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Treatment with tacrolimus enhances alveolar bone formation and decreases osteoclast number in the maxillae: A histomorphometric and ultrastructural study in rats

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Summary. Recent studies have suggested that tacrolimus monotherapy is a beneficial therapeutic alternative for the normalization of cyclosporin-induced bone loss in animal models and humans. The mechanism accounting for this action is unclear at present. In the present study, we attempted to determine the effect of tacrolimus monotherapy on alveolar bone using histological, histomorphometrical and transmission electron microscopy (TEM).

Groups of rats (n=10 each) were treated with either tacrolimus (1mg/kg/day, s.c.) or drug vehicle for 60 days. Fragments containing maxillary molars were processed for light microscopy to investigate the alveolar bone volume, trabecular separation, number of osteoclasts and osteoblasts, and transmission electron microscopy to investigate their ultrastructural basic phenotype.

Treatment with tacrolimus monotherapy during 60 days may induce increases in alveolar bone volume (BV/TV,%; $P<0.05$) and a non-significant decrease in trabecular separation (Tb.Sp,mm; $P>0.05$), represented by a decrease in osteoclast number (N.Oc/BS; $P<0.05$) and maintenance of osteoblast number (N.Ob/BS; $P>0.05$). Osteoblasts were often observed as a continuous layer of active cells on the bone surface. Osteoclasts appeared to be detached from the resorbed bone surface, which was often filled by active osteoblasts and collagen-rich matrix. Moreover, osteoclasts in the treated group were frequently observed as inactive cells (without ruffled border, clear zone and detached from the bone surface).

Within the limits of the present study, we conclude that tacrolimus leads to an increase in alveolar bone formation, which probably exerts action on osteoclasts. Tacrolimus could, therefore, play a crucial role in the control of both early osteoclast differentiations from precursors, as well as in functional activation.

Key words: Tacrolimus, Osteoclasts, Osteoblasts

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Introduction

Tacrolimus (FK-506) is a non-steroidal immunosuppressant drug, based on calcineurin inhibition, isolated from *streptomycees tsukubaensis* (Kino et al., 1987), which is widely used for organ transplantation (Scott et al., 2003) and atopic dermatitis (Krueger et al., 2007). Following the use of new and more powerful immunosuppressive drugs, such as tacrolimus, survival rates have significantly improved in solid organ transplantation due to an effective control of acute rejection episodes (Horslen et al., 2007). Recent clinical studies have also demonstrated the efficacy of tacrolimus in the treatment of rheumatoid arthritis (Miyata et al., 2005), resistant external otitis (Caffier et al., 2007), refractory adult Still's disease (Murakami et al., 2007) and Wiskott-Aldrich syndrome (WAS) (Bienemann et al., 2007).

Some studies have reported that calcineurin inhibitors (CIs), such as tacrolimus, have serious effects, causing rapid and severe bone loss in animal models and

humans (Sass et al., 1997; Tamler and Epstein, 2006). In humans, acute, rapid, severe bone loss ensues with a fracture incidence approaching 65% (Epstein et al., 2003). However, other recent studies have reported that tacrolimus is not osteotoxic (Marcen et al., 2006; Guimaraes et al., 2007; Spolidorio et al., 2007).

Tacrolimus potently inhibits the phosphatase activity of calcineurin by interacting with domains of the calcineurin A subunit (Aramburu et al., 2000) and reducing the expressions of interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-10 (IL-10), interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α), as well as the activation of the T lymphocytes (Scott et al., 2003; Taylor et al., 2005). In line with these findings, we recently showed, in an animal model, that tacrolimus treatment does not induce alveolar bone loss and, more specifically, tacrolimus suppresses the expression of serum interleukin-1 beta (IL-1,), tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), cytokines well known to be involved in bone resorption. Although the role of the calcineurin pathway was first described in T-lymphocytes, some studies have demonstrated evidence of the critical role of this protein in regulating the genesis and function of osteoclasts (Hirotani et al., 2004; Takayanagi et al., 2005; Sun et al., 2007) and its expression in osteoblasts, with a direct implication on the control of osteoblastic bone formation (Sun et al., 2005). In the present study, we attempted to determine the effect of tacrolimus monotherapy on the alveolar bone of rats by analyses of histomorphometrical parameters and ultrastructural aspects by transmission electron microscopy (TEM).

