

SCREW VS CEMENT-IMPLANT-RETAINED RESTORATIONS: AN EXPERIMENTAL STUDY IN THE BEAGLE. PART 2. IMMUNOHISTOCHEMICAL EVALUATION OF THE PERI-IMPLANT TISSUES

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Crestal bone loss has been reported to occur around dental implants. Even if the causes of this bone loss are not completely understood, the presence of a microgap between implant and abutment with a possible contamination of the internal portion of the implants has been suggested. The aim of this study was to see if there were differences in the vascular endothelial growth factor (VEGF) expression, microvessel density (MVD), proliferative activity (MIB-1), and inflammatory infiltrate in the soft tissues around implants with screwed and cemented abutments. Sandblasted and acid-etched implants were inserted in the mandibles of 6 Beagle dogs. Ten 3.5- × 10-mm root-form implants were inserted in each mandible. A total of 60 implants (30 with screwed abutments and 30 with cemented abutments) were used. After 12 months, all the bridges were removed and all abutments were checked for mobility. A total of 8 loosened screws (27%) were found in the screwed abutments, whereas no loosening was observed in cemented abutments. A gingival biopsy was performed in 8 implants with cemented abutments, in 8 implants with screwed abutments, and in 8 implants with unscrewed abutments. No statistically significant differences were found in the inflammatory infiltrate and in the MIB-1 among the different groups. No statistically significant difference was found in the MVD between screwed and cemented abutments ($P = .2111$), whereas there was a statistically significant difference in MVD between screwed and unscrewed abutments ($P = .0277$) and between cemented and unscrewed abutments ($P = .0431$). A low intensity of VEGF was prevalent in screwed and in cemented abutments, whereas a high intensity of VEGF was prevalent in unscrewed abutments. These facts could be explained by the effects induced, in the abutments that underwent a screw loosening, by the presence of bacteria inside the hollow portion of the implants or by enhanced reparative processes.

INTRODUCTION

Peri-implantitis is a site-specific, plaque-induced infection with progressive loss of the bone supporting a functioning implant.¹ In peri-implantitis, it is possible to observe pocket formation, radiographic bone destruction, suppuration, swelling, color changes, and bleeding upon gentle probing.²⁻⁴ Angiogenesis consists of the formation of new capillaries by the budding of endothelial cells⁵ and is a complex process that involves epithelial cell division, selective degradation of vascular basement membranes and of surrounding extracellular matrix, and endothelial cell migration.⁶⁻¹² It is physiologically important in developing organs, inflammation, and wound healing, but its role in the development and progression or in the healing of periodontal lesions has not been elucidated.¹²⁻¹⁶ In periodontal tissues, angiogenesis plays a role in the maintenance of tissue health and in chronic inflammatory periodontal disease.¹³ Moreover, angiogenesis takes part in the development and maintenance of chronic inflammatory diseases such as diabetic retinopathy, rheumatoid arthritis, psoriasis, age-related macular degeneration, and tumors.^{13,16,17} Changes in the relative balance of inducers and inhibitors of angiogenesis probably contribute to the angiogenic process.¹³ The major angiogenic activators are basic fibroblast growth factor-2 and vascular endothelial growth factor (VEGF), and the most important inhibitors are angiostatin and endostatin.^{11,13}

Vascular endothelial growth factor is a glycoprotein that shares homology with platelet-derived

growth factor and is a potent inducer of microvascular permeability.^{11,12,17,18} It has been shown to have a remarkable potency in inducing specific endothelial cell proliferation *in vitro* and also to have an angiogenic activity *in vivo*.^{5,13,19} It induces microvascular permeability with a potency of about 50 000 times that of histamine.¹⁸ Induced angiogenesis and vasculogenesis are impaired in mice with mutated and nonfunctional VEGF gene.¹⁹ It is regulated by hypoxia-mediated control of gene transcription, alternative messenger-RNA splicing, and proteolytic processing.¹⁷ Inflamed tissues enhance the expression of inflammatory mediators, which, in turn, may promote angiogenesis.¹⁷ Vascular endothelial growth factor has been detected in vascular endothelial cells; inflammatory cells; and in the junctional, sulcular, and gingival epithelium^{17,20} and is expressed in a variety of highly vascularized tissues.^{11,21} A correlation between VEGF expression and neovascularization assessed by factor-VIII immunostaining and evaluated by microvessel density (MVD) has been found.¹⁰ Moreover, MVD is correlated to poor clinical outcome in several malignant tumors.¹⁰

