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ORIGINAL ARTICLE

Histological and immunohistochemical evaluation of the peri-implant soft tissues around machined and acid-etched titanium healing abutments: a prospective randomised study

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Abstract A close spatial correlation has been described between the roughness of intraoral materials and the rate of bacterial colonisation. The aim of the present study in man was to conduct a comparative immunohistochemical evaluation of the inflammatory infiltrate, microvessel density, the nitric oxide synthases 1 and 3 and the vascular endothelial growth factor expression, the proliferative activity, and the B and T lymphocyte and histiocyte positivity in the peri-implant soft tissues around machined and acid-etched titanium healing caps. Ten patients participated in this study. The patients were enrolled consecutively. All patients received dental implants left to heal in a non-submerged mode. Healing caps were inserted in all implants. Half of the implants were supplied randomly with machined caps of titanium (control), while the other half were provided randomly with acid-etched titanium caps (test). After a 6-month healing period, a gingival biopsy was performed with a circular scalpel around the healing caps of both groups. The inflammatory infiltrate was mostly present in *test* specimens. Their extension was much larger than that of the *control* samples. A higher number of T and B lymphocytes were observed in *test* specimens. Higher values of microvessel density and a higher expression of vascular endothelial growth factor intensity were observed in the *test* samples. Furthermore, the Ki-67, NOS1 and NOS3 expression was significantly higher in the *test* specimens. All these results showed that the tissues around *test* healing caps underwent a higher rate of restorative processes, most probably correlated to the higher inflammation processes observed in these tissues.

Keywords Acid-etched surface · Healing caps · Machined surface · Peri-implant soft tissues

Clinical relevance Caution should be paid to the roughening of abutment surfaces as if the rougher part of the abutment should become exposed to the oral cavity due to a soft tissue recession or shrinkage, there may be an increased risk of a higher degree of inflammation in the peri-implant tissues.

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Introduction

The mechanisms of bacterial interactions with implant materials located in the oral cavity are still not completely known [1]. The abutment surface characteristics may have a relevant role in promoting or delaying plaque accumulation and subsequent pathology of the peri-implant soft tissues [2, 3]. A close spatial correlation has been described between the roughness and surface free energy of intraoral materials and the rate of bacterial colonisation [2, 4–6]. Indeed, surfaces used in the transmucosal portion of the abutment should help in the reduction in the number of adhering bacteria, minimising in such a way plaque formation [2, 7, 8] and peri-implant soft tissue inflammation. On the other hand, a microtextured surface will help

soft tissue integration [2]. It is well known that the surface topography may influence the orientation and proliferation of cells on titanium surfaces [9]; therefore, the topography of an abutment could minimise bacterial adhesion and allow epithelial and connective tissue attachment [2]. The adhesion of bacteria on oral cavity surfaces occurs in four steps: transport of the bacterium to the surface, initial adhesion with a reversible and irreversible stage, attachment by specific interactions and colonisation in order to form a biofilm [10]. Rough surfaces accumulate up to 25 times more subgingival plaque than do smooth ones, and moreover, rough surfaces are more difficult to clean [6, 11, 12]. Surface roughening increases by a factor of 2–3 the area available for bacterial adhesion [12].

The plaque build-up may favour the development of inflammatory lesions of the peri-implant mucosa [13]. Moreover, it has been reported that if an implant with a rough surface is exposed to the oral cavity, a greater amount of plaque, leading to perimucositis and peri-implantitis, may be present [14]. Previous research has demonstrated that a correlation exists between plaque accumulation and peri-implant bone resorption [15]. Plaque accumulation on implant surfaces or abutments induces an inflammatory reaction within the gingiva and alveolar mucosa in the same manner as around teeth [16]. Peri-implantitis is a site-specific, plaque-induced infection leading to progressive bone loss [1, 5, 17, 18]. Pocket formation, radiographic bone destruction, suppuration, swelling, colour changes and bleeding on gentle probing are signs and symptoms present in cases of peri-implantitis [1, 5, 17, 18].

During organ development, wound healing and inflammatory processes, an important role is played by angiogenesis. It consists in the formation of new capillaries due to the budding of endothelial cells [19–27]. This process involves epithelial cell division, selective degradation of vascular basement membranes and endothelial cell migration [19–27]. Inflamed tissues tend to increase the inflammatory mediators expression, which in turn may promote angiogenesis [25]. One of the major angiogenic activators is vascular endothelial growth factor (VEGF), which has been detected in vascular endothelial cells, inflammatory cells, junctional, sulcular and gingival epithelium during reparative processes [22, 26]. Further factors of critical importance in the inflammation process are Ki-67 and nitric oxide synthases (NOS). Ki-67 antigen expression has been detected in the nuclei of proliferating cells in the G1, S, G2 and M phases of the cell cycle, but is absent in quiescent cells (GO phase) [28]. Nitric oxide (NO) is a free radical that has been implicated in osteoclast function under normal and pathologic conditions [29]. Three different isoforms of NOS have been described: Endothelial NOS (eNOS or NOS1) and neuronal NOS (nNOS or NOS3) are called constitutive because they are essentially expressed and continuously and functionally modulated [20, 25–36]. The third isoform is an inducible isoenzyme (iNOS or NOS2). NOS2 is a mediator of the tumoricidal and bactericidal action of macrophages [9, 29–35, 37–39].