Materials and methods

Animals

Twenty male Holtzman rats (*Rattus Norvegicus Albinus*), weighing approximately 100g, were selected and randomly distributed into two groups of ten animals each. The rats were housed in polypropylene cages in groups of five animals per cage at controlled temperature (23±2°C) and humidity (55%±10%) and 12/12 hours light/dark cycles, beginning at 7:00 a.m. Standard chow and tap water were available ad libitum. All the experimental protocols were approved by the local Ethics Committee for Animal Experimentation and performed in accordance with the guidelines from the Brazilian College for Animal Experimentation (COBEA).

Tacrolimus treatment

One group of rats was treated with Tacrolimus (Prograf® - Janssen Cilag, Brazil), injected subcutaneously in a daily dose of 1mg/kg body weight. According to Jiang et al. (1991) and Li et al. (2003), this dosage provides plasma peak and therapeutic concentrations of 11.2 and 13.1 ng/mL, respectively. Control rats were injected daily subcutaneously with the drug vehicle. The subcutaneous route was chosen to avoid the variability and difficulty of oral dosing and because this method has been used successfully previously (Spolidorio et al., 2004a,b).

All rats were weighed weekly and monitored for any abnormal appearance of their coats and abnormal levels of activity, and were sacrificed 60 days after the beginning of the treatments.

Histology

The upper maxilla was carefully removed, dissected free of soft tissue and fixed in 10% formalin for 48h. The right side was decalcified in Morse solution (50 mL formic acid and 50 mL of 20% sodium citrate) for approximately 20 days. Serial transversal paraffin sections of 5µm were obtained from the buccal-lingual aspects of the whole 1st right molar and were subsequently stained with haematoxylin and eosin. Stereological studies were performed on the interradicular region to a level where the mesial and distal roots of the first upper molar were visible. Each upper 1st molar has a mesial-distal diameter of approximately 0.4 mm, thus resulting in approximately 60 sections.

Trabecular bone histomorphometry of alveolar bone

The following stereometrical parameters were quantified, according to the methods described by Spolidorio et al. (2007). Nomenclature and abbreviations follow the recommendations of the American Society for Bone and Mineral Research (Parfitt, 1988). For structural parameters, the following were measured: Bone volume (BV/TV; % - bone hits/total hits x 400), which represents bone volume (BV; mm^3) per total tissue volume (TV; mm^3); trabecular separation (Tb.Sp; mm, the shortest distance between trabeculae); osteoblast surface (N.Ob/BS/mm) and osteoclast surface (N.Oc/BS/mm), values that indicate the number of osteoblasts and osteoclasts per bone surface, respectively. Measurements were performed with the aid of a Zeiss microscope at a magnification of x400, coupled to a Pentium IV Intel computer, and a morphometry program named "stereology" (KSS Computer Engineer, Magma, UT). The distance between the selected sections was 50 µm.

The counted cells considered as osteoclasts were large multinucleated cells found next to excavated surfaces of alveolar bone (Baron, 1989), and the counted cells considered as osteoblasts, were typically secretory osteoblasts that were cuboidal cells, smaller than osteoclast cells, with a round nucleus and usually found in a single layer adherent to bone surfaces (Cerri, 2005).

Ultrastructural analysis

Fragments of the left upper maxilla containing the first molar were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (from paraformaldehyde),

buffered at pH 7.2 with 0.1 M sodium cacodylate at room temperature for 24 hours. After decalcification for 45 days in 7% EDTA containing 0.5% formaldehyde, buffered at pH 7.2 in sodium cacodylate 0.1 M, the specimens were washed in 0.1 M cacodylate buffer (pH 7.2). They were then transferred to cacodylate-buffered 1% osmium tetroxide at pH 7.2 for 1-1.5 h at room temperature and subsequently treated with aqueous 2% uranyl acetate for 2 h. After dehydration in graded concentrations of ethanol, the specimens were treated with propylene oxide and then embedded in Araldite. Toluidine-stained semithin sections were examined under a light microscope for selection of regions to be trimmed. Ultrathin sections were collected onto grids and stained with uranyl acetate and lead citrate before examination in a Zeiss EM10 transmission electron microscope.