The aim of the present study in Beagle dogs was to conduct a comparative immunohistochemical evaluation of VEGF, inflammatory infiltrate, proliferative activity (MIB-1) expression, and MVD in the peri-implant soft tissues surrounding dental implants with cemented and screwed abutments.

MATERIALS AND METHODS

Sandblasted and acid-etched implants (Bone System, Milano, Italy) were placed in the mandibles of 6 male Beagle dogs of at least 2 years of age. The Ethics

Committee of the University of Madrid, Spain, approved the protocol. The 2 premolars and the first molars had been extracted 3 months beforehand. Each dog received ten 3.5- × 10-mm root-form implants in the mandible (5 on the right side and 5 on the left side). All surgical procedures were performed under general anesthesia (premedication with acepromazine 0.5 mg/kg subcutaneously, with nembutal 15 mg/kg intravenously) and antibiotic prophylaxis. The implant sites were prepared with drills under generously chilled, saline irrigation. The implants were then inserted with a tapping instrument. All implants were placed by a submerged approach, and the top of each implant was located clinically at the alveolar crest. The mucosal tissues were closed with 3-0 silk sutures. In the first 2 postsurgical weeks, the oral cavities were rinsed daily with chlorhexidine-digluconate 0.12% (Peridex, Procter & Gamble, Cincinnati, Ohio). In addition, the dogs were fed a soft diet. The sutures were removed after 1 week. Three months after implantation, second-stage surgery was performed for abutment connection. After a midcrestal incision, the peri-implant soft tissues were evaluated with exposure of the peri-implant bone crest, and abutments were inserted in all implants. The abutments were either screwed by applying a total strength of 30 Ncm or cemented with Panavia 21 (J. Morita USA Inc, Tustin, Calif). The cement was mixed according to the manufacturer's recommendations and applied on the axial surface of the internal portion of the implants to minimize hydrostatic pressure during seating. Abutments were cemented on the implants with a load of 5 kg maintained for 10 minutes. Excess cement was

removed with a scaler. One investigator (B.A.) carried out mixing and cementing procedures at room temperature. After cementation of the implant-abutment connection, a bridge of 6 elements was cemented with a load of 5 kg maintained for 10 minutes. After cementation of the bridge, the excess cement was removed with a scaler. A total of 60 implants were inserted, 30 with cemented abutments and 30 with screwed abutments. No postoperative complications or deaths occurred.

After 12 months, all the bridges were removed and all screwed and cemented abutments were checked clinically for mobility, alternatively pressing the facial and lingual surfaces of the abutments with the nonworking ends of 2 instrument handles.

A total of 8 loosened screws (27%) were found in the screwed abutments after 12 months. No loosening was observed in cemented abutments. A statistically significant difference was present between the incidence of the loosening in the screwed and cemented abutments ($P = .0001$).

A gingival biopsy was performed near the top of the implant in 8 of the implants with cemented abutments, in 8 of the implants with screwed abutments, and in 8 of the implants where the abutments were found to be unscrewed after 12 months. The dimensions of the gingival biopsy were 1 mm in thickness and 1 mm in length. The dogs were not killed in this study.

All specimens were immediately fixed in 10% neutral buffered formalin and then embedded in paraffin. Three-micrometer sections were then obtained with a Leitz 1512 microtome and stained with hematoxylin and eosin.

