

Bone remodeling at microscrew interface near extraction site in the beagle dog mandible-histologic and immunohistochemical analyses

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ABSTRACT

Extraction is often used as part of orthodontic therapy, and good control of anchorage is a key step after extraction. Although microscrews can be implanted close to the extraction site in order to achieve orthodontic support, the efficiency of bone remodeling at the implant-bone interface near the extraction region is dubious. Objective: The purpose of this study was to investigate bone remodeling of the bone-microscrew interface near the tooth extraction site, in the absence of loading. Material and Methods: Third and fourth premolars were extracted from the mandibles of beagle dogs, followed by placement of test microscrews near the extraction sites. Control microscrews were placed further away from the extraction site. All samples were collected after 1, 3, 8, or 12 weeks of healing following extraction. The bone remodeling process at the interface was evaluated using histologic and immunohistochemical analyses. Results: Initially, a large number of inflammatory cells were aggregated at the interface. The expression levels of core binding factor (Cbfa1), osteocalcin (OC) and transforming growth factor beta (TGF- β) were inconspicuous in both groups, whereas tumor necrosis factor alpha (TNF- α) was strongly expressed, especially in the test groups ($P < 0.05$). Subsequently, the expression levels of Cbfa1, OC and TGF- β were found to increase significantly, and active osteogenesis was observed. Conclusions: During week 1, inflammatory reaction is a major concern at the bone-microscrew interface near the extraction site. However, with healing, the influence of extraction on the remodeling of bone surrounding the microscrews decreases, thus facilitating successful treatment.

Keywords: Bone remodeling. Core binding factor. Osteocalcin. Transforming growth factor alpha. Transforming growth factor beta.

INTRODUCTION

Anchorage control is a challenge to nearly every orthodontist. Since the first successful attempt to move teeth against a fixed screw by Linkow¹² (1969), the application of microscrews in orthodontic treatment has become a mainstay in contemporary orthodontics^{7,14}. Owing to its miniature size, the interradicular site is the most common choice for microscrews in orthodontic clinical treatment⁹. Moreover, palatal implants,

the implant system for orthodontic anchorage, have shown promising results in recent years by achieving maximal intraoral orthodontic anchorage purposes¹. However, orthodontists are often faced with complicated challenges, such as: 1) the low-order maxillary sinus that hinders implantation in the molar area, 2) the maxillary tubercle and external oblique line where implanting microscrews is a greater challenge compared to the flat areas in the jaw bone, 3) the complicated surgical placement for palatal implants¹⁸, and

4) too many missing teeth or rampant caries/periodontitis within the same quadrant. These factors make it very difficult to identify the ideal location for implants. Although microscrews can be implanted close to the extraction site in order to achieve orthodontic support, the stability of the microscrews and the efficiency of bone remodeling at the implant-bone interface near the extraction region is dubious. To our knowledge, this issue has not been resolved.

Literature reports^{11,17} show that extraction undoubtedly leads to a decrease of bone density in the extraction vicinity. Miyawaki and his colleagues¹⁴ (2003) proved that the decrease in bone density increases the risk of non-integration at the implant-bone interface. Zheng's examination indicated that the risk of loosening of microscrews near extraction site was the most severe in the first week following implant placement²¹. In addition, there are several factors associated with the stability of microscrews, such as the diameter of the miniscrew, proximity with dental roots and inflammation within peri-implant tissue. To understand and overcome these challenges, numerous studies have been conducted, with the aim of promoting bone tissue remodeling at the implant-bone interface to increase stability of microscrews under diverse conditions^{13,19,21}. However, the various animal and clinical studies have focused on the stability of microscrews under loading in order to mimic the actual clinical process as closely as possible. This leads to the evaluation of microscrew stability under the influence of iatrogenic factors such as intention, direction and occasion of loading.

In order to determine the ideal implantation strategy for loading, clinical orthodontists certainly need to understand the state of the bone-device interface during the healing process. In accordance with the change rule of bone healing¹⁵, the bone remodeling process at the interface was evaluated at 1, 3, 8 or 12 weeks following implantation. In this study, we hypothesized that tooth extraction will influence bone tissue remodeling near the microscrews, and we evaluated this effect *via* histologic and immunohistochemical analyses.

MATERIAL AND METHODS

Animals and surgical procedures

Twelve male beagles meeting the following criteria were selected: 18 months of age, 10 kg in weight, presence of fourth premolars on mandible, and healthy with no malocclusion and periodontal diseases. They were handled according to the experimental protocol approved by the Bioethics Committee of Sichuan University, China (Number of permit: SYXIC111 2009-045, China).