Statistical analysis

Comparisons between the untreated group (control) and the tacrolimus treated group, at 60 days, for the morphometric parameter data (BV/TV(%), Tb.Sp(mm), N.Ob/BS/mm and N.Oc/BS/mm) were made using the unpaired Student t-test. Regarding the body weight of the rats over time, comparisons were made using one-way analysis of variance (ANOVA) and, when ANOVA test showed significant differences, pair-wise multiple comparisons were used (Tukey test). Significance level was always set at 1%.

Results

In-life phase

All animals survived the whole experimental period. Treatment with tacrolimus did not affect body weight. At the end of the study, rats in the control and tacrolimus groups weighed 380±109.6g and 324±89.5g, respectively (Fig. 1). During the experimental periods no alterations in the buccal mucosa were observed, e.g. gingival overgrowth (Fig. 2A,B).

Histomorphometric findings

Histological examination of decalcified sections of the upper maxillae of tacrolimus-treated animals revealed a discreet increase in the size of bone trabeculae, associated with an increase in typical reversal lines, when compared to the control group. In the treated animals, osteoclasts characterized by the presence of various nuclei were scarce, while osteoblasts were aligned as a single-cell layer on the trabecular bone surface (Fig. 2C,D). Quantitative analysis of morphometric parameters revealed a statistically higher BV/TV (%) in the tacrolimus-treated group, when compared to the control group (1.95±0.36 versus 1.45±0.49) and intergroup analysis showed that there were no significant differences in Tb.Sp (mm) between the two groups (0.12±0.66 mm versus 0.14±0.04 mm, respectively) (Fig. 3A,B).

The number of osteoclasts (N.Oc/BS) was decreased significantly in the tacrolimus-treated group (0.9N.Oc/BS ±0.43 versus 2.5N.Oc/BS ±0.77 for the control group). Maintenance of the osteoblast number (N.Ob/BS) was observed between the tacrolimus-treated rats and control rats (28.26N.Ob/BS±7.4 and 32.60N.Ob/BS±8.0, respectively) (Fig. 4A,B).

Ultrastructural analysis

The ultrastructural analysis showed that the alveolar bone of the drug vehicle and the tacrolimus-treated rats revealed numerous secretory osteoblasts apposed to the bone surface. Frequently, the polarized osteoblasts containing several profiles of rough endoplasmic reticulum and well-developed Golgi sacules were in close juxtaposition to the unmineralized bone layer, the osteoid. The unmineralized layer exhibited predominantly cross-sectioned collagen fibrils; in some areas, these fibrils were densely packed, forming bundles of collagen fibrils, which appeared to be in continuity with the bone surface. Bundles of longitudinal or obliquely sectioned collagen fibrils penetrated into the bone, forming Sharpey´s fibers. The Sharpey´s fibers, orientated perpendicular to the bone surface, perforated the continuous layer of osteoblasts that covered the osteoid (Fig. 5A). The scarce multinucleated osteoclasts were also observed on the bone surface; some of these cells appeared to detach from the previously reabsorbed bone lacuna and these excavated bone surfaces were often filled by active osteoblasts and collagen–rich matrix (Fig. 5B). In these areas, in which active bone formation took place, the electron-opaque lines present in the bone that were typical of reversal lines confirmed the histological analysis. Osteoblasts exhibiting short cytoplasmic projections were often surrounded by bundles of densely packed collagen fibrils (Fig. 5C).

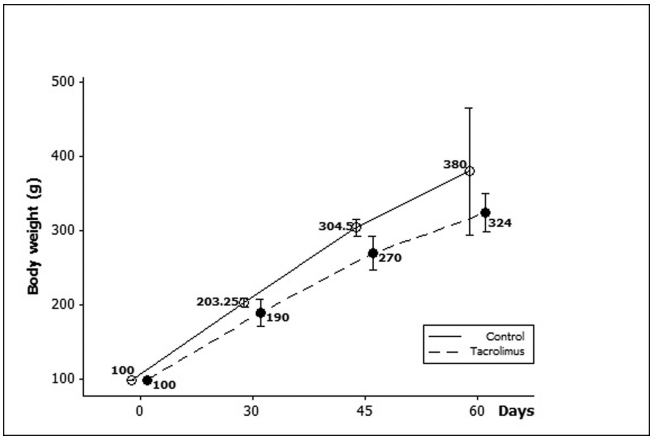

Fig. 1. Time course of the body weight of tacrolimus-treated rats and control rats. There were no statistical differences between the groups, over time. P<0.01 (Tukey test).

