CLINICAL ORAL IMPLANTS RESEARCH

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The relationship between interimplant distances and vascularization of the interimplant bone

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Abstract

Background: Long-term success of the implant restorations is based upon the biology and vasculature of the bone surrounding the implants, especially for the bone between two implants.

Purpose: The aim of this study was to evaluate how loaded implants placed 2 or 3 mm apart influence bone vessel organization.

Material and methods: Six mongrel dogs were used for the study. The four mandibular premolars were extracted and 3 months later, four 4.5×10 mm implants were placed on each side of the mandible. The implants were placed so that two adjacent implants were 2 mm (group 1) or 3 mm (group 2) distant from each other. After 12 weeks, the implants were loaded with provisional prostheses, then metallic crowns were placed 4 weeks later. Both temporary and metallic restorations were made so that the distance between the contact point and the bone crest was 5 mm. The animals were sacrificed after 8 weeks. The hemi-mandibles were removed and prepared for analysis. The interimplant bone vasculature of the two groups was studied using scanning electron microscopic images fractal analysis. The fractal dimension (D_f) was calculated using the box-counting method.

Results: The values of the D_f for the blood vessels were significantly higher ($P<.05$) in the specimens of the group 2 (1.969 ± 0.169) than the group 1 (1.556 ± 0.246).

Conclusion: The presence of more blood vessels in the group 2 is another indication that 3 mm is a preferable distance for contiguous implants than the 2 mm distance.

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Multiple contiguous implants pose a greater challenge for aesthetic outcome than single implants between teeth, especially the formation of interimplant papillae and the related crestal bone remodeling. There are many factors that play a role in this situation and interimplant distance is an important one. Scarano et al. (2004) studied bone remodeling around interimplant distances of 2, 3, 4 and 5 mm in dogs and concluded that vertical bone loss was greater with 2 mm of interimplant distance, (1.98 mm) and decreased as the distance between the implants increased, the 5 mm group had only 0.23 mm of crestal bone loss. Their results considered the distances of 2 and 3 mm as the critical distances for crestal bone remodeling. Recent studies in dogs (Novaes et al. 2006a, 2006b; Oliveira et al. 2006; Papalexiou et al. 2006) have compared interimplant distances of 1, 2 and 3 mm with bridges made with a distance from the contact point to the bone crest of 5 mm and evaluated how this would influence interimplant papillae formation and crestal remodeling. Their results did not obtain either satisfactory papillae formation or differences in crestal bone remodeling and suggested that the distance from the contact point to the bone crest should be about 3 mm to compensate for the crestal bone resorption that occurs for the formation of the biologic seal. This was confirmed very recently in a study by Novaes et al. (2009a), where good papillae formation was obtained around implants with bridges with a contact point to the bone crest of 3 mm.

The issue of interimplant distance has focused on the cervical region of the implant and whether or not papillae were obtained. Although these issues are very important, the effect that loaded implants with different interimplant distances could have on the biology of the bone surrounding the entire implants are also important for the long-term success of the treatment. Novaes et al. (2009b) evaluated through fluorescence analysis the effect that loaded implants with interimplant distances of 1, 2 and 3 mm would have on the rate of bone remodeling when comparing with the control bone, the bone on the free ends of the bridges. It was concluded that the 3 mm distance presented similar bone remodeling rates to the control bone and that the rate was slightly increased for the 2 mm distance and highly increased for the 1 mm distance. It was concluded that regarding the bone remodeling rate, the 3 mm interimplant distance was recommended and that the 1 mm distance should be avoided. In another study, Traini et al. (2008) evaluated the influence that loaded contiguous implants placed 2 or 3 mm apart may have on the bone microstructure and also concluded that implants should be placed at least 3 mm apart because they presented better collagen fiber orientation, relevant larger numbers of marrow cavities and larger quantities of bone of high mineral density. Regardless of all the studies cited, the influence that this situation may have on the blood vessels has not yet been evaluated.

The amount and the distribution of the bone vasculature network should be considered a part of the host defence system.

During peri-implant site preparation and healing, blood vessels are injured, which results in an inversion of the blood circulation from the Volkmann's canals and in the formation of a blood clot around the implants. Blood clot resorption and replacement show the importance of angiogenesis, which provides the nutrient supply for peri-implant healing. The vascular supply has been shown to be important for the *de novo* bone formation, contact osteogenesis and bone remodeling. For a detailed review, see Davies (2003).

