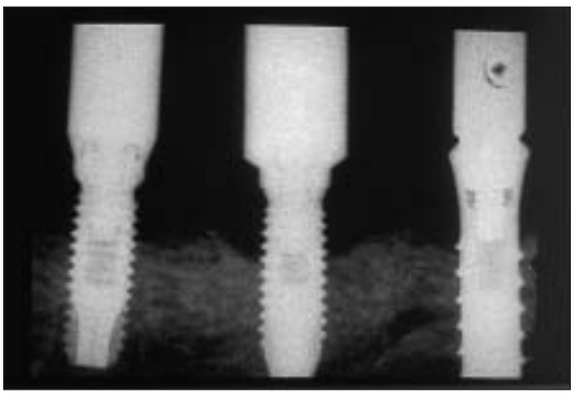

Fig 4b Periapical radiograph taken at 60 days after ligature placement.

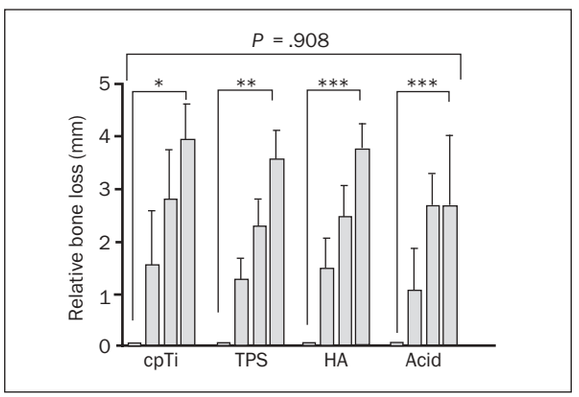

Fig 5 Means and standard deviations of radiographic bone loss on different dental implant surfaces at 20, 40, and 60 days. *P = .005; **P < .0001; ***P = .0001.

Campylobacter spp was not identified at baseline. However, it was detected at days 20, 40, and 60 in 4 (2 cpTi, 1 TPS, and 1 acid-etched); 6 (4 cpTi, 1 TPS, and 1 acid-etched); and 3 (1 cpTi, 1 TPS, and 1 acid-etched) dental implants, respectively. Therefore significant differences were not observed among dental implant surfaces (P = .425) or time periods ($P > .05$) (Fig 3f).

Candida spp was isolated at only 6 dental implants (2 cpTi, 1 TPS, 2HA, and 1 acid-etched) at day 20.

Radiographic Analysis

At the start of ligature-induced peri-implantitis, the linear distance between a fixed point and first relative peri-implant bone loss was measured to avoid interference from the different macrostructures of the dental implants utilized in this study. The radiographically measured mean bone loss was observed at days 20, 40, and 60 (Figs 4a and 4b). No dental implant exhibited peri-implant radiolucencies at baseline.

Significant differences were not found between surfaces (P = .908), despite the fact that the relative means of the TPS (1.79 ± 1.52 mm) and the acid-etched surfaces (1.62 ± 1.32 mm) were lower than those of the HA (1.94 ± 1.59 mm) and cpTi (2.09 ± 1.70 mm) implants among the time periods (Fig 5). Significant differences ($P \leq .005$) were found between baseline and the other time points.

DISCUSSION

In this study, it was observed that ligature-enhanced bacterial biofilm accumulation around different dental implant surfaces resulted in rapid peri-implant tissue breakdown. Radiographically significant peri-implant bone loss was established within 60 days.

Tissue breakdown around different dental implant surfaces was accomplished by bacterial shift in a relatively short period (20 days), in agreement with Schou and coworkers[9] and Nociti and associates.[22] Other reports evaluated just the microbiota for longer periods: Hanisch and coworkers[10] for 10 months and Tillmanns and colleagues[14] for 3 months. However, all reports found similar microbiota before and after ligature placement. The increase in radiographic bone loss takes place between days 0 and 60 in dogs, not because of mechanical trauma from the ligature, but as the result of peri-implant microbiota. These data are in accordance with those of Zappa and Polson[23] and Schou and associates.[24] However, Tonetti[25] disagreed with this statement, and further studies could answer this question. Persson and coworkers[26] reported that 6 weeks after ligature placement, about 20% bone loss was observed. Ligature placement was (in 8 weeks) able to rapidly induce significant peri-implant bone loss, comparable with the studies of Hanisch

and coworkers,[10] Tillmanns and coworkers,[14] Lang and associates,[27] and Hurzeler and colleagues.[28]

Some important differences between the types of surfaces that affect dental implant microstructure and ultrastructure seem to influence adsorption and bacterial colonization. Statistical difference was not observed for TVC, although lower counts for TPS and acid-etched implants were observed. Several studies[29,30] have shown that the presence and density of periodontal pathogens were influenced more by oral status than by the implant surface characteristics. Although this study used ligatures to facilitate bacterial colonization, TVC means were slightly higher on the cpTi than on the TPS surface. It is speculated that the smooth surface present on the neck of TPS dental implants could explain these data. In the case of the acid-etched surface, which has a machined surface on the first 3 threads and a treated surface on the other threads, the difference in results versus the cpTi surface could be explained by oxide present after acid treatment. The presence of different oxides could influence the affinity of bacterial lipopolysaccharide for these components.[31] In addition, the sample size and the short period evaluated in the present study could explain these microbiologic and radiographic data.

In this study, *P gingivalis* and *P intermedia/nigrescens* were associated with the induction and progression of peri-implantitis, as well as with periodontal diseases.[32] *Fusobacterium* spp and *Campylobacter* spp, which were also identified on some dental implant surfaces, have also been associated with peri-implant diseases, according to Papaioannou and coworkers,[33] Macuch and Tanner,[34] and Mombelli and associates.[35] The greatest increase in bone loss was accomplished when these microorganisms were detected. These microbiologic results confirm those of Mombelli and coworkers,[3,35,36] Schou and associates,[9] Hanisch and colleagues,[10] Tillmanns and coworkers,[14] Lee and colleagues,[7] and Listgarten and Lai.[37]

The presence of beta-hemolytic *Streptococcus* agrees with the results of Hanisch and associates,[10] although these bacteria were not found in the same proportion. This bacterium was detected on 5 implants (62.5%) at 10 months in the study by Hanisch and associates,[10] in comparison to 30 implants (83.3%) at 20 days after ligature placement in the present study. The absence of this microorganism in the buccal cavity or at low frequency[38] indicates the existence of a low pH resulting from the induced peri-implantitis.[39] Leonhardt and coworkers[40] reported the presence of *Candida* spp in association with failing implants, in accordance with the present data.

The absence of *A actinomycetemcomitans* and *Capnocytophaga* spp is not in accordance with the reports of Renvert and coworkers,[41] Schou and coworkers,[9] Hanisch and associates,[10] and Tillmanns and associates.[14] The difference between the results of this study and the aforementioned studies is possibly related to diet, time of evaluation, marginal inflammation, ligature materials, use of chlorhexidine and antibiotics, and different microbiologic methods (culture media, polymerase chain reaction, and DNA probes).

CONCLUSIONS

In the present investigation, the association between increased viable counts of periodontal pathogens and peri-implant bone loss was evident. Thus, within the limits of this study, it can be concluded that: *(1)* there was no quantitative significant statistical difference, considering the TVC on the different implant surfaces, without qualitative difference; *(2)* a bacterial shift had taken place by 20 days after ligature placement; and *(3)* coated dental implant surfaces may be as susceptible as smooth surfaces to ligature-induced peri-implantitis in 60 days.

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