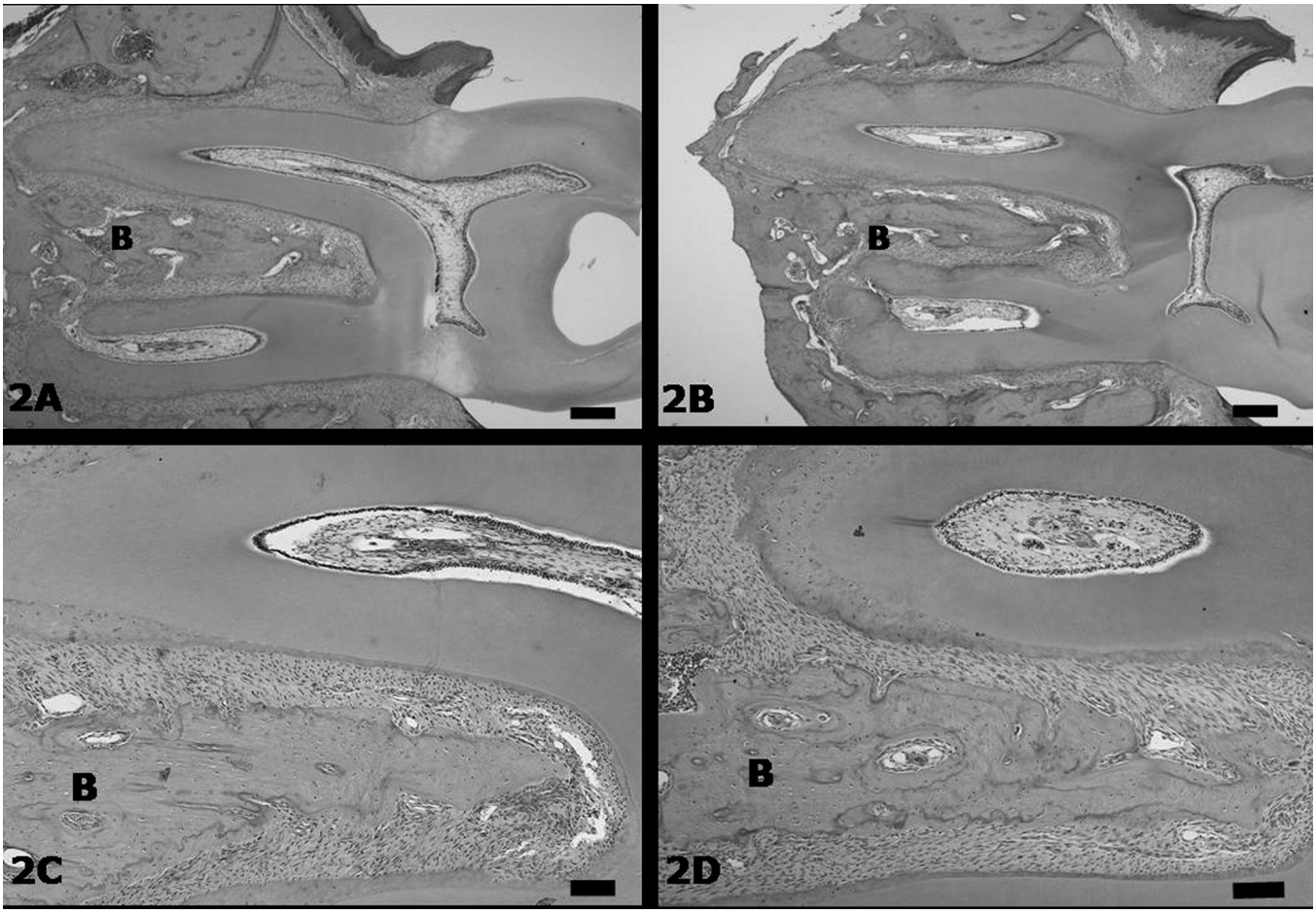

Fig. 2. Decalcified transversal sections of the interradicular region of the 1st upper maxilla molar from controls (**A, C**) and tacrolimus treated rats (**B, D**) at 60 days of treatments, stained by hematoxylin and eosin. A significant increase in bone mass and an increase in typical reversal lines can be observed in tacrolimus-treated rats (**D**). B: alveolar bone. Bars: A, B, 250 µm; C, D, 625 µm.