The aim of the present study in man was to conduct a comparative immunohistochemical evaluation of the inflammatory infiltrate, microvessel density (MVD), VEGF, NOS1 and NOS3 expression, the proliferative activity and the B and T lymphocyte and histiocyte positivity in the peri-implant soft tissues around machined and acid-etched titanium healing caps.

Materials and methods

Eleven patients, six men and five women (age 30–66 years, mean 49), participated in this study. The patients were enrolled consecutively. The protocol was approved by the Ethics Committee of the University of Guarulhos (UnG), Sao Paulo, Brazil, and all patients signed an informal written consent. The inclusion criteria were controlled oral hygiene, absence of any lesions in the oral cavity, sufficient residual bone volume to receive implants of at least 3.8 mm in diameter and 9.5 mm in length and a wide band of keratinised tissue. In addition, the patients had to agree to participate in a post-operative control program. The exclusion criteria were insufficient bone volume, bone quality type 4, a high degree of bruxism, smoking more than five cigarettes/day, excessive consumption of alcohol, localised radiation therapy of the oral cavity, antitumour chemotherapy, liver diseases, blood diseases, kidney diseases, immunosupressed patients, patients taking corticosteroids, pregnancy, inflammatory and autoimmune diseases of the oral cavity and poor oral hygiene. All patients received 3.8 mm in diameter and different length XiVE® plus dental implants (DENTSPLY Friadent, Mannheim/Germany). All implants were left to heal in a non-submerged (single-stage) mode (Fig. 1). Healing caps (3.8 mm in diameter and 3.0 mm in height) were inserted in all implants. Half of the implants were randomly supplied with standard, machined, prefabricated caps of commercially pure titanium (FRIADENT® Gingiva Former, DENTSPLY Friadent, Mannheim, Germany) (control), while the other half were randomly provided with acid-etched commercially pure titanium caps (DENTSPLY Friadent, Mannheim/Germany) (test) (Figs. 1a–d and 2a). The test healing caps were acid-etched and were provided by DENTSPLY-Friadent (Mannheim, Germany). The surface roughness thus obtained was similar to that of the upper coronal portion of the XiVE® plus implants. For allocation concealment, prenumbered identical containers were administered serially to the participants. The soft tissues were then sutured around the healing caps (Fig. 2b).

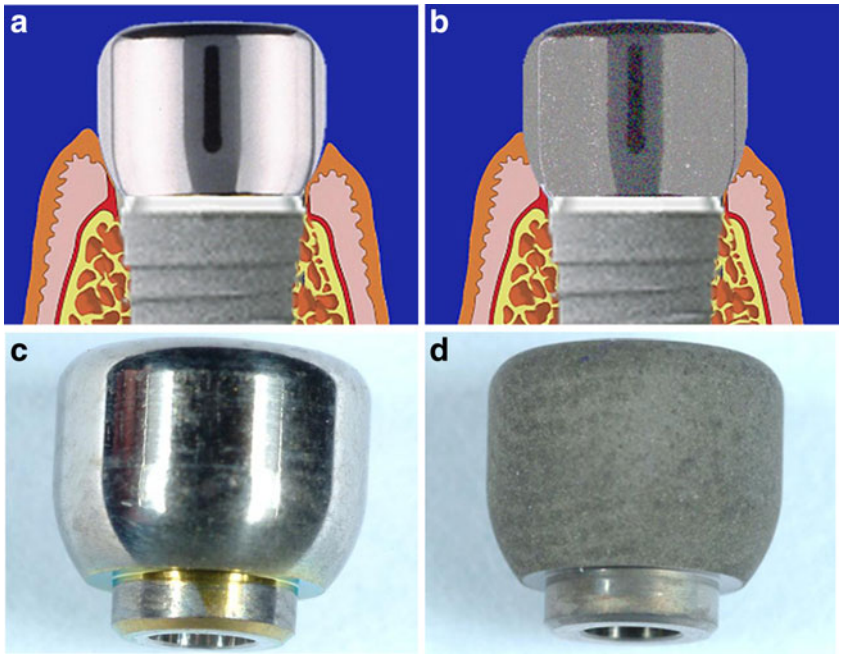

Fig. 1 **a** Machined healing cap. **b** Acid-etched healing cap. **c** Surface characteristics of the machined surface. **d** Surface characteristics of the acid-etched healing cap