For the immunohistochemical staining of VEGF, factor VIII, and

MIB-1, 3- μ m sections were cut and preincubated in phosphate-buffered saline (PBS) for 5 minutes and then with rabbit polyclonal antibodies (Santa Cruz Biotech Inc, Santa Cruz, Calif), which were diluted 1:100 in PBS and applied for 30 minutes at room temperature. Slides were then washed twice in PBS for 5 minutes and in Tris-HCl buffer, pH 7.6, for 10 minutes. Sections were incubated in the presence of biotinylated-specific secondary antibody, and avidin-biotin peroxidase was developed with diaminobenzidine chromogen (Biomedica Corp, Foster City, Calif) and the nuclei were counterstained with hematoxylin. The secondary antibody was an alkaline phosphatase-labelled monoclonal calf antibody, and the detection antibody was a monoclonal anti-calf-mouse antibody. After incubation with a chromogen alkaline phosphatase substrate (Fast Red, Dako Cytomation, Hamburg, Germany), specimens were counterstained with Mayer hematoxylin and were coverslipped.

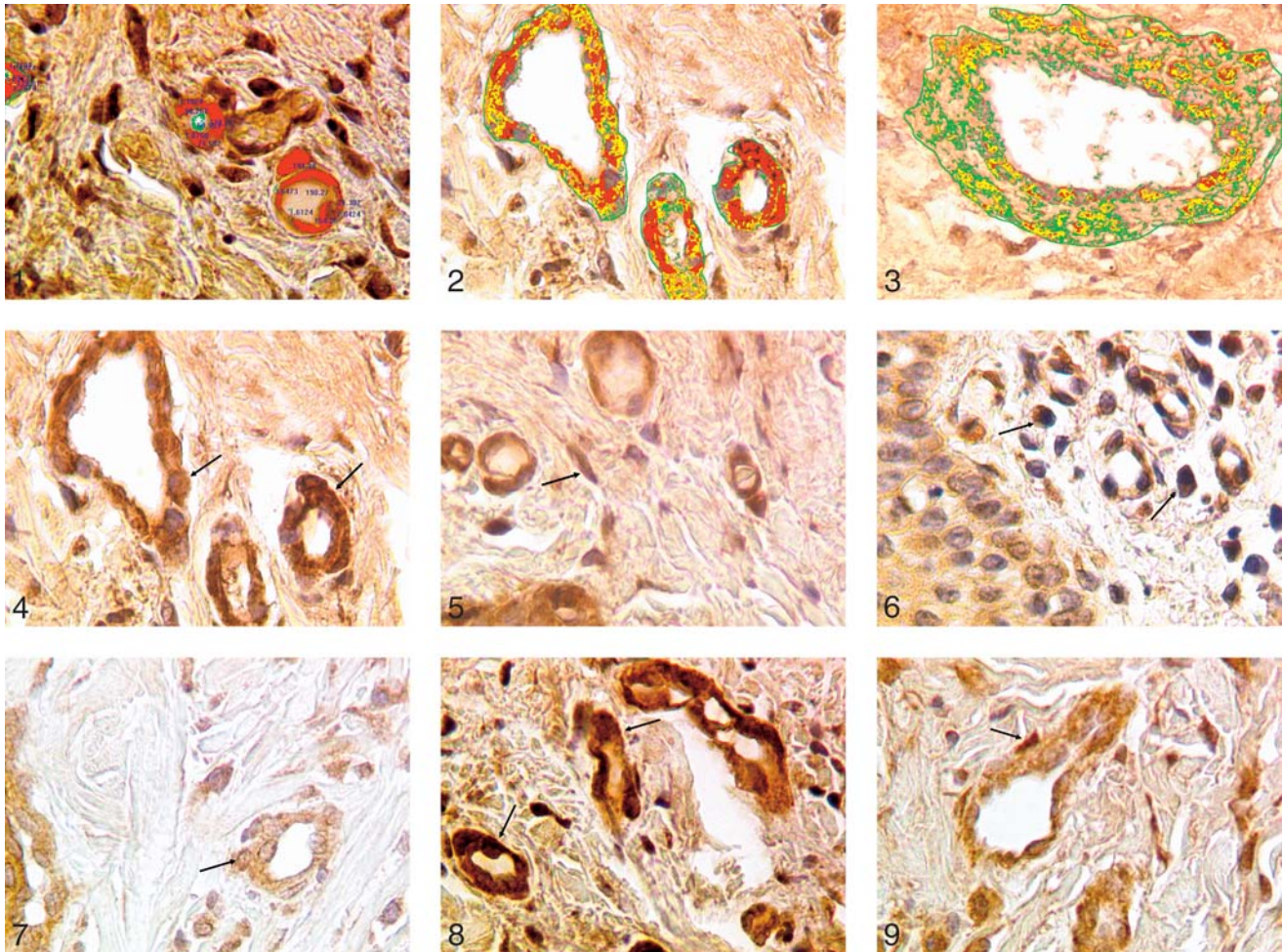
Vascular endothelial growth factor has been evaluated in the vessels, in the cells of the inflammatory infiltrate (mainly lymphocytes, plasma cells, and neutrophils), and in the stromal cells (fibroblasts). Inflammatory infiltrate, factor VIII, and MIB-1 expressions were evaluated by a semiquantitative analysis: low (+), intermediate (++), and high (+++). The value was considered low (+) when fewer than 10% of the cells were positive for these factors, intermediate (++) when the percentage of positive cells was between 10% and 50%, and high (+++) when the percentage of positive cells was more than 50%. The antibody against 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. Two pathologists (L.A. and C.R.) performed the microvessel count in a blinded and independent fashion with an IBAS-AT image analyzer (Kontron, Munich, Germany). For the evaluation, a $\times 400$ magnification was used and the individual microvessel profiles were circled to prevent the duplication or omission of microvessel count. For each case, 10 high-power fields corresponding to 1.1 mm² were measured. The values were expressed as the number of microvessels per square millimeter of peri-implant soft tissues surrounding screwed, cemented, and unscrewed abutments (MVD).