The animal model was described as our previous study²¹. All the third and the fourth premolars were surgically extracted from the mandibles. Ninety-six microscrews (diameter 1.6 mm, length 6 mm) (Medicon Company, Tuttlingen, Germany), were placed between the mesial and distal roots of P2, P3, P4, and M1, on the buccal side of the mandible, 6 mm beneath the top of the alveolar crest (Figures 1a and 1b). The test implants (48 microscrews) were placed near the extraction sites (between the mesial and distal roots of P3 and P4), and the control implants (48 microscrews) were placed at the normal sites (between the mesial and distal roots of P2 and M1) (Figures 2a and 2b). Microscrews in both the test and control groups experienced no loading. The beagles were fed liquid diet in order to avoid the impact of hard food on the microscrews, and were finally executed at 1, 3, 8 or 12 weeks following implantation.

Histologic analysis

The mandibles were removed from the executed animals and carefully sectioned into small tissue blocks (10x10x6 mm). Each tissue block contained one microscrew surrounded by at least 4 mm-thick bone tissue. The blocks were fixed in cold buffered formalin, pH 7.0, for 3–6 days and then demineralized in 20% tetrasodium ethylenediaminetetraacetic acid (EDTA, pH 7.0) for about 4 to 6 months until the microscrews could be easily removed without breaking the implant-bone interface. All paraffin-embedded blocks were cut

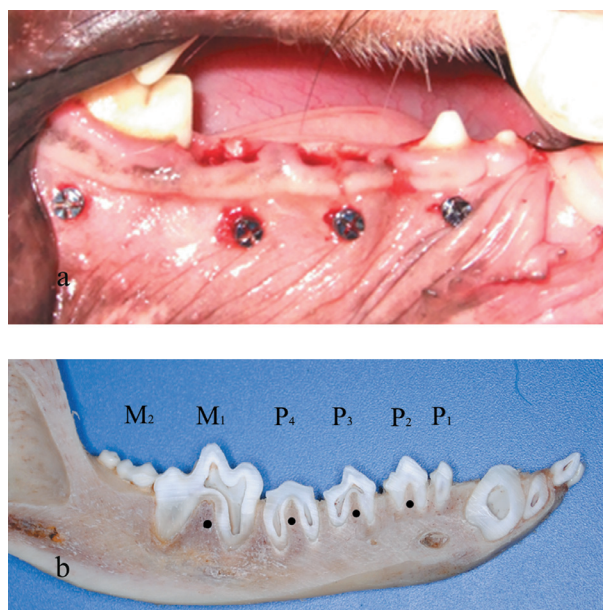


Figure 1- Images illustrating placement sites of microscrews in the mandible of beagle dogs. (a) Clinical picture of microscrews in mandible. (b) Transverse section of mandible with black dots indicating the precise site of microscrews

into 4 μm -thick slices. Some tissue sections were stained with Masson's Trichrome for descriptive analysis and determination of neutrophil and osteoblast densities. For the determination of cell density, 5 histological fields of the implant-bone interface were randomly selected and the number of neutrophils and osteoblast was counted by manual method using 200 \times magnification coupled to Nikon E600 microscope (Nikon Instruments Inc, Melville, USA). The cell-density was calculated by the mean of cell number *per* field.

Immunohistochemistry analysis

Following paraffin removal from the tissue sections, they were hydrated by incubation in 95%, 90%, 80%, and 70% ethanol for 5 minutes. After antigen retrieval with TRIS EDTA (pH 9.0) solution for 30 min, sections were immersed in PBS-H2O2 (0.01 ml, pH 7.0, PBS 99 ml + 30% H2O2 1 ml) for 20–30 minutes, at room temperature (23°C) to eliminate the endogenous peroxidase. The sections were first washed in distilled water for 5 min, and then washed in phosphate-buffered saline

(PBS) for another 5 minutes. Before incubation in primary antibody, sections were immersed in non-immune serum (5% bovine serum albumin, BSA) and diluted 1:5–1:20 for 30 minutes without wash. Following the above step, the sections were incubated with mouse anti-dog OC antibodies (1:75, R&D Systems, Minneapolis, MN), rabbit anti-dog TGF- β antibodies (1:100, Santa Cruz Biotechnologies, Inc, Santa Cruz, CA, USA) and caprine anti-dog TNF- α antibodies (1:200, R&D Systems, Minneapolis, MN, USA) at 4°C overnight. Next, the sections were sequentially incubated with secondary biotinylated goat anti-mouse antibodies (Santa Cruz Biotechnologies, Inc, Santa Cruz, CA, USA), mouse anti-rabbit antibodies (1:200, R&D Systems, Minneapolis, MN, USA) and pig anti-caprine antibodies (1:100, R&D Systems, Minneapolis, MN, USA) for 30 minutes at 23°C. The sections were washed and the specific antibody binding reaction was amplified using streptavidin peroxidase. Diaminobenzidine (DAB 0.5 mg/ml) staining and counterstaining with hematoxylin were performed to provide enhanced orientation of the tissue topography. Finally, the sections were dehydrated in an ethanol gradient and mounted for microscopic observation. As negative controls, slides were incubated with PBS 1% instead of primary specific antibodies. The images were acquired at 200 \times magnification using a Nikon E600 microscope (Nikon Instruments Inc, Melville, USA). All files were saved in tagged-image file format (TIFF). The integral optical density (IOD) of the target protein was measured with Image-Pro Plus 5.0 (Media Cybernetics, Rockville, MD, USA). In the process of measurement, the value was defined firstly by determining the positive staining of control sections, and was used to automatically analyze images of all samples that were stained