The purpose of this study was to evaluate how loaded implants placed 2 or 3 mm apart influenced bone vessel organization; the bone vessel network was analyzed as bone vessels volume density (BVVD) through fractal analysis.

Material and methods

In the present animal study, adequate measures were taken to minimize pain or animal discomfort. The experiments were carried out in accordance with the US guidelines of the National Institute of Health (NIH) in accordance with local laws and regulations for experimental procedures on animals.

Six young adult male mongrel dogs, weighing approximately 10 kg were used. They had intact maxillas and mandibles and no general occlusal trauma. The animals were in good general health and had no viral or fungal oral lesions. The animals were kept fasting since the night before surgery.

They received an intramuscular injection of preanesthetic (2% Rompun, 20 mg/kg, 0.5 ml/10 kg) and were then anesthetized intravenously with thiopental (1 ml/kg; 20 mg/kg thiopental diluted in 50 ml saline). A total flap was raised in the region of the four mandibular premolars and the teeth were sectioned in the buccal-lingual direction and extracted with forceps. The flaps were repositioned and sutured with absorbable 4-0 sutures.

After a healing period of 3 months, the animals received 20,000 IU penicillin and streptomycin (1 g/10 kg) the night before surgery. This dose provides antibiotic coverage for 4 days; thus, another dose was given 4 days later to provide coverage for a total of 8 days. This large spectrum antibiotic is commonly used to treat infections in small animals. After repeating the same sedation and anesthesia as in phase 1, a horizontal crestal incision was made from the distal region of the canine to the mesial region of the first molar and implants were placed according to the manufacturer's instructions. Four 4.5×10 mm Frialit (DENTSPLY Friadent, Mannhein, Germany) implants, blasted and acid-etched surfaces, were placed on each side of the mandible of each animal, totaling 48 implants in the experiment. The implants were placed so that two adjacent implants were 2 mm (group 1) or 3 mm (group 2) distant from each other. For this, a stainless steel device was made to standardize both the angle and the distance between implants. Contralaterally, the distances between implants were repeated, however, with variation in the position of the implants, respecting the random *cross-location* method.

The flaps were repositioned and sutured with absorbable sutures, so that the implants were totally submerged. Sutures were removed after 10 days.

During the healing period, the animals received monthly prophylaxis with ultrasonic points. After 12 weeks, prosthetic restoration was begun. The same method of sedation and anesthesia described previously was followed; the implants were exposed and loaded with provisional acrylic prostheses. These temporary restorations were made so that the distance between the contact point and the bone crest was 5 mm. After 4 weeks, metallic crowns substituted the provisional prostheses, maintaining the distance of 5 mm from the contact point to the bone crest. Prophylaxis continued on a weekly basis.

Following a period totaling 8 weeks of prosthetics, the animals were sedated and then sacrificed with an overdose of thiopental. The hemi-mandibles were removed, dissected and fixed in 4% formalin pH 7 for 10 days, and then transferred to a solution of 70% ethanol until processing. The specimens were dehydrated in increasing concentrations of alcohol up to 100%, infiltrated and embedded in LR White (London Resin Company, Berkshire, UK) resin. Undecalcified longitudinal cut sections of 50 μm were prepared by using a cutting and grinding TT system (TMA2, Grottammare, AP, Italy). The sections were double stained with toluidine blue and fuchsine acid for some samples and silver-enhanced stains for others to be analyzed by the means of an Axiolab microscope (Carl Zeiss, Jena, Germany) under transmitted and polarized light. In addition to the histomorphometric analysis, the presence of blood vessels found in the interproximal bone when the implants were placed 2 or 3 mm apart were studied using fractal analysis.

Special processing for specimens

All the samples were etched with 0.1 N HCl for 24 h, and treated with trypsin (80 U/ml, pH 7.4 at 39°C for 48 h. By this way, the mineralized matrix was degraded and the vessels, the cells and the tissue around vessels remained as these structures were infiltrated by resin so they did not dissolve and could be seen.

Samples belonging to the two different groups, were mounted onto aluminum stubs and sputter gold coated in Emitech K 550 (Emitech Ltd, Ashford, Kent, UK) for surface characterization using a scanning electron microscope (SEM) Zeiss EVO 50 XVP with LaB6 (Carl Zeiss SMY Ltd, Cambridge, UK). From all samples, SEM images at higher magnification and resolution were collected for the analysis.