Patients underwent oral hygiene sessions and were enrolled in a strict maintenance program. Ten additional healing caps (five control and five test), provided by the same Company, were used for an evaluation of the surface roughness characteristics. The surface roughness was evaluated under a by white-light confocal microscope (NanoFocus AG, Oberhausen, Germany). Mean roughness (R_a) and 10-point average roughness (R_z) were calculated as typical height parameters. Surface topography of the specimens was also evaluated with a 3D focus-variation scanning microscopy (Alicona Imaging, Graz, Austria). Two topographical measures representing the surface area

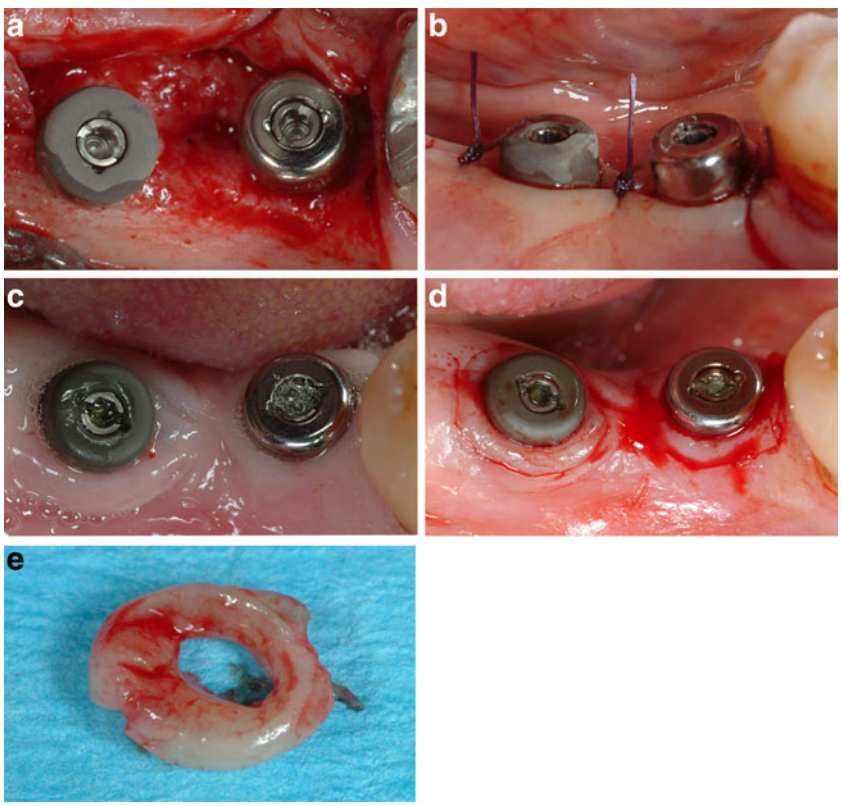

Fig. 2 **a** The healing cps (machined and acid-etched) have been inserted on the implant. **b** The flaps have been sutured. **c** Healing after 6 months. **d** A cuff of soft tissue is removed. **e** The issue removed

roughness (S_a) and surface maximum peak-to-valley distance (S_z) were included in the analyses. S_a is measured as the arithmetic mean (micrometres) of the absolute values of the surface departure (either as peaks or valleys) from the mean plane of the sampling area. S_z is the mean distances (micrometres) from five highest peaks to the five lowest valleys in the sampling area, representing the mean maximum peak-to-valley distance of the sampling area. In addition, a height and spatial descriptive (hybrid parameter) was measured: S_{dr} is the developed surface area ratio, which is the percentage of additional surface area contributed by the texture as compared to an ideal plane the size of the measurement region.

Three readings were performed, in average, for each type of surface. Two areas of 200 μm in diameter were evaluated for each healing cap surface. A Gaussian filter was used to separate roughness from error of form and waviness. The filter size was set at 50×50 μm.

After a 6-month healing period, a gingival biopsy was performed with a circular scalpel (5.5 mm in diameter) around the healing caps of both groups, without unscrewing or removing the healing caps (Fig. 2c, d). The dimensions of the gingival biopsies were a mean 2.1 mm (5.5–3.8 mm) in thickness and 3 mm in height (Fig. 2e). A total of 24 specimens (12 test and 12 control) were retrieved.

All specimens were immediately fixed in 10% neutral buffered formalin and accordingly embedded in paraffin. Three-micrometre sections were subsequently obtained with a Leitz 1512 microtome and stained with haematoxylin–eosin.