Quantitative analysis was performed for VEGF. A light microscope (Laborlux S, Leitz, Wetzlar, Germany) connected to a high-resolution video camera and interfaced to a monitor and personal computer was used to evaluate VEGF in the vessels of screwed, cemented, and unscrewed abutments. This optical system was associated with a digitizing pad and a histometry software package with image-capturing capabilities (Image-Pro Plus 4.5, Media Cybernetics Inc, Immagine & Computer, Milano, Italy). Five random fields were chosen for each specimen. An evaluation was performed in each field after 3 different intensities of the expression of VEGF were distinguished, which the personal computer software recognized as red (low), yellow (intermediate), and green (high) (Figures 1 through 3).

Statistical evaluation

The Friedman test was used to evaluate the presence of statistically significant differences of VEGF within and among the 3



FIGURES 1-9. FIGURE 1. High intensity of vascular endothelial growth (VEGF) expression. FIGURE 2. Intermediate intensity of VEGF expression. FIGURE 3. Low intensity of VEGF expression. FIGURE 4. VEGF positivity of the endothelial cells lining the blood vessels (arrow) (magnification $\times 30$). FIGURE 5. VEGF positivity of the stromal cells (arrow) (magnification $\times 30$). FIGURE 6. VEGF positivity of the cells of the inflammatory infiltrate (arrow) (magnification $\times 30$). FIGURE 7. Implant with a cemented abutment: low intensity of VEGF positivity of the endothelial cells lining the blood vessels (arrow) (magnification $\times 30$). FIGURE 8. Implant with an unscrewed abutment: high intensity of the VEGF positivity of the endothelial cells lining the blood vessels (arrow) (magnification $\times 30$). FIGURE 9. Implant with a screwed abutment: intermediate intensity of VEGF positivity of the endothelial cells lining the blood vessels (arrow) (magnification $\times 40$).

groups considered. The percentages have been expressed as a mean \pm SD. Statistically significant differences were set at $P < .05$. Inflammation was evaluated by the χ^2 test. Finally, the Wilcoxon test was used to evaluate MVD and MIB-1.