Basic principles for fractal-based image analysis

Measurements applying the Euclidian geometry equations in a stochastic growth system, such as the bone vasculature, appear to be very difficult and inaccurate, if not impossible. On the other hand, the fractal dimension (D_f) has often been applied as a measure of complexity (Sandau & Kurz 1997). Yet, the D_f of a natural object reveals information not otherwise apparent. A fractal image of a microvascular structure has the advantage to be scale invariant in a given range. The geometry is independent over both the image magnification and resolution (in a given range). Fractal analysis is then a procedure that compares the size or the outline of an object for each scale of measurement. This can be performed using an overimposed square grid. Briefly, grids of various size length (ε), from 1 pixel to 45% of the image area, were used to cover the entire image, and the number of grids that contained a vessel segment, $N(\varepsilon)$, was registered for each grid size. D_f can be obtained by taking the negative of the slope of a double-logarithmic plot between $N(\varepsilon)$ and ε. Stated mathematically, D_f is estimated using the following equation

$$D_f = \lim_{\varepsilon \to 0} \left[\frac{\ln N(\varepsilon)}{\ln(1|\varepsilon)} \right] \tag{1}$$

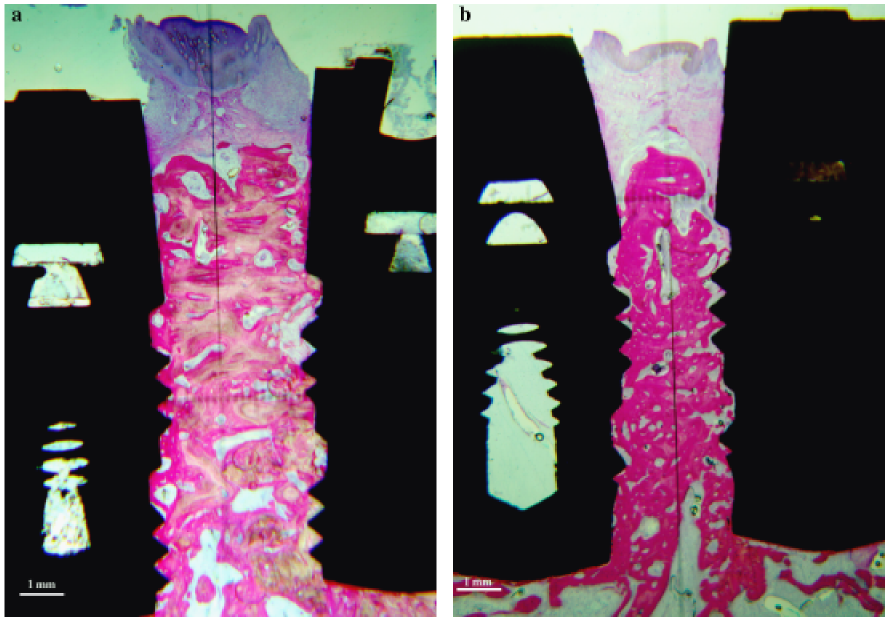

Fig. 1. Representative histological images of the 3 mm interimplant distance (a) and of the 2 mm interimplant distance (b). Original magnification × 12, stain toluidine blue and fuchsine acid.