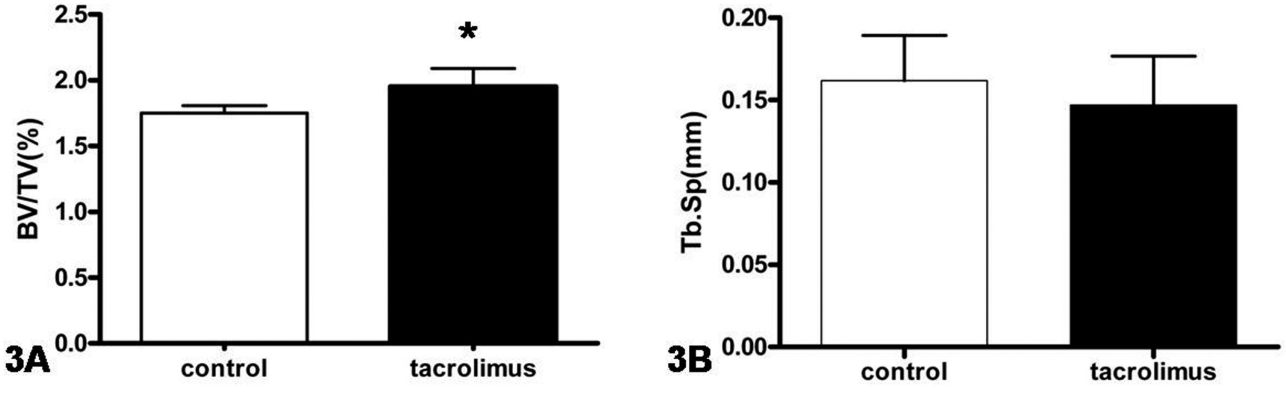

Fig. 3. Average stereometric measurements from maxillae obtained from rats under the different treatments: control (untreated), Tacrolimus (1 mg/kg/day, s.c., during 60 days). **A.** Bone volume (BV/TV; %). **B.** Trabecular separation (Tb.Sp; mm). All values are expressed as ± SEM (n=10 animals per group). *: $p<0.01$ vs. the other group at 60 days (Student t-test).

Discussion

The deleterious effects of tacrolimus on bone mineral metabolism, in vivo, were first described by Cvetkovic et al. (1994). Since then, other studies have shown controversial results and incited great discussion. Some studies have demonstrated that the systemic administration of calcineurin inhibitor drugs, e.g. tacrolimus, to rats causes a dramatic osteoporosis (Cvetkovic et al., 1994; Sass et al., 1997; Stempfle et al., 2002; Tamler and Epstein, 2006), while in humans, acute, rapid, severe bone loss ensues with a fracture incidence approaching 65% (Stempfle et al., 2002; Epstein et al., 2003). It has been speculated, but never proven in either animals or humans, that cyclosporine-A or tacrolimus use the calcineurin pathway to exert their skeletal effects. In fact, the independent effects of tacrolimus monotherapy on bone have been difficult to characterize in humans. This is mainly due to the fact that transplant patients usually undergo combined therapy regimes (i.e. tacrolimus together with corticosteroids or other drugs) (Stempfle et al., 2002; Scolapio et al., 2003; Marcen et al., 2006), as well as unavoidable sources of variability, such as differences in drug dosage, gender and age among the subjects that take part in these studies. Furthermore, the genetic capacity of the host that determines the bioavailability and disposition of the administered drug, and the individual responsiveness of the bone to this drug, may also hinder the evaluation of tacrolimus on bone. As a consequence, and in order to evaluate the effects of tacrolimus monotherapy on specific cells or tissues, experimental animal protocols are a convenient alternative to attain more uniform results by strictly controlling the above-mentioned variables. The results obtained on tacrolimus-induced gingival overgrowth and bone loss in rats are much more uniform than those observed in humans (Spolidorio et al., 2005; Guimarães et al., 2007; Nassar et al., 2008). To our knowledge, no study directly involving tacrolimus monotherapy and upper maxilla has been performed to date.