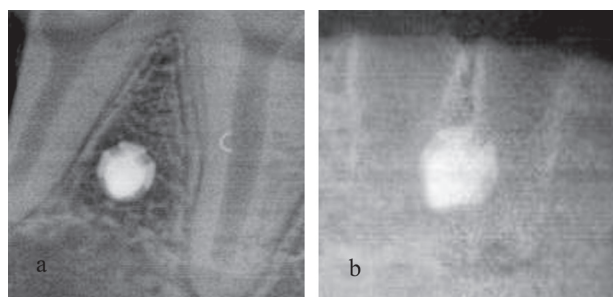


Figure 2- Radiographs illustrating implanted microscrews. (a) Radiograph of micro screw implanted between the roots of first molar. (b) Radiograph of a micro screw implanted near an extraction site

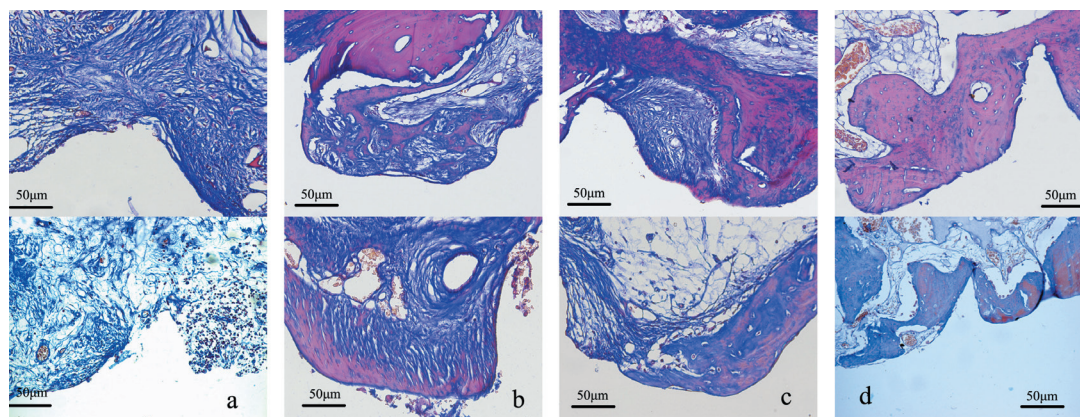


Figure 3- Histologic analysis at the implant-bone interface in the test group (lower panel) and control group (upper panel) using Masson staining. (a) In the test group, a large number of neutrophils were aggregated at the interface at week 1 while a large amount of fibrous tissue was aggregated in the control group. (b) the new bone layer in the test group at week 3. (c) A large-scale dematrix bone (DB) excreted by osteoblasts around the micro screw was observed in both groups at week 8. (d) A mass of mature lamellar bone in the implant-bone interface in both groups at week 12 (Masson stain). Magnification: a, b, c, d; 200 \times

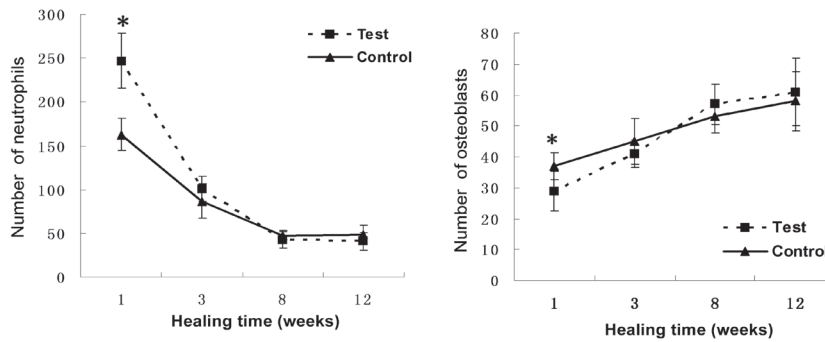


Figure 4- Graphs showing the changes in the density of neutrophil and osteoblast in both groups at 1, 3, 8 and 12-weeks healing time. * indicates statistically significant differences