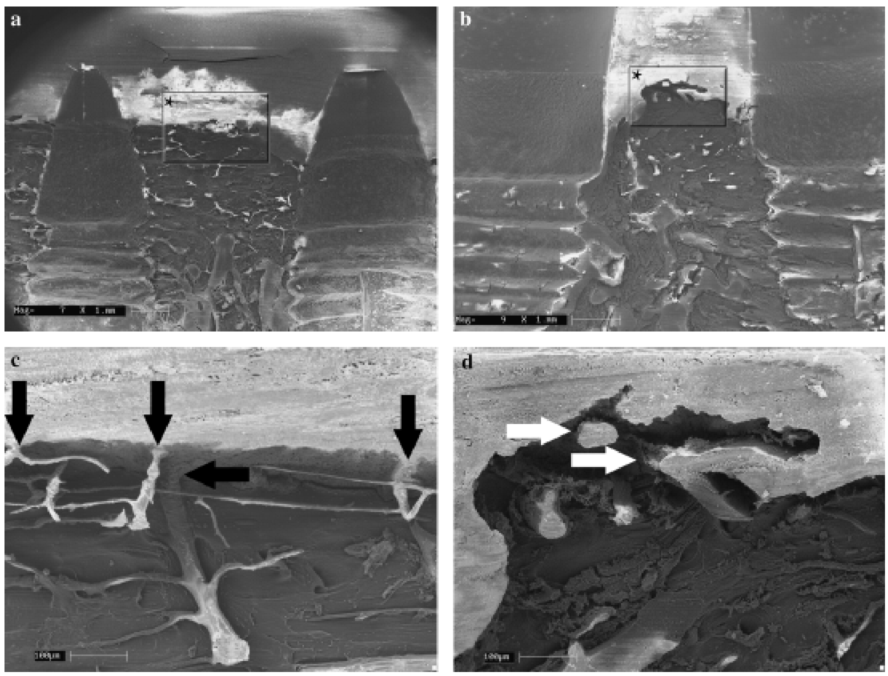

Fig. 2. Scanning electron microscopic images of the vessel network in the interimplant bone of the two groups. (a) The interimplant distance of 3 mm; the square rectangle (*) was reported at a higher magnification (× 500) in Fig. c. (b) The interimplant distance of 2 mm; the square rectangle (*) at higher magnification (× 500) was reported in (d). Black arrows in (c) indicate the exchange vessels between connective tissue (top of the picture) and the bone (bottom of the picture) in the group of 3 mm. White arrows in (d) indicate the vessels in the group of 2 mm of distance. Note the vessel that run only inside the bone tissue without penetrating the connective tissue.

which transforms it into a power law relationship

$$N(\varepsilon)^{D_f} = 1 \tag{2}$$

where the exponent D_f is an index of the complexity of the object and also an indicator of how space filling a structure is.

There are several computer software, such as Fractalyse, Whinrhizo, Image-Pro Plus and FracLac; between them, only FracLac evaluates better D_f for all models (Mancardi et al. 2008).

D_f was calculated using the box-counting method (Baish & Jain 2000) by FracLac, 2.5

release 1b5j, a plugin of Image J (A. Karperien, Charles Sturt University, Australia).

Stated formally, D_f is the derivative of structural detail with respect to magnification (Roberts et al. 1989). For images that consist of lines, D_f always varies between 1 and 2, with the higher value representing greater complexity. In our study, the images were binarized and skeletonized using ImageJ 1.42 g (Wayne Rasband, NIH, Bethesda, MD, USA) and D_f for each image was obtained as the average of four global scans initiated at randomly selected initial points. Because nonlinearity of the double-logarithmic plot occurs as å, it approaches its minimum and maximum, a slope-corrected D_f was used and only the linear portion of the plot in the calculations of complexity was included. This measuring procedure was calibrated with shapes of a known fractal dimension with an inaccuracy of <3%.

Statistical analysis

Statistical analysis of the results was performed using the package Sigma Stat 3.5 (SPSS Inc., Ekrath, Germany). Two sets of tests, one for 3 mm and one for 2 mm, were applied to the D_f data originating from each dog as the mean variation $\pm$ SD of the four sites were evaluated. Comparison was carried out using a nonparametric test as the Mann–Whitney *U*-test as it is more conservative. A Pearson product moment correlation was used to quantify the strength of the association between the variables. A two-sided analysis was considered appropriate and a *P*-value under 0.05 was considered statistically significant.

Results

Representative histological images under a light microscope of both the 3 and 2 mm interimplant distances were reported (Fig. 1a and b). The comparison of vessels organization in the two groups imaged under SEM and measured for fractal dimension are reported in Fig. 2a–d. Results are presented as means $\pm$ standard deviation (SD). The values of the D_f (mean $\pm$ SD) for the blood vessels were significantly higher ($P<.05$) in the specimens of the group with an interimplant distance of 3 mm (1.969 ± 0.169) vs. 2 mm (1.556 ± 0.246) (Fig. 3). The distribution of the exchange in vasculature between the crestal bone and the covering connective tissue in the 3 mm group distance was much more expressive (Fig. 4a and b) while in the 2 mm interimplant distance, it appeared less expressive (Fig. 5a and b).