One-month-old rats were selected since modeling processes prevail over remodeling, making them a good model to study the metabolism of upper maxilla, and intense modeling is an important feature of the alveolar bone and occurs throughout the lifetime of the animal (Misawa et al., 2007). The selected dose of tacrolimus was based on previous studies (Guimarães et al., 2007; Nassar et al., 2008) and, according to Jiang et al. (1991) and Li et al. (2003), this dosage provides plasma peak and therapeutic concentrations of 11.2 and 13.1 ng/mL, respectively. The subcutaneous route was chosen to avoid the variability and difficulty of oral dosing and because this method has been used successfully previously (Spolidorio et al., 2004ab).

Based on these premises, quantitative analysis of morphometric parameters in alveolar bone revealed a statistically significant increase in BV/TV (%), but there were no statistical differences in Tb.Sp (mm) in the tacrolimus-treated rats when compared to the control group. Consistent with these results, other studies evaluating the effect of tacrolimus in other bone sites and using a similar dose of tacrolimus revealed similar results in rats (Voggenreiter et al., 2000; Guimarães et al., 2007; Spolidorio et al., 2007; Nassar et al., 2008). On the other hand, the present findings are not in line with those from other studies with tacrolimus on bone, confirming controversial results (Cvetkovic et al., 1994; Sass et al., 1997; Stempfle et al., 2002; Tamler and Epstein, 2006).

It is well documented that homeostasis of the bone system is maintained by a bone remodeling process, which is dependent on a delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts (Takayanagi 2005; Guimarães et al., 2007). In the present study, treatment with tacrolimus maintained the number of osteoblasts and induced a significant decrease in the number of osteoclasts compared to the control group; paradoxically, we recently showed that tacrolimus treatment did not alter

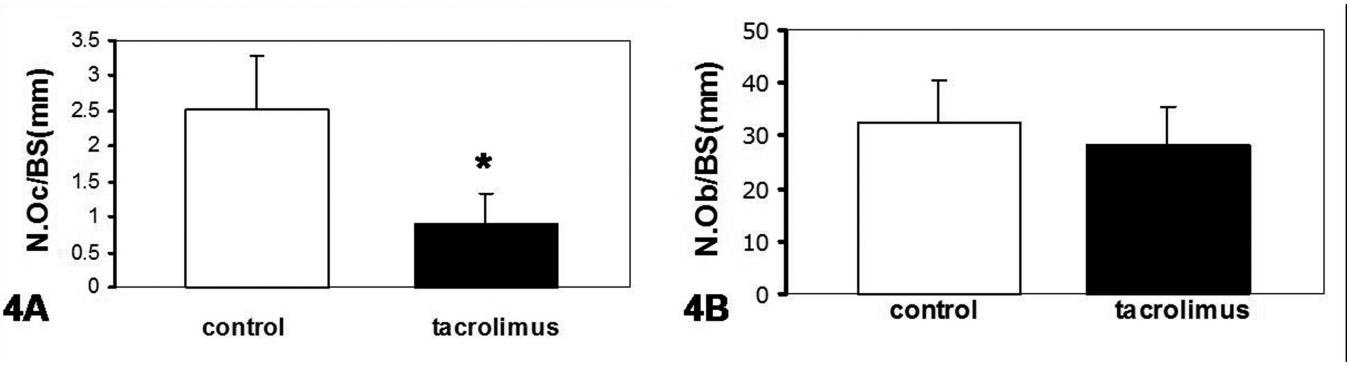

Fig. 4. Average stereometric measurements from maxillae obtained from rats under the different treatments: control (untreated), Tacrolimus (1 mg/kg/day, s.c., during 60 days). **A**. Number of osteoclasts per bone surface (N.Oc/BS). **B.** Number of osteoblasts per bone surface (N.Ob/BS). All values are expressed as ± SEM (n=10 animals per group). *: $p<0.01$ vs. the other group at 60 days (Student t-test).

osteoclast number in the mandible of rats treated with the same dose of tacrolimus (Spolidorio et al., 2007). Huja et al. (2006) studied the remodeling dynamics in the alveolar process in skeletally-mature dogs and verified that, under physiological conditions, there is a variation in bone turnover between maxilla and mandible. It is intriguing that there are differences in the remodeling rates of the alveolar process between the jaws, but the reason for this turnover is not clearly understood. The authors suggested, however, that at a tissue level the differences between the jaws probably involve a complex interplay of bone turnover, mass, and architecture. In fact, future studies associating morphologic studies and molecular biology are