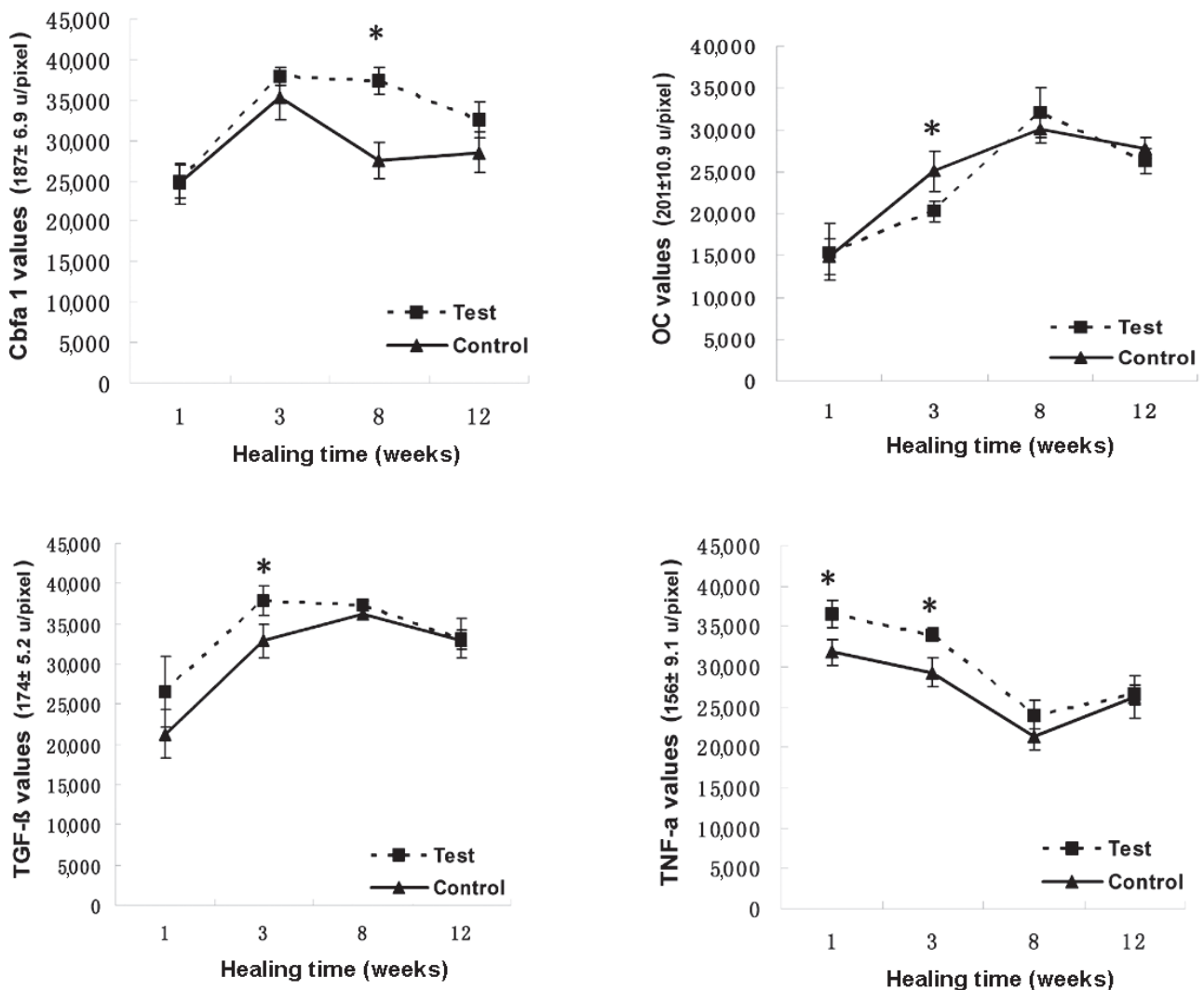


Figure 5- Graphs showing the changes in expression of Cbfa1, osteocalcin (OC), TGF-β and TNF-α, in both groups at 1, 3, 8 and 12-week healing time. * indicates statistically significant differences. (a) At week 8, the expression in the test group was significantly higher than in the control group ($p < 0.01$). (b) The expression of OC reached peak values at week 8 and subsequently decreased. Significant differences were observed at week 3 and values of OC in the control group were significantly higher than in the test group ($p < 0.01$). (c). The peak of TGF-β in the control group appeared at week 8, and subsequently went down to the level as in week 3. At week 3, significant differences were observed between both groups, but the expression of TGF-β in test groups was stronger than in control groups ($p < 0.01$). (d) Significant differences in expression between two groups were observed at week 1 and week 3, and the values in the test group were higher than those in the control group ($p < 0.01$)