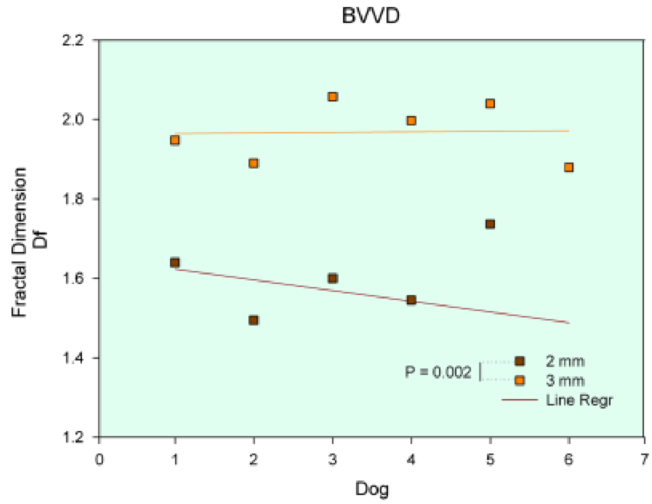

Fig. 3. Comparison of the bone vessels volume density (BVVD) (D_f) between the groups of 2 and 3 mm interimplant distances. (Mann–Whitney *U*-test).

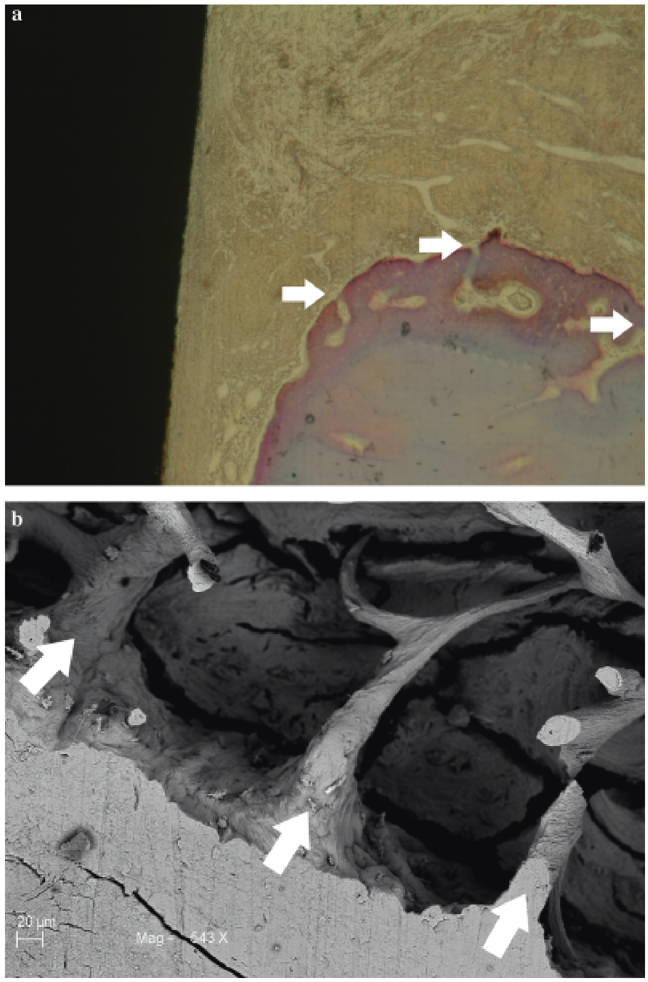

Fig. 4. Comparison between light and scanning electron microscopic images of the 3 mm group. (a) The white arrows indicate some exchange between vessels in the crestal bone and overlaying connective tissue. (b) The white arrows indicate the three dimensional aspect of the interaction of the blood vessels of the connective tissue and the bone.

Statistical results

The Kolmogorov–Smirnov (K–S) test for normality distribution of the data showed a K–S distribution of 0.169 for the group 1 and a K–S distribution of 0.186 for the group 2, both passed with $P > 0.2$. Moreover, the data also passed the equal variance test ($P = 0.321$). Notwithstanding the normality distribution of the data, we preferred to use a more conservative test (Mann–Whitney *U*-test) in light of the relatively small sample size. The following statistical results summarized the comparisons (Table 1). The present results were also graphically summarized (Fig. 6) with data reported previously for the same group of specimens (Novaes et al. 2006b; Papalexiou et al. 2006; Traini et al. 2008). The linear correlation was used to quantify the strength of the association between the BVVD and both the papillae heights and bone loss values reported by Novaes et al. (2006b) and Papalexiou et al. (2006), respectively. The results for BVVD and papillae heights showed correlation coefficients of 0.31 for the group 1 and 0.06 for the group 2, while the correlation between BVVD and bone loss showed correlation coefficients of 0.09 for the group 1 and 0.27 for the group 2. Both the correlation were not statistically significant ($P > .05$).