The immunohistochemical staining of VEGF, factor VIII, NOS1, NOS3, CD3, CD20, CD68 and Ki-67 was performed using the streptavidine–biotine–peroxidase (strep-ABC) method. Three-micrometre sections were cut and mounted on poly-L-lysine-coated slides. Paraffin sections were dewaxed by xylene, rehydrated and finally washed in phosphate-buffered saline (pH 7.4) for 10 min. In order to unmask the antigens, a microwave oven and a 2.1% content of citric acid were used related to the antibodies CD3, Ki67, NOS1 and NOS3. It was not necessary to submit the sections to prior treatment. The subsequent steps were optimised by automatic staining (Optimax, BioGenex, San Ramon, CA, USA). Sections were incubated with primary antibody for 30 min at room temperature. Slides were rinsed in buffer, and immunoreaction was completed with the strep-ABC-peroxidase method, applying the "Super sensitive immunodetection" kit by BioGenex (San Ramon, CA, USA) and utilising a multi-link as a secondary biotinilated antibody. After incubation with a chromogen employing "liquid DAB substrate pack" (BioGenex, San Ramon, CA, USA), the specimens were counterstained with Mayer's haematoxylin and coverslipped. The VEGF was evaluated in the vascular endothelial cells. The inflammatory infiltrate was evaluated using a semi-quantitative analysis: *low* = +; *intermediate* = ++; *high* = +++. The value was be considered low (+) in case <10%, intermediate (++) in case 10–50% and high (+++) in case >50% of the cells were positive for the investigated factors. The antibody against the human factor VIII-related antigen was used to highlight the blood microvessels. All the morphologic structures with a lumen surrounded by factor VIII-positive endothelial cells were considered as blood microvessels. For the evaluation, counting of the microvessels was performed by a pathologist (LA) in a blind manner. A 200-fold magnification was used, and the individual microvessel profiles were circled to prevent the duplicates or to omit counting. For each case, five high power fields, corresponding to 1.1 mm^2, were measured. The values were expressed as number of microvessels per square millimetre of peri-implant soft tissues (MVD). The same technique was used to count the cells positive to Ki-67.

Quantitative analysis was performed for VEGF, NOS1 and NOS3. VEGF was evaluated employing a light microscope (Laborlux S, Leitz, Wetzlar/Germany) connected to a high resolution video camera and interfaced to a monitor and PC. This optical system was linked to a digitising pad and a histometry software package with image capturing capacity (Image-Pro Plus 4.5, Media Cybernetics Inc., Immagine & Computer, Milan, Italy). The assessment was carried out at the level of the endothelial cells lining the vessels. Five random fields were chosen for each specimen. After having distinguished two different intensities of the expression of VEGF, low and high (Fig. 3a–c), the evaluation was conducted in each field. The intensities were respectively recognised by the PC software as red and yellow.

For statistical evaluation, differences between low and high intensity of NOS1, NOS2 and VEGF were evaluated by means of the Kruskal–Wallis test, followed by Dunn's multiple comparisons post-test. Data on Ki-67, MVD, CD3, CD20 and CD68 were analysed by Mann–Whitney test. All the results were presented as means±standard deviation (SD), and differences at $p \leq 0.05$ were considered statistically significant.

Results

No clinically visible plaque accumulation, suppuration or bleeding on probing was present in the control group. A large amount of bacterial plaque was present on the healing caps of the test group. No suppuration or bleeding on probing was, however, present in this group. Probing depth was <3 mm in both groups.

The description of surface topography is reported in Table 1. The immunohistochemical results were shown in Figs. 4, 5 and 6.

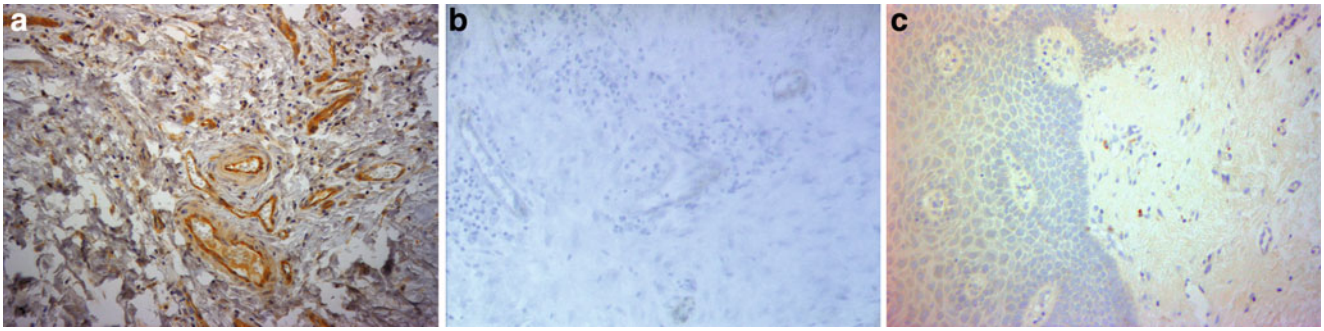

Fig. 3 Different intensities of the expression, *high* (**a**) and *low* (**b**), and unstained cells. H&E, ×20