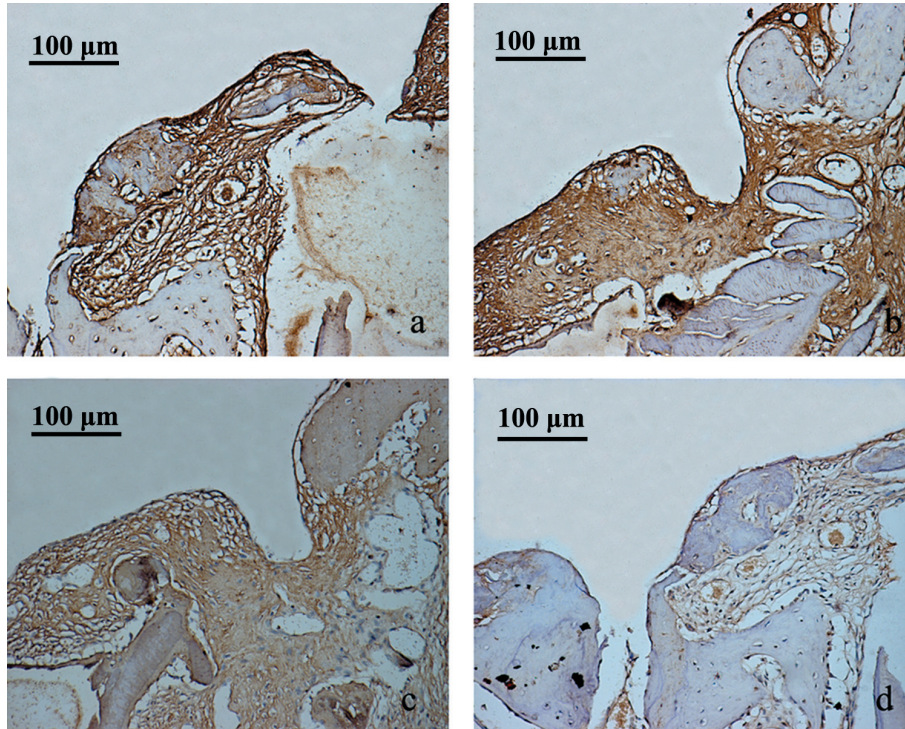


Figure 6- Immunohistochemical staining of Cbfa1 in sections from beagle mandible from test (a and c) and control groups (b and d), during weeks 3 and 8. The expression of Cbfa1 in both groups reached peak values at week 3, but there were no statistically significant differences between test group (a) and control group (b). At week 8, significant differences were observed between both groups, and the expression of Cbfa1 in test group (c) was stronger than in control group (d). Magnification: 200x

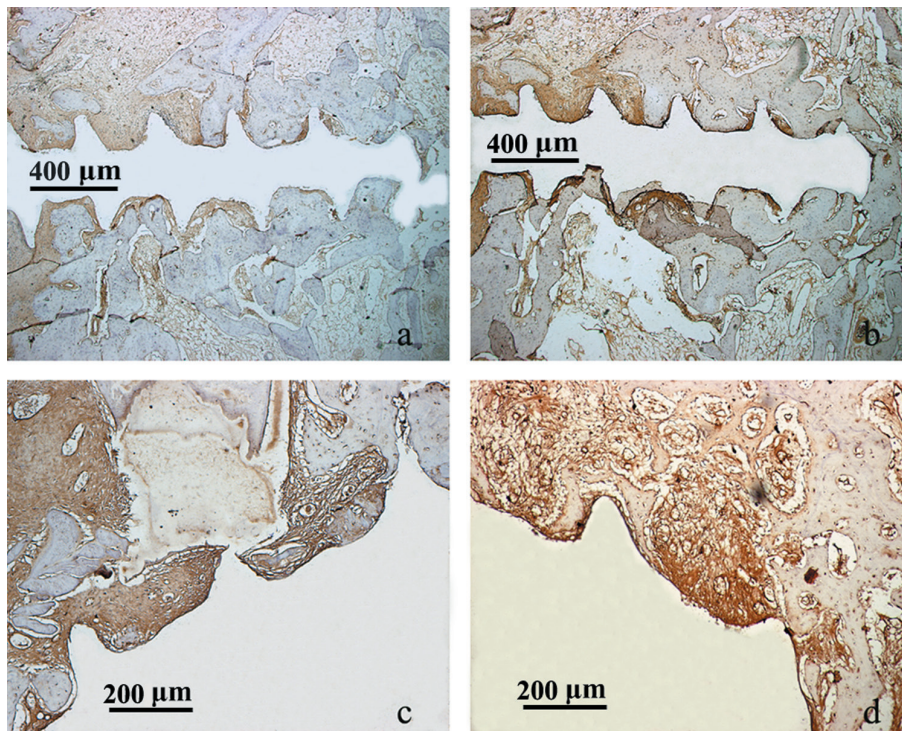


Figure 7- Immunohistochemical staining of osteocalcin (OC) in beagle mandible sections from test (a and c) and control groups (b and d), during weeks 3 and 8. The expression of OC in control group (b) was stronger than in test group (a) at week 3. The expression levels of OC in both groups reached peak values at week 8 (c, d). No statistically significant differences were observed between the two groups at week 8. Magnification: a and b; 40x, c and d; 100x

under identical conditions.