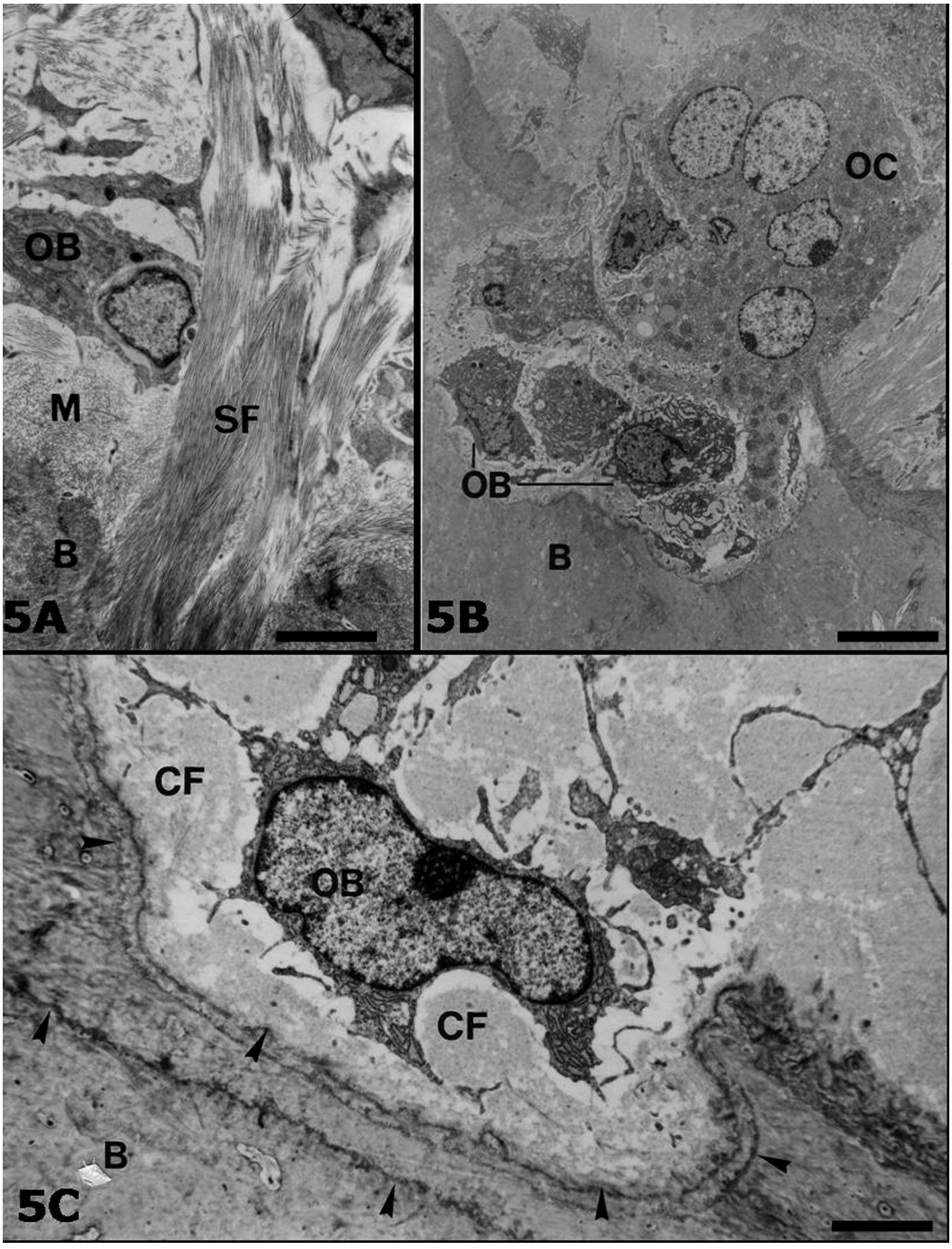

Fig. 5. Electron micrographs showing portions of alveolar bone from tacrolimus-treated rats and control rats. In fig. **5A** (control rats), a bundle of longitudinal or obliquely-sectioned collagen fibrils (SF) penetrate into the bone (B); portion of osteoblast (OB) is apposed to a layer of unmineralized matrix (M) that covers the surface of the bone (B) of the control animal. Bar: 2.5 µm. In fig. **5B** (tacrolimus treated rats), a deep excavation in the surface of the bone contains some apparently active osteoblasts (OB); a multinucleated osteoclast (OC) showing irregular shape appears to be detaching from the excavated surface of the bone (B). Bar: 2.0 µm. In fig. **5C** (tacrolimus-treated rats), an excavation of the surface of the alveolar bone contains an irregular osteoblast (OB). Numerous collagen fibrils (CF) are packed around the osteoblast (OB). In the excavated region, the alveolar bone exhibits three electron-opaque lines (arrowheads) indicating the continuous deposition of the bone matrix. Bar: 1.0µm.

necessary to elucidate the mechanism underlying the difference between mandible and maxillae following tacrolimus treatment. Thus, a limitation of this study is that it examines the remodeling dynamics using morphologic techniques.

In addition, the ultrastructural examination revealed that most of the osteoclasts were detached from the bone surface, indicating that these cells were inactive. Multinucleated osteoclasts exhibiting typically ruffled borders, clear zones and numerous mitochondria distributed throughout the cytoplasm, suggestive of typically active osteoclasts, were rarely found. According to Igarashi et al. (2005), tacrolimus mainly inhibits the late stages of the osteoclast life cycle, largely through induction of osteoclast apoptosis. However, these authors also infer that, although an apoptotic effect appears to be the predominant action of the tacrolimus on the osteoclasts, other mechanisms may be involved in the inhibitory effects on these cells, since other signaling pathways have been shown to be involved in osteoclast activation and survival.

The osteoblasts were frequently observed as a continuous layer on the bone surface. In addition, the osteoblasts were found on excavated bone surfaces and showed features of active cells, such as a fairly rough endoplasmic reticulum and well-developed Golgi sacules (Mackie, 2003; Cerri, 2005). However, a newly-formed bone matrix was observed between these active osteoblasts and the irregular bone surfaces. Therefore, these ultrastructural findings indicate that the osteoblasts are producing new bone matrix on previously reabsorbed bone surfaces; this idea is reinforced by the significantly higher BV/TV (%) and a non-significant decrease in Tb.Sp (mm).