RESULTS

In all cases in all 3 groups, there was an inflammatory infiltrate composed mainly of lympho-

cytes, plasma cells, and granulocytes. There was no statistically significant difference in the amount of the inflammatory infiltrate between screwed and cemented abutments ($P = .548$), between screwed and unscrewed abutments ($P = .391$), and between cemented and unscrewed abutments ($P = .682$) (Table 1). There were no statistically significant differences in MVD between screwed and cemented abutments ($P = .2111$). On the con-

trary, there was a statistically significant difference in MVD between screwed and unscrewed abutments ($P = .0277$) and between cemented and unscrewed abutments ($P = .0431$) (Table 2). There was no statistically significant difference in MIB-1 among the 3 groups considered (Table 3).

Vascular endothelial growth factor was expressed in all groups but with different intensity in vascular endothelial cells. Vascular endothelial cells (Figure

TABLE 1

Inflammatory infiltrate χ^2 test				
Abutment*	Score			
	0	1	2	3
Screwed (n)	6	4	8	2
Cemented (n)	3	7	7	5
Unscrewed (n)	0	0	2	4

*Unscrewed vs screwed abutments $P = .391$, unscrewed vs cemented abutments $P = .682$, screwed vs cemented abutments $P = .548$. All P values are nonsignificant.

4), stromal cells (fibroblasts) (Figure 5), and the cells of the inflammatory infiltrate (Figure 6) were positive for VEGF. A low intensity of VEGF expression was prevalent in screwed and cemented abutments (Figure 7). There was a statistically significant difference among the 3 degrees of intensity ($P < .0001$). However, a high intensity of VEGF expression was prevalent in unscrewed abutments (Figure 8). In this group, there was no statistically significant difference ($P = .0805$) because the 3 degrees of intensity were always expressed, even if it was possible to observe a tendency from low to high intensity (Figure 9, Table 4). Moreover, high and intermediate intensities were significantly different among the 3 groups ($P < .0001$), whereas low intensity was not statistically significant (Table 4). No quantitative analysis of VEGF expression was performed in the stromal cells and in the cells

TABLE 2

Microvessel density Wilcoxon test		
Abutment*	Mean	SD
Screwed	78.95	16.18
Cemented	89.13	22.03
Unscrewed	117.33	15.47

*Unscrewed vs screwed abutment $P = .0277$, unscrewed vs cemented abutment $P = .0431$, screwed vs cemented abutment $P = .2111$ (nonsignificant).

TABLE 3

Proliferative activity Wilcoxon test		
Abutment*	Mean	SD
Screwed	22.30	11.81
Cemented	22.77	9.41
Unscrewed	22.16	8.93

*Unscrewed vs screwed abutment $P = .345$, unscrewed vs cemented abutment $P = .600$, screwed vs cemented abutment $P = .840$. All P values are nonsignificant.

of the inflammatory infiltrate because of technical difficulties.

DISCUSSION

The majority of studies in implant dentistry have focused more on the bone integration of dental implants and much less on the dimensions and relationship of the peri-implant soft tissues.²² Crestal bone loss has been reported to occur in the first year after implant insertion. This initial bone loss forms a V- or U-shaped pattern.²³ The precise causes of this bone loss are unknown, but several hypotheses have been suggested, such as surgical trauma, overload, peri-implantitis, microgap, reformation of the biologic width, and implant crestal module.²⁴ The determination of the etiology of this crestal bone loss is important to obtain an improved environment for long-term peri-implant health.²³ Occlusal overload and peri-implantitis are 2 of the main causative factors for implant failure in later stages.²⁴ Moreover, a correlation between plaque ac-