Inflammatory infiltrate

Control

The inflammatory infiltrate was predominantly consisted of lymphocytes, plasma cells and histiocytes. The semi-quantitative analysis showed that, in the control group, the inflammatory infiltrate was evaluated as + in four and as ++ in one samples, while in the test group, the values were higher: In four samples, the inflammatory infiltrate was evaluated as +++ and only in one samples as ++. Regarding the extension, in the control group, the inflammatory infiltrate penetrated into one third of the mucosa in all the cases. On the contrary, test samples showed an extension of inflammatory infiltrate for two thirds in four samples, for one third in one. Moreover, microulcerations of the overlying mucosa were present in one third of the test cases.

Microvessel density

In control samples, only a few microvessels were present within the submucosa. The mean number of vessels was 16.16±2.22%. In test samples, many microvessels were present within the submucosa; they were predominantly located in the areas where a wider inflammatory infiltrate was present. The mean number of vessels was 28.66± 4.74%. Extremely significant differences between control and test groups were found (p=0.0004).

Nitric oxide synthase 1

NOS1 expression was present in the tissues both around test and control specimens. The intensity of the expression differed, however, in the two groups. NOS1 was expressed at the level of the cells of the superficial epithelium and of the vascular endothelium.

Control

Cells presented a higher expression of lower intensity (20.01±1.44) than higher intensity (9.62±0.89). This difference was statistically significant (p=0.01).

Test

Cells presented a prevalently a higher intensity of expression (36.97±1.22) than a lower intensity of expression (15.42± 1.27). This difference was statistically significant (p=0.01).

A statistically significant difference of the high intensity of expression of NOS1 in both group was present (p=0.001), while a not statistically significant difference in the low intensity of expression of NOS1 in both groups was present ($p \geq 0.05$).

Nitric oxide synthase 3

NOS3 was expressed in tissues around test and control specimens; a difference of the intensity of the expression was

Table 1 Surface topography

Amplitude and height parameters	Control healing caps	Test healing caps
R_a (μm)	0.05195	0.8135
R_z (μm)	0.356	3.733
S_a (μm)	0.575	1.427
S_z (μm)	4.7	13.025
S_{dr} (%)	0.956	69.9