Yoshikawa et al. (2000) and Takayanagi (2005) showed that nuclear factor of activated T cells (NFAT) activity is a crucial transcription factor for osteoclast differentiation, and that tacrolimus inhibits osteoclastogenesis *in vitro* by suppressing NFATc1 induction; NFATc1 is stimulated by a key cytokine for osteoclastogenesis, the receptor activator of NF-κß ligant (RANK). Moreover, Yoshikawa et al. (2005) suggest that the inhibition of calcineurin by the tacrolimus-FKBP complexes could induce the in vitro osteogenic differentiation of marrow mesenchymal cells and, consequently, an ossification-inducing effect. Tang et al. (2002) also observed a positive effect with respect to tacrolimus on bone metabolism and suggest that this immunosuppressor directly induces differentiation of osteoblastic and mesenchymal cell lines, resulting in bone formation. However, given these processes, it is expected that the in vivo administration of tacrolimus would suppress osteoclastic bone resorption and increase bone mass. In contrast, Koga et al. (2005) showed that tacrolimus administration induces a reduction of bone mass, despite blocking osteoclast differentiation.

In summary, this study showed, in the alveolar bone of rats, a significant increase in BV/TV(%), as well as a non-significant decrease in Tb.Sp (mm), with a maintenance of osteoblast number and a slight decrease in the number of osteoclast cells under tacrolimus treatment, when compared to the control group. At the same time, an overview of the ultrastructural characteristics, which is scarce in the related literature, could be provided. Thus, within the limits of this study, we may conclude that tacrolimus provides a beneficial therapeutic alternative that favors the normalization of immunosuppressor-induced alterations in bone metabolism, probably through the alterations in osteoclast metabolism. Moreover, these results may provide important insights into the management of post-transplantation alveolar bone loss. Further studies are being designed and conducted to address the mechanisms of action of tacrolimus on osteoclasts and osteoblasts that are relevant for bone turnover.

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