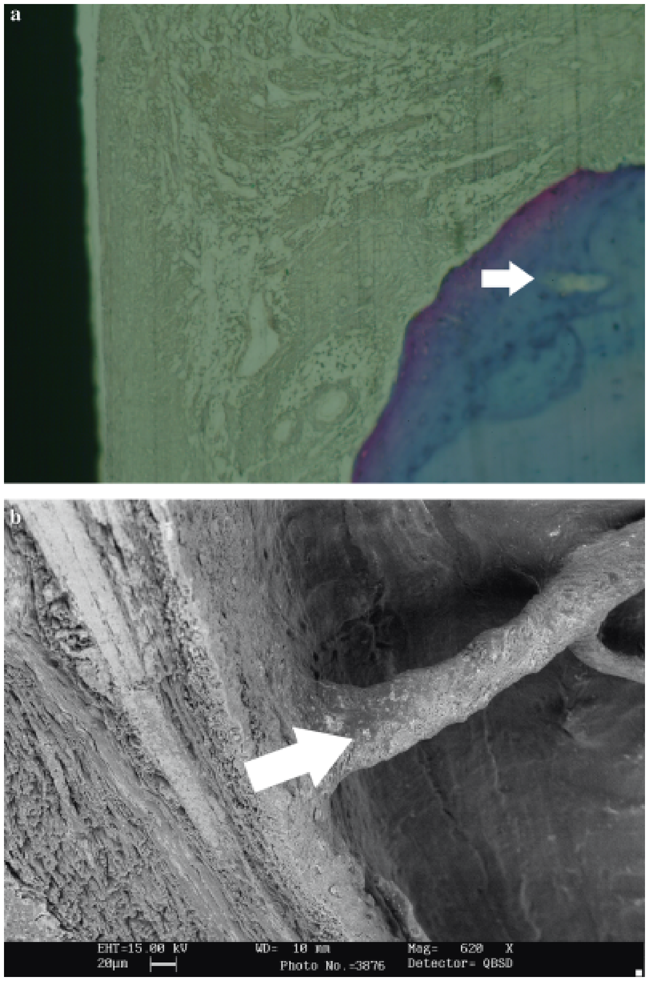

Fig. 5. Comparison between light and scanning electron microscopic images of the 2 mm group. (a) The white arrow indicates only one vessel between the crestal bone and the overlaying connective tissue. (b) The white arrow indicate the three dimensional aspect of the interaction of the blood vessels of the connective tissue and the bone. This group showed a significant reduction in the interactions between the blood vessels.

Discussion

Previous studies have shown that interimplant distance can have different effects on the biology and microstructure of the implant surrounding tissues (Novaes et al. 2006a, 2006b, 2009a, 2009b; Papalexiou et al. 2006; Oliveira et al. 2006; Traini et al. 2008).

Novaes et al. (2009b) using fluorescence microscopy showed that the interimplant distance of 3 mm presented remodeling results similar to bone that was on the free ends of the bridges when compared with 1 or 2 mm interimplant distances.

In another study, Traini et al. (2008) also concluded that implants should be placed at least 3 mm apart because they presented better collagen fiber orientation, relevant larger numbers of marrow cavities and larger quantities of mineral of high density in relation to implants placed 2 mm apart.

Previous studies on the same group of specimens reported the height of the interimplant papilla that had a mean ($\pm$ SD) of 3.07 ± 0.23 mm for group 1 and 3.55 ± 1.42 mm for group 2 without any statistically significant difference ($P > .05$) (Novaes et al. 2006b). Yet, the bone density appeared to be significantly increased in the interimplant bone area only if compared with the distal extension area (Papalexiou et al. 2006). Meanwhile, significant differences in the bone microstructure such as an increase of longitudinally oriented collagen fiber, a relevant extension of marrow spaces, and a higher level of mineralization were reported in group 2 (Traini et al. 2008). Regardless of all the studies cited, the influence that this situation may have on the bone blood vessels has not yet been evaluated.

In this study, the values of the D_f (mean $\pm$ SD) is directly related to the BVVD, which was significantly higher ($P < .05$) in the specimens of the group 2 (1.969 ± 0.169) vs. group 1 (1.556 ± 0.246). The presence of more blood vessels, supported by Davies (2003), is important for *de novo* bone formation, contact osteogenesis and bone remodeling.

Table 1. **The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference ($P = 0.002$)**

Group name	N	Median	25%	75%
2 mm	6	1.573	1.495	1.64
3 mm	6	1.973	1.89	2.04

Mann–Whitney *U* statistic = 36.
$T = 21$; n (small) = 6; n (big) = 6; P (test) = 0.005; P (exact) = 0.002.