***In situ* hybridization**

In situ hybridization for Cbfa1 was performed using digoxigenin-labeled riboprobes. Before unsealing, the Cbfa1 probes were briefly centrifuged and immersed in ddH₂O. These probes were then stored at -20°C until needed. Deparaffinage, hydration and deactivation of endogenous enzymes in the paraffin sections were performed as mentioned in the previous section. Tissue sections were dropped in pepsin diluted 3% citric acid for 30 min at 37°C, fixed for 10 min (1% paraformaldehyde (0.1 M PBS, PH 7.0–7.6)), and washed in distilled water 3 times. The sections were pre-hybridized for 2 hours at 37–42°C using 20 µL pre-hybridization solution *per* sections, and then they were hybridized with the probe (2 µg/ml) diluted in hybridization buffer and in 2×SSC (standard saline citrate) for 16–18 h at 38–42°C. The sections were washed sequentially in 0.2×SSC, blocked with blocking solution and then incubated with anti-mouse antibody for 1 h at 37°C, washed in PBS. These sections were then exposed to SABC (Strept Avidin-Biotin Complex) and Biotin peroxidase for 30 min at 37°C, and washed again in PBS. Finally, sections were

stained, counterstained, dehydrated and mounted. The expression of cbfa1 was quantified using the same methodology for immunohistochemical analysis.

Statistical analysis

All statistical analyses were performed with SPSS software (SPSS, Chicago, Ill). Student's t-test was used to determine statistical differences in the values between the test groups and the control groups. Data were presented as means with standard deviations. A difference of $P < 0.05$ was accepted statistically significant.

RESULTS

Histologic and immunohistochemical sections from the samples are shown in Figures 4–9. Twelve male beagles received 93 samples. Three samples were omitted due to the loss of microscrews in the test group at week 1.

Histologic findings

At week 1, a large number of neutrophil were aggregated at the bone-screw interface (Figure 3a). In the test group, the neutrophil density was higher ($p < 0.01$) while the osteoblast density was

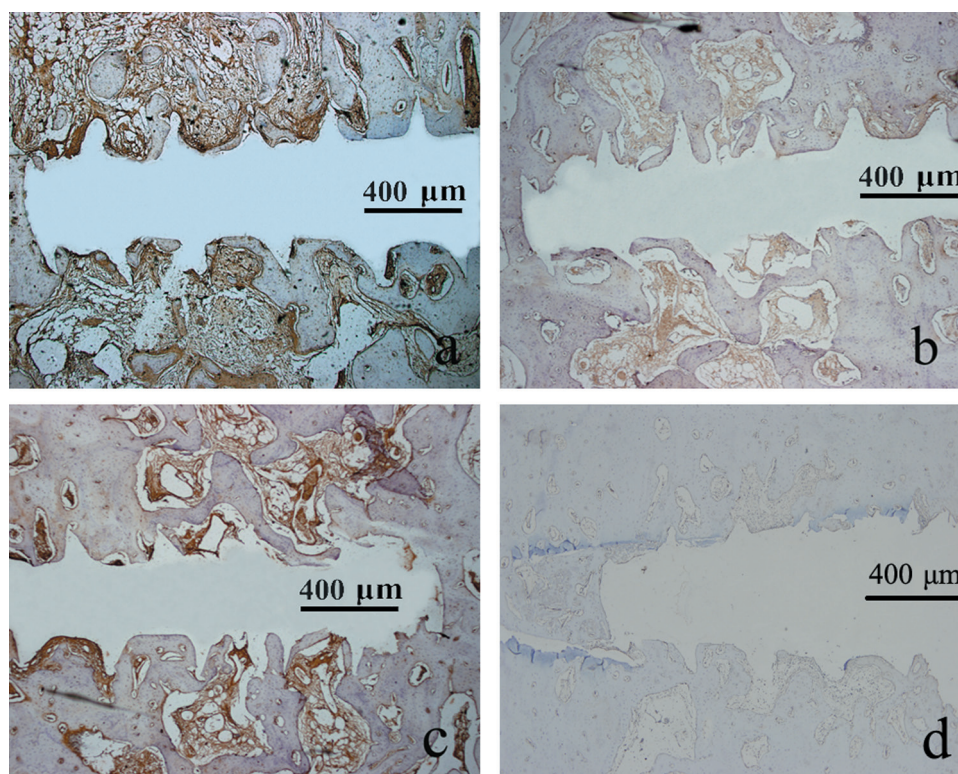


Figure 8- Immunohistochemical staining of TGF- β in sections from beagle mandible from test (a and b) and control group (c and d), during weeks 3 and 8. The expression of TGF- β in test group reached the peak values at week 3 (a). The expression in control groups was low at week 1 (d) and reached the peak values at week 8 (c). There were statistically significant differences between two groups at week 3. The values in test group were higher than in control group (b). Magnification: 40x

