Supplementation with Melatonin Prevents Alveolar Bone Resorption in Rats with Periodontal Disease and Pinealectomy

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Abstract

Aims: To determine the effect of melatonin supplementation on alveolar bone metabolism in rats with periodontal disease (PD) and pinealectomy.

Materials and methods: Eighty rats were distributed into 8 groups: control (CN); pinealectomized (PNX); PD; pinealectomized with PD (PNXPD); control with melatonin (CNMEL); PD with melatonin (PDMEL); pinealectomized with melatonin (PNXMEL); pinealectomized with PD and melatonin (PNXPDMEL). After 20 days of pinealectomy, PD was induced and the melatonin supplementation was starded. After 28 days, experiments were performed on animals; then, the sample analyses were performed.

Results: An increase in the proinflammatory mediator TNF-α was observed in both PD and PNX animals. PD and PNXPD groups demonstrated greater alveolar bone resorption due to a higher activity of the osteoclasts compared to the osteoblasts evidenced by the increase of the resorption marker, and decrease of the formation marker as well as histomorphometric results. However, in animals treated with melatonin, there was no change in plasma TNF-α concentration and alveolar bone loss.

Conclusion: Supplementation with melatonin decreased TNF-α in pinealectomized and PD animals. In groups with PD, melatonin protected the alveolar bone resorption. Therefore, these results demonstrate that melatonin is efficient in controlling both alveolar bone resorption and TNF-α in rats (PD and PNX), demonstrating its local and systemic importance.

Keywords. Melatonin; pineal gland; periodontal diseases; inflammation; bone resorption

Introduction

Periodontal disease (PD) is a chronic inflammatory disease of the periodontal tissues, involving a series of pathological changes in the periodontium that lead to the gradual destruction of these tissues (Di Benedetto *et al.*, 2013). PD is one of the main causes of tooth loss among adults due to loss of alveolar bone support, reducing oral function, and quality of life (Kumar *et al.*, 2014).

It is known that lipopolysaccharide (LPS), a component present in the outer membranes of gram negative bacteria residing in the oral biofilms (Queiroz *et al.*, 2008; Choi *et al.*, 2011), membrane-associated vesicles,

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and other soluble and particular fractions promote the activation of monocytes, macrophages and fibroblasts (Queiroz *et al.*, 2008; Darveau *et al.*, 1997; Yamazaki *et al.,* 1997). This activation can produce proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Morrison and Ryan, 1987; Hernández *et al.*, 2011), and free radicals (Bartold *et al.*, 1984; Battino *et al.*, 1999). These products accentuate the inflammatory process, promoting osteoclastogenesis, greater destruction of the fibers of the periodontium ligament, and collagenase synthesis, leading to the destruction of alveolar bone (Ali *et al.*, 2011; Lalla *et al.*, 1998; Lalla *et al.*, 2000; Taubman *et al.*, 2005).

In addition to the influence of cytokines on alveolar bone loss, studies indicate that the absence of melatonin (N-acetyl-5-methoxytryptamine), a hormone of the pineal gland, may alter bone metabolism (Amstrup *et al.*, 2013; Maria *et al.*, 2014), thus compromising the balance between bone formation and resorption and contribute to increased bone resorption (Egermann *et al.*, 2011). This hormone has also been recognized as a potent free radical scavenger with antioxidant properties (Maria and Witt-Enderby, 2014; Sánchez-Barceló *et al.*, 2010; Galano *et al.*, 2011), immunomodulatory (Cengiz *et al.*, 2012), and anti-inflammatory effects (Mayo *et al.*, 2005). Therefore, melatonin may have implications in PD because of its anti-inflammatory, antioxidant, fibroblast proliferation, and bone remodeling activities (Galano *et al.*, 2011; Cutando *et al.*, 2007). Thus reducing oxidative stress, limiting tissue damage, stimulating the immune response, and reducing the progressive loss of alveolar bone.

Studies have shown that pinealectomized (PNX) animals have bone loss and significantly decreased bone mineral density, reinforcing the importance of sufficient levels of melatonin (Egermann *et al.*, 2011; Amstrup *et al.*, 2013). In addition, melatonin has a direct effect on bone remodeling, benefiting bone metabolism through bone anabolism, and anti-resorptive effects (Amstrup *et al.*, 2013; Tresguerres *et al.*, 2014).

Therefore, considering that both PD and absence of melatonin promote an imbalance in the process of bone remodeling causing bone loss due to a greater osteoclast activity (Egermann *et al.*, 2011; Amstrup *et al.*, 2013; Cochran, 2008; Kajiya *et al.*, 2010), and that melatonin contributes to bone remodeling (Amstrup *et al.*, 2013) it is necessary to better understand the effects of pinealectomy in association with PD on bone markers, inflammatory cytokines and the action of melatonin supplementation on these parameters in the alveolar bone. Thus, we hypothesized that PNX would aggravate alveolar bone resorption in PD animals and that melatonin supplementation prevents alveolar bone resorption.