To best address the discussion we report to in Fig. 6, all the data belonging to the same group of specimens which have been previously published (Novaes et al. 2006b; Papalexiou et al. 2006; Traini et al. 2008). Any correlation between the papillae height measured radiographically (PH_{Rx}) and the interimplant BVVD was noted ($P > .05$); thus, appears to be independent from the BVVD (Fig. 7). Yet, no significant correlation ($P > .05$) was noted for the crestal bone loss measured both radiographically (BL_{Rx}) and histologically (BL_{Histo}) and the BVVD (Fig. 8). Nevertheless, with relation to the lack of correlation between the BVVD and the crestal bone resorption, it is necessary to state that both the modeling and remodeling processes start as a consequence of the bone fatigue damage related to the occlusal load. The process involves three different mechanisms: Activation of osseous precursor cells (A), Resorption (R) and bone Formation (F). The entire process, called sigma phase, in dogs occurs in 12 weeks (3 weeks for A → R segment and 9 weeks for (F) (Roberts et al. 1989). Considering that the the animals were sacrecifed 12 weeks after implant loading the differences in BVVD appears to be an independent factor in crestal bone resorption after 4 weeks with provisional restoration and 8 weeks with final restorations (12 weeks of loading). Finally, about the mineralization of the newly formed bone and the collagen bundles organization in the bone matrix, both factors appear to be positively correlated to the levels of the BVVD.

Hence, the higher BVVD in the group 2 is another indication that 3 mm is a preferable distance for contiguous implants when compared with 2 mm, Especially if we consider the bone microstructure. At the same time, there was no difference in bone level or papilla formation in the short term, but perhaps the difference in bone vessels might affect it in the long term.

Besides the significant organization of the vessels inside the bone tissue, it was possible to observe some differences in the distribution of the exchange vasculature (perforating canals) between the crestal bone and the covering connective tissue. In the group with an interimplant distance of 3 mm (Fig. 4), much more perforating vessels were noted compared with 2 mm (Fig. 5) in which they appeared to be almost absent. Kramer (1987) reported that for plaque-induced periodontal disease, the emergence from the crestal bone of the blood vessels determines the vulnerability to inflammation of the interdental bone itself. This aspect could play a role in the long term also, notwithstanding the apparent lack of correlation between bone loss and BVVD after 12 weeks. Considering that vessels are housed within the mineralized tissue, the blood supply of the cortical bone is entirely dependent on the arterioles. In cases of inflammation, the limited number of vessels are unable to eliminate the irritant due to the vessel congestion also, this in turn would result in hypoxia and necrosis. Blood vessels play a key role in the bone regeneration and remodeling at peri-implant sites (Mair et al. 2007; Kanczler & Oreffo 2008) and adequate vascularization is a prerequisite for bone development (Roncalli et al. 2008). The development of a microvasculature with microcirculation is of critical importance for the bone tissue homeostasis. In an experimental study using titanium caps in rabbit calvarium, it was found that the generation of new bone was dependent on the blood supply and that the formation

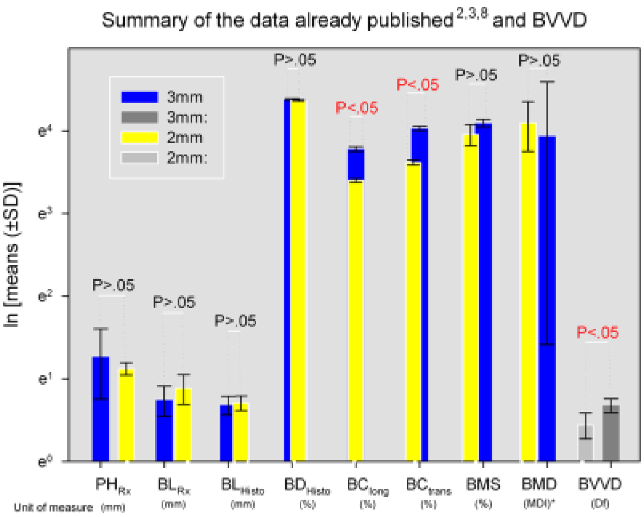

Fig. 6. Summary of the data. PH_{Rx} is the papillae height measured radiographically in Novaes et al. (2006b) – BL_{Rx} is the crestal bone loss measured radiographically in Novaes et al. (2006b) – BL_{Histo} is the crestal bone loss measured histologically in Papalexiou et al. (2006) – BD_{Histo} is the bone density measured histologically in Papalexiou et al. (2006) – BC_{long}, BC_{trans}, BMS and BMD are the bone collagen longitudinally oriented fibers, the bone collagen transversally oriented fibers, the bone marrow spaces and the mean of bone mineral density respectively, in Traini et al. (2008) – BVVD is the bone vessels volume density. (*) for the definition of the mineral density index (MDI) we refer to Traini et al. (2008).