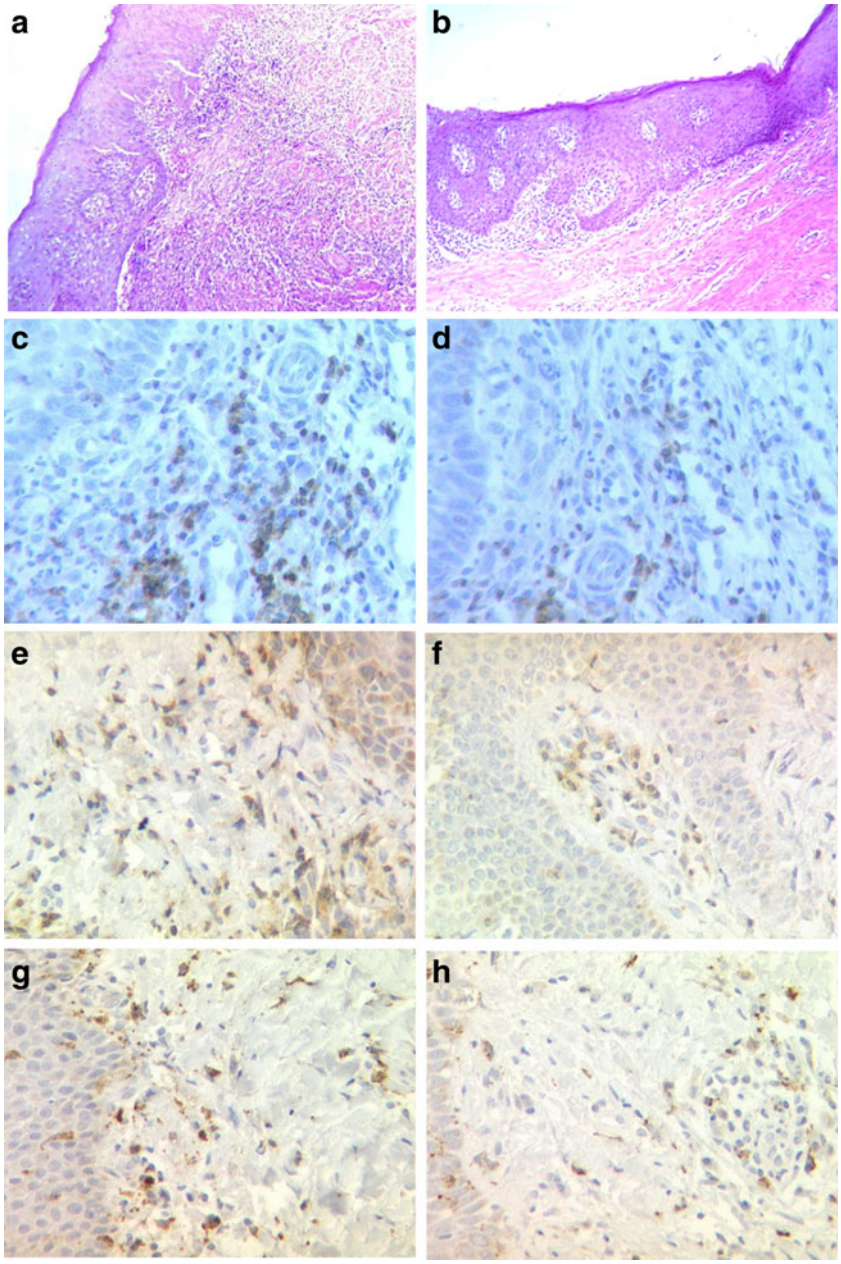

Fig. 4 **a** Test: severe inflammatory infiltrate in the superficial, middle and deep portions of the submucosa. H&E, ×4. **b** Control: a small inflammatory infiltrate is present in the superficial portion of the submucosa. H&E, ×4. **c** Test: moderate inflammatory infiltrate. Many positive B lymphocytes are present. CD20, ×20 ABC. **d** Control: moderate inflammatory infiltrate. Only few B lymphocytes are positive. CD20, ×20 ABC. **e** Test: many T lymphocytes are present in the submucosa. CD3, ×20 ABC. **f** Control: moderate inflammation. Only a few T lymphocytes are positive. CD3, ×20 ABC. **g** Test: many histiocytes infiltrate the mucosa. CD68, ×20 ABC. **h** Control: moderate inflammation. Only a few histiocytes are positive. CD68, ×20 ABC

observed in the two groups. NOS3+ cells were present in the superficial epithelium and in the vascular endothelium.

Control

Cells presented a lower intensity of the expression (14.47±1.24) than a higher intensity of expression (5.15±0.78). This difference was statistically significant (p=0.01).

Test

Cells presented a prevalently higher intensity of the expression (39.21±0.93) than a lower intensity of expression (12.61±0.96). This difference was statistically significant (p=0.01).

A statistically significant difference was present on the high intensity of expression of NOS3 (p=0.001) between control and test samples. A not statistically significant difference was present, on the contrary, in the low intensity of expression of NOS3 ($p \geq 0.05$).

Vascular endothelial growth factor

VEGF+ cells were present in the tissues around control and test healing caps. The intensity of the expression differed, however, in the two groups. VEGF+ cells were found in the