The study aimed to verify the effect of melatonin supplementation and pinealectomy on alveolar bone metabolism and plasma concentration of inflammatory cytokines in rats with PD and PNX rats. To this end, we evaluated pro-inflammatory cytokines (TNF-α and IL-6), bone markers such as tartrate-Resistant acid phosphatase (TRAP), osteocalcin (OCN), and alveolar bone histomorphometry in control and pinealectomized adult rats with and without periodontal disease and treated with melatonin.

Material and methods

Animals

Eighty male Wistar rats weighing 200-220g aged 40 days were used in the study. The animals were kept in a room with a light/dark cycle of 12 h (light period starting at 7 h) and controlled temperature of 23 ± 2°C, with access to water and food (23% protein, 4% fat, and 58% carbohydrates) (Presença, Paulínia, São Paulo, Brazil) ad libitum. This study followed the Ethical Principles and Guidelines for Animal Experimentation and was approved by the Ethics Committee on the Use of Animals (CEUA) of the São Paulo State University (Unesp), Araçatuba (protocol number 00544-2016).

The animals were randomly divided into 8 groups (n=10/group): 1) control (CN); 2) pinealectomized (PNX); 3) periodontal disease (PD); 4) pinealectomized and periodontal disease (PNXPD); 5) control treated with MEL (CNMEL); 6) pinealectomized treated with MEL (PNXMEL); 7) periodontal disease treated with MEL (PDMEL), and (8) pinealectomized and periodontal disease treated with MEL (PNXPDMEL). Animals were fasted for 14 h prior to experiments and anesthetized with sodium thiopental (Thiopentax®, Cristália, Itapira, Brazil) (3%, 50 mg/kg body weight, intraperitoneal) preceded by the application of regional/local blocking anesthetic (Lidocaine, Cristália, Itapira, Brazil) (4 mg/kg body weight, intraperitoneal), 10 minutes before the application of barbiturate anesthesia. The experiments were performed between 7:00 p.m. and 8:00 p.m.

Pinealectomy

The animals in the PNX, PNXPD, PNXMEL, and PNXPDMEL groups at 40 days of age were anesthetized with a combination of ketamine hydrochloride (Ketamina, Agener, Embu-Guaçu, Brazil) (10%, 80 mg/kg body weight, intraperitoneal) and xylazine (Xilasina, Dorcipec®, Monte Carlos, Brazil) (2%, 10 mg/bw and intraperitoneal) and subjected to pinealectomy according to Hoffman and Reiter (1965). Briefly, after anesthesia, the animal was placed on a stereotaxic apparatus for small animals by opening in a sagittal scalp. The lambda suture was exposed by pushing away the skin and muscles. Around the lambda perforation was performed by means of a circular drill, delicately removing the shaped

piece of the bone disk. Thus, with the aid of fine forceps, the pineal gland was removed, which is located just below the junction of the cerebral sinuses). After brief hemostasis, the skull was closed by returning the disc-shaped bone, and the scalp was sutured with cotton yarn. As a prophylactic measure after surgery, 0.2 ml of a veterinary antibiotic penicillin-streptomycin-1.200.000 IU (Forte Dodge Saúde Animal Ltda, Campinas-SP, Brazil) intramuscularly was injected. After all the experiments, the effectiveness of pinealectomy was verified by macroscopic postmortem brain examination to confirm the absence of the pineal gland.

Periodontal Disease

Periodontal disease (PD) was induced in the animals 20 days after pinealectomy in the PD, PNXPD, PDMEL, and PNXPDMEL groups. The animals were anesthetized by the combination of ketamine (10%, 80 mg/kg body weight) and xylazine (2%, 10 mg/kg body weight) administered intraperitoneally, and periodontal disease was induced by installing a ligature with a 4-0 silk thread (Seda-Silk Locked Ethicon-Johnson & Johnson, São José dos Campos, Brazil) around the lower first molars at the level of the gingival sulcus, bilaterally, as described by Rodini *et al.* (2008).

Treatment with melatonin

The animals in the CNMEL, PDMEL, PNXMEL, and PNXPDMEL groups received solubilized melatonin (Melatonin, Sigma-Aldrich, St. Louis, MO, USA) orally. Melatonin was prepared twice a week by dissolving 1g of melatonin in 20 mL of absolute ethanol (2%) and 980 mL of distilled water. Every day the melatonin solution was adjusted according to previous night ingested volume and the bodyweight of each animal, and Melatonin solution was added to drinking water during the 12-hr dark period, starting at 7:00h p.m. and ending at 7:00h a.m. at a dosage of 5 mg/kg body weight based on similar studies (Tresguerres *et al.*, 2014; Kose *et al.*, 2016; Kara *et al.*, 2013), for 28 days immediately after induction of PD. The untreated animals received a vehicle solution prepared with absolute ethanol dissolved in water added to the drinkers following the same criteria for treated animals.

Determination of plasma concentrations of IL-6 and TNF-α

Blood samples from the inferior vena cava were collected after median laparotomy to measure plasma concentrations of IL-6 and TNF-α. Blood samples were