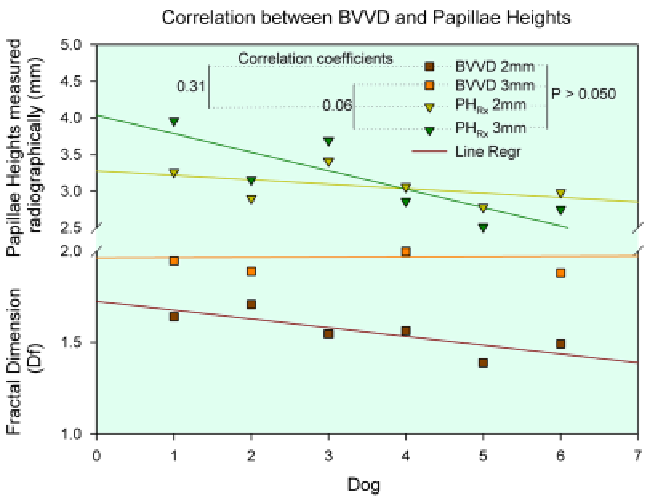

Fig. 7. A Pearson product moment correlation between bone vessels volume density and papillae height.

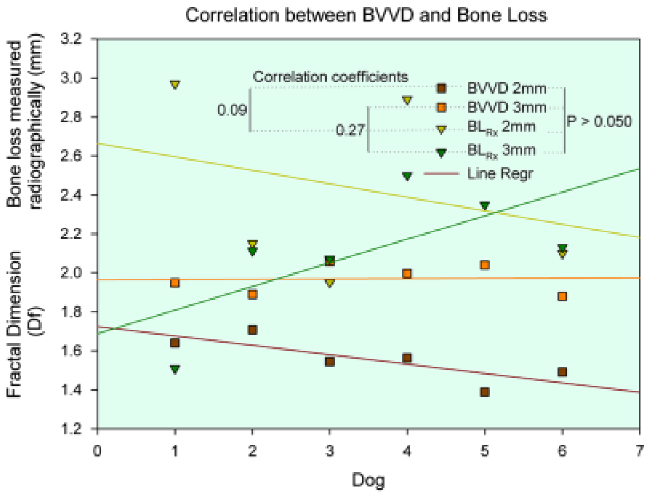

Fig. 8. A Pearson product moment correlation between bone vessels volume density and bone loss.

of mineralized bone along the inner portion of the caps depended on angiogenesis preceding bone formation (Yamada et al. 2008). Deficient angiogenesis is thought to contribute in a significant way to a failure of bone formation or remodeling (Matsumoto et al. 2008), and osseointegration may be impaired in the presence of compromised blood vessels formation, e.g. diabetics, smokers, radiotherapy (Mair et al. 2007), severe skeletal injuries (Roncalli et al. 2008). Yet, the use in a rabbit study of an anti-angiogenic substance decreased peri-implant bone formation (Mair et al. 2007). In two human histologic and immunohistochemical studies of sinus augmentation procedures using autogenous bone and anorganic bovine bone, a close temporal and spatial relationship was found between angiogenesis and newly formed blood vessels, which were in close contact with the newly formed bone trabeculae (Degidi et al. 2006, 2007). We have finally to consider as a limit of the present paper that the vascularity of the dog is not directly correlated with that of humans; nevertheless, the dog model was used to study the arterial supply of both the palatine mucoperiosteum (Fernandes et al. 2002) and in bone grafts (Sunagawa et al. 2000). The observations reported, even if not speculative in some aspects, are confirmed in unpublished data on human specimens.

In conclusion, the presence of more blood vessels in the 3 mm group is another indication that 3 mm is a preferable distance for contiguous implants when compared with 2 mm, as already mentioned in the studies cited above. Also, further, in depth researches are needed to focus on the interrelationship between bone vasculature and bone loss around dental implants, especially in the long term.

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