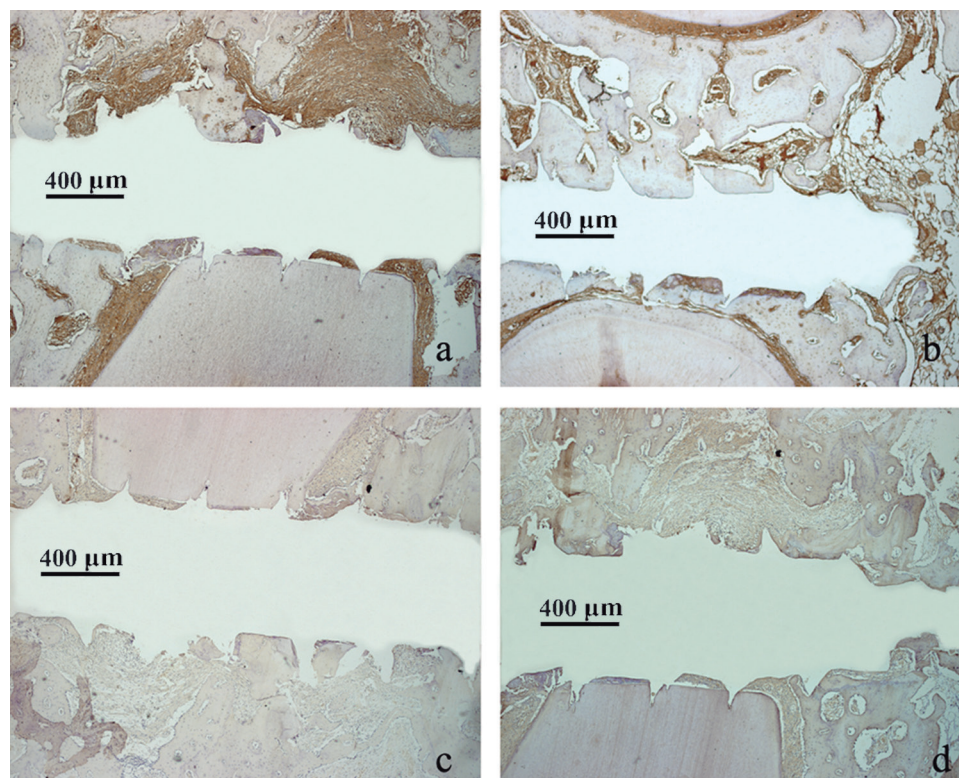


Figure 9- Immunohistochemical staining of TNF- α in beagle mandible sections from test (a and c) and control group (b and d), during weeks 3 and 8. Statistically significant differences were observed between the two groups at week 3. The TNF- α values in test group (a) were higher than in control group (b). The expression in test group (c) and control group (d) presented a downward trend at week 8. Magnification: 40x

lower ($p < 0.01$) in relation to control group (Figure 4). There were new bone layers in the test group at week 3 (Figure 3b). By week 8, many active osteoblasts gathered along the interface and excreted a large-scale bone matrix around the microscrew (Figure 3c). By week 12, there was a mass of mature lamellar bone in the implant-bone interface, calcified to a degree close to that of normal bone tissue (Figure 3d). However, the amount of dematrix bone in the control group was greater than in the test group at week 3 and week 8.

Immunohistochemistry analysis

The expression of Cbfa1 in both groups reached a peak at week 3 (Figure 5). At week 8, the expression in the test group was higher significantly than in the control group (Figure 6) ($p < 0.01$). The expression of OC reached peak values at week 8 and subsequently decreased (Figure 5). Significant differences were observed at week 3 and values of OC in the control group were significantly higher than in the test group (Figure 7) ($p < 0.01$). The TGF- β values in test groups reached a peak at week 3. At week 3, significant differences were observed between both groups, but the expression of TGF- β in test groups was stronger than in control groups (Figure 8) ($p < 0.01$). The mean levels of

TNF- α in both groups were high in the first three weeks after implantation. Significant differences in expression between two groups were observed at week 1 and week 3, and the values in the test group were higher than those in the control group (Figure 9) ($p < 0.01$).