cumulation and progressive bone loss around implants has been reported in experimental and clinical studies.²⁴ In most 2-stage implants, after abutment connection, a microgap is produced at or below the alveolar crest.²⁴ This microgap represents a site of infection and the host reacts with an inflammatory response.²⁵⁻²⁹ Several studies have demonstrated a second discrete inflammatory cell infiltrate, in addition to that located in the marginal portion of the soft tissues, in the connective tissue lateral to the abutment fixture junction.³⁰⁻³⁴ This infiltrated connective tissue (abutment ICT) is believed to arise as a result of a microbial contamination of the internal portion of the implant.³⁰⁻³⁴ In nonsubmerged implants, however, the implant itself extends above the alveolar crest level, and the microgap does not exist at the level of the bone.^{23,24} Minimal or no resorption was found in these implants.²⁵⁻²⁹ One-piece implant designs seem to determine the least inflammation and the smallest resultant tissue changes.²⁵⁻²⁹ The degree of inflammation has been reported to be less around 1-piece implants as compared with 2-piece implants.²⁵⁻²⁹ In 2-piece implants, the crestal bone levels appeared dependent on the location of the microgap.^{25-29,35} The least bone resorption and peri-implant inflammation was re-

TABLE 4

Vascular endothelial growth factor Friedman test				
Abutment	Intensity (Mean \pm SD)			P Value
	High	Intermediate	Low	
Screwed	10.0 \pm 11.8	31.7 \pm 32.3	120.4 \pm 41.3	<.0001
Cemented	11.9 \pm 22.0	19.1 \pm 31.0	86.9 \pm 45.9	<.0001
Unscrewed	132.1 \pm 69.9	126.7 \pm 71.0	90.4 \pm 38.4	.0805*
P value	<.0001	<.0001	.0872*	

*Indicates nonsignificant.

ported to occur if the microgap was located 1 mm above the alveolar crest.²⁸ From the literature, it can be speculated that though the microgap may not be considered as the only cause of early implant bone loss, it might cause implant crestal bone loss during the healing phase if it is placed at or below the alveolar crest.^{23,24} In a previous in vitro study from our laboratory, we demonstrated that a bacterial penetration in implants with screwed abutments was observed at the implant-abutment interface, whereas no bacteria were observed in the hollow portion of the implants with cemented abutments.³⁶ This bacterial reservoir could interfere with the peri-implant tissue health and produce an inflammation of the peri-implant tissues, which could interfere with the long-term success of the implants.³⁷⁻⁴⁰

Angiogenesis is a process in which new blood vessels are produced by sprouting from established vessels.¹⁵ An enhanced expression of inflammatory mediators, many of which can promote angiogenesis, can be found in inflamed tissues.¹⁵ Angiogenesis can also contribute to the severity of the inflammation as the result of the ability of the new blood vessels to transport proinflammatory cells to the lesion and supply nutrients and oxygen to the inflamed tissues.^{15,19}

Vascular endothelial growth factor 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 the blood flow.^{15,17} Aberrant angiogenesis and persistent granulation have been reported in periodontitis, and these phenomena may contribute to its

progression (ie, the aberrant angiogenesis enhances leukocytes infiltration into the lesion and formation of persistent granulation tissue).¹⁶

The aim of the present study was to see if there were differences in the MVD, inflammatory infiltrate, MIB-1, and VEGF expression in the soft tissues around implants with screwed and cemented abutments. No statistically significant difference was found in the MVD, inflammatory infiltrate, and MIB-1 between screwed and cemented abutments, and the significance was prevalently low in the VEGF intensity between screwed and cemented abutments. However, it was interesting to note that the MVD was statistically significantly higher compared with the screwed and cemented abutments in the peri-implant soft tissues around implants whose abutments had become unscrewed after a loading period of 12 months. In the unscrewed specimens, it was possible to find a prevalence of a high intensity of VEGF. These results could point to the fact that though screw loosening in the screwed abutments did not occur, no differences in the soft tissue vasculature between the screwed and the cemented abutments exist. However, when there was a loosening of the screws, which is frequently reported in the literature,⁴¹ we observed a significant increase in the MVD and in the intensity of VEGF expression in the peri-implant tissues. These facts could be explained by the effects induced by the presence of bacteria inside the hollow portion of the implants or by the presence of enhanced reparative processes in which VEGF is implicated.^{13,15}

Further studies are certainly needed to try to understand the significance of the possible re-

lationship between bacterial contamination of the internal portion of the implants and long-term peri-implant soft tissue health.

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