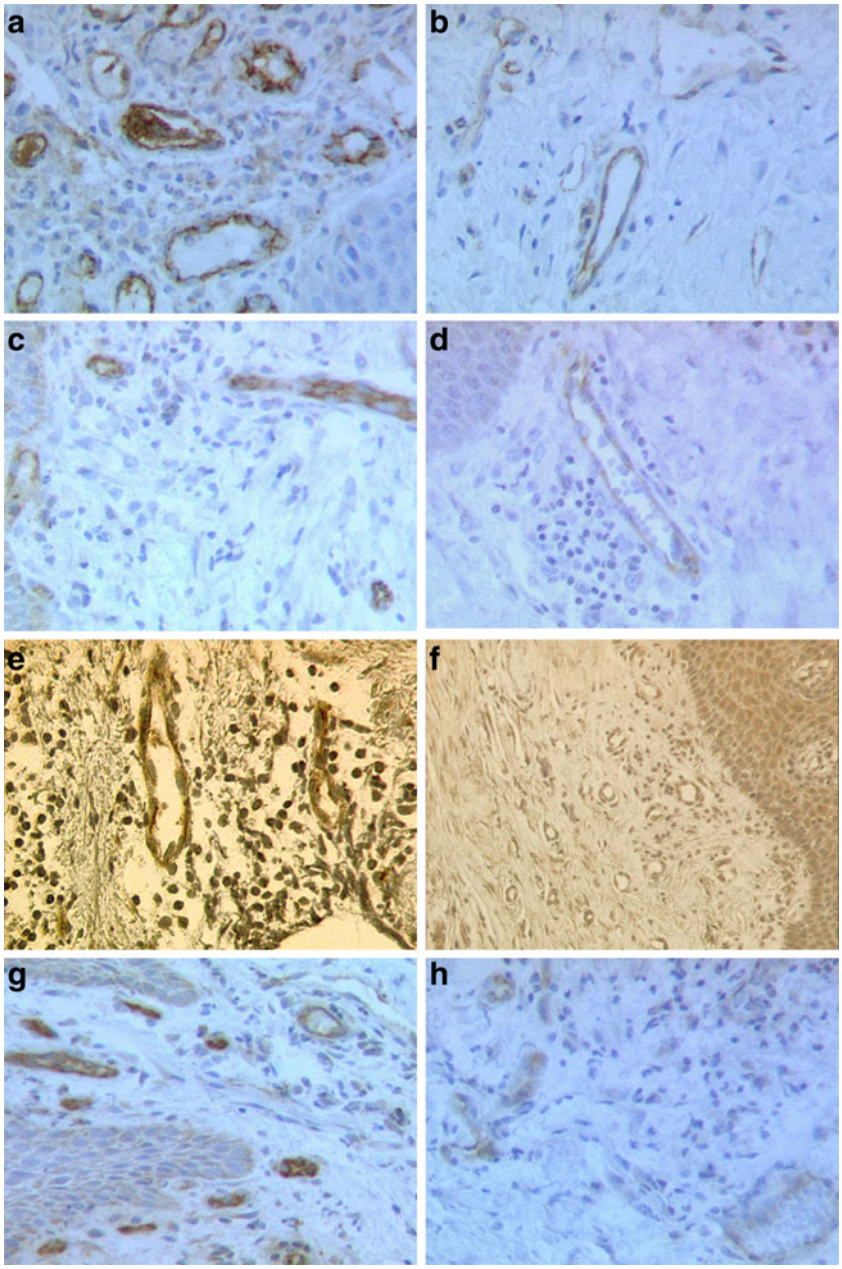

Fig. 5 **a** Test: MVD +++ factor VIII ×20 ABC. **b** Control: MVD+ factor VIII ×20 ABC. **c** Test: high expression in vessels. VEGF, ×20 ABC. **d** Control: low expression of vascular expression. VEGF, ×20 ABC. **e** Test: high expression in vessels. NOS1, ×20 ABC. **f** Control: low expression in vessels. NOS1, ×20 ABC. **g** Test: high expression in vessels. NOS3, ×20 ABC. **h** Control: low expression in vessels. NOS3, ×20 ABC

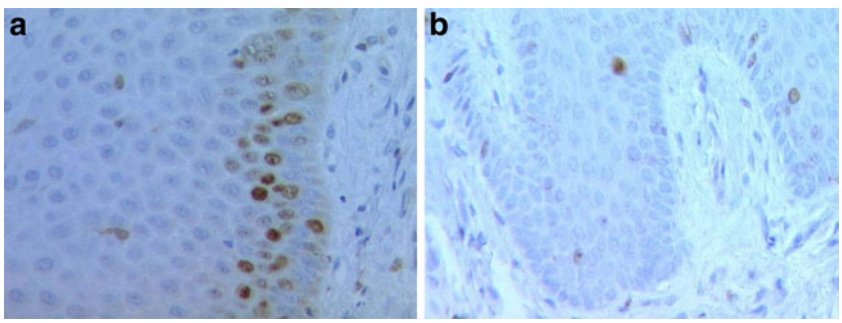

Fig. 6 **a** Test: many basal cells are positive. Ki67, ×20 ABC. **b** Control: only a few basal cells are positive. Ki67, ×20 ABC

vascular endothelium, stroma and inflammatory infiltrate. The evaluation was performed at the level of the endothelial vascular cells.

Control

Cells presented a prevalence of higher intensity of the expression (27.33±1.35) than a lower intensity of expression (17.23±2.96) with no statistically significant differences ($p \geq 0.05$).

Test

Cells presented a prevalently higher intensity of the expression (43.38±2.81) than a lower intensity of expression (28.72±1.82) with no statistically significant differences ($p \geq 0.05$).

A statistically significant difference of the high and low intensities of expression of VEGF in both groups was present ($p \leq 0.05$).

Proliferative activity

The positivity to Ki-67 was nuclear. The positivity was located at the level of the basal and superficial epithelial layers.

In controls, 19.35±5.06% of the cells in the basal layer were positive. No positivity was observed in the superior layers of the epithelium. In tests, 71.11±3.33% of the cells of the basal layer were positive to Ki-67. A positivity was observed also in many cells of the superior epithelial layers. A statistically significant difference between control and test specimens in the number of Ki-67-positive cells was observed ($p \leq 0.0001$).