DISCUSSION

Owing to its miniature size and simple surgical placement, miniscrews are easy to place in the maxillae and mandibles, with the aim of providing skeletal anchorage for orthodontic patients. However, in face of the variety of oral conditions seen clinically, orthodontists often need to choose the most suitable miniscrew site, and at present, interradicular sites are the most common choice. In this study, all miniscrews were placed between the mesial and distal roots of P2, P3, P4 and M1 at the buccal side of the mandible of beagles. In order to avoid damaging the roots of neighboring teeth, as reported in a study by Asscherickx, et al.² (2005), radiographs of the beagle mandibles were taken to confirm that the furcation angles of the roots of P2, P3, P4, and M1 of all beagles were above 50 degrees. Moreover, the radiographs, at a later stage, had revealed that implantation between the mesial and distal roots of P2, P3, P4 and M1 were

accurate, and these sites had not interfered with the roots of neighboring teeth or other important structures of the mandible.

In this study, histologic findings from the test group revealed that, at week 1, the original bone was destroyed, with aggregation of a large number of inflammatory cells at the screw-bone interface. Active osteoblasts were gathered around the new bone by week 3. Moreover, by week 8, osteoblasts had secreted a large-scale bone matrix around the microscrew. Literature had reported that, during the first week, pull-out strength of the miniscrews was significantly lower near the extraction site than it was at a distance away from it, followed by an increase in strength during weeks 3 and 8. This indicates that inflammatory reaction and bone resorption at the implant-bone interface were 2 major initial events following implantation. This likely explains why 3 microscrews in the test group failed at this stage. However, subsequent to longer healing time and formation of new bone, the risks surrounding the stability of microscrews decreased significantly, as can be confirmed by the ensuing molecular regulation of osteogenesis around the miniscrews.

At week 1, the expression of Cbfa1, OC and TGF- β was inconspicuous in control and test groups. In contrast, TNF- α expression in both groups was most robust following implantation. This suggests that there emerged a mass of macrophages and osteoclast mediated by TNF- α ^{3,10}, which aggravated directly the damage of interface bone, especially in the test group, which likely caused 3 microscrews to fail in this group. However, due to the low-expression of Cbfa1, OC and TGF- β , bone formation and bone mineralization triggered by osteoblasts were still inconspicuous during this stage^{4,5,8}. In addition, literature reports have suggested that extraction leads to a decrease in bone density in the surrounding vicinity, which increases the risk of non-integration at the implant-bone interface^{11,14,17}. Thus, it is safe to assume that infiltration of numerous inflammatory cells had reduced the stability of the microscrews at week 1, and that the area near the extraction site was not suitable for implantation, even without loading.

Tu, et al.¹⁶ (2007) discovered that alveolar bone defects were largely filled with fibrous connective tissues 3 weeks after surgery in normal mice. In contrast, wound healing was dramatically delayed in Cbfa1-deficient mice. Therefore, with the increasing level of Cbfa1, the most active period of bone remodeling possibly occurred at week 3 post-implantation. Although the high-intensity expression of Cbfa1 was not significantly different at week 3 between the two groups in our study, it maintained its intensity until week 8 in the test

groups, and decreased significantly in the control groups. Esposito, et al.⁶ (2010) showed that the most active period of bone remodeling following extraction was week 8, which may explain why the level of Cbfa1 was higher in test groups at week 8. Likewise, the expression of TGF- β and OC was high from week 3 to week 8. Thus, active osteoblasts and large-scale new bone were formed at this stage. Osteogenesis was observed at the implant-bone interface during this stage, and the expression of TNF- α , as well as the inflammation mediated by it, began to decline significantly. On the other hand, the expression of OC in the control group was higher than in the test group at week 3. TNF- α can likely inhibit the expression of matrix protein genes at week 3¹⁰. However, by week 8, the expression level of TGF- β and OC was the same in both the test and control groups. Literature²¹ reported that values of microscrew pull-out strength were similar between the test and the control groups at week 8. Thus, these findings suggest that, with a longer period of healing, the risk of microscrew instability decreased significantly, and that, by week 8, the remodeling of the interface bone, both in test and control groups, tended to be similar.

As for week 12, there was a large amount of mature lamellar bone at the implant-bone interface, calcified to a degree that was similar to that of normal bone tissue. The expression of TGF- β , OC and Cbfa1 began to decline, which illustrated a decline in bone tissue remodeling. TNF- α expression had begun to rebound, which suggests that lack of corresponding bone stimulation aggravates bone resorption²⁰.

CONCLUSIONS

After investigating the remodeling of the bone-microscrew interface near extraction sites via histologic and immunohistochemical analysis, we conclude that:

In the early days, the bone remodeling of extraction will affect stability of microscrew near extraction;

Subsequent to a longer healing period, the influence of extraction on the remodeling of interface bone surrounding microscrews decreases;

Irrespective of the location of the interface, near or away from an extraction site, microscrews are suitable for implantation.

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