CD20

B lymphocytes constituted 14.44±3.90% of the whole inflammatory infiltrate in control group and 14.16±3.76% of that of the test specimens. The difference was not statistically significant ($p=0.95$).

CD3

T lymphocytes constituted 42.5±6.89% of the whole inflammatory infiltrate in control group and 42.22±8.70% of that of the test specimens. The difference was not statistically significant ($p=0.95$).

CD68

Histiocytes constituted 43.33±8.16% of the whole inflammatory infiltrate in control group and 44.44±9.82% of that of the test specimens. The difference was not statistically significant ($p=0.99$).

Discussion

Only a few studies have investigated the effects of different implant surfaces on the inflammatory process or on the nature of the infiltrating cell types in the peri-implant soft tissues [39]. The search for an optimal implant surface to decrease the bacterial adhesion and to improve the soft tissues adhesion is still ongoing [39]. Different results have been reported in the literature. Even if it is conceivable that the implant surface may have some influence on the nature of the inflammatory infiltrate in the peri-implant tissues [39], no relationship was found between the inflammatory response and the abutment surface roughness when investigating the early tissue response (i.e. after 1 month of function) in man [14]. Another factor that may have contributed to the abovementioned result could be that the inclusion criteria required patients with healthy mucosa and that all abutments were surrounded by attached mucosa. Thus, the resistance to inflammation may have been enough to withstand any effects of increased abutment surface roughness. Furthermore, no differences were found, after 6 months of undisturbed plaque formation in dogs, in the peri-implant soft tissues around acid-etched or turned abutments [13]. In a study comparing four surfaces (acid-etched, mild anoxic oxidation, machined, machined with a groove), it was reported that tissues surrounding all types of abutments showed some degrees of inflammation after 6 months, with the machined surface presenting the smallest area of inflammation [39]. These results are similar to those reported in the present study.

In the specimens of the present study, the inflammatory infiltrate was mostly present in test specimens. Their extension was much larger than that of the control samples. The inflammatory infiltrate consisted of lymphocytes, plasma cells and histiocytes. A higher number of T lymphocytes (CD3+) and of B lymphocytes (CD20+) were observed in test specimens.

In most cases of the test specimens, the inflammatory infiltrate tended to ulcerate the overlying mucosa. Higher values of MVD were observed in the test samples. In addition, a higher expression of VEGF intensity was observed in the peri-implant tissues of the test specimens, while predominantly lower expressions of the VEGF intensity were noted around the control healing caps. In addition, the Ki-67 expression was significantly higher in the test specimens. All these results showed that the tissues around test healing caps underwent a higher rate of restorative processes, most probably correlated to the higher inflammation processes observed in these tissues. A higher

intensity expression of NOS1 and NOS3 was recorded in the tissues around test specimens, while, on the contrary, a lower intensity of expression was found in the tissues around control specimens. These latter results may indicate that the higher expression of these two mediators could be correlated to a higher amount of bacteria present around the test samples. In the present specimens, T lymphocytes were the dominant infiltrating cells; these results are in accord to those reported by Pongnarisorn et al. [39].

Surface properties of transgingival implant components are important determinants in bacterial adhesion. The long-term survival of dental implants depends, in part, on the control of bacterial infection in the peri-implant region. Bacterial adhesion to implant or abutment surfaces is a critical issue [2, 4, 6]. It is considered a first stage of peri-implant mucositis and peri-implantitis. A positive correlation has been confirmed between oral hygiene and peri-implant marginal bone loss in the edentulous mandible [1, 40]. Moreover, a correlation between plaque accumulation and progressive bone loss around implants has been reported in experimental and clinical studies [15, 41]. Surface modifications or the utilisation of different materials have shown to play a relevant role in the bacterial adhesion of implant surfaces. VEGF is probably involved in the etiology of gingivitis and its progression to periodontitis by an expansion of the vascular network, an increase in tissue edema, and a decrease in the rate of blood flow [23]. VEGF also seems to be involved in tissue reparative processes [43].

Conclusion

Acid-etching of the lower part of the abutment has been suggested due to the fact that an improved connective tissue adhesion has been reported for some rougher surface in a study performed in the rat subcutaneous tissue [42]. In the present study, the connective tissue parameters were not evaluated; however, the present results suggest caution in roughening the abutment surface. In fact, if the rougher part of the abutment should become exposed to the oral cavity due to a soft tissue recession or shrinkage, there may be an increased risk of a higher degree of inflammation in the peri-implant tissues.

Conflict of interest The authors declare that there is no conflict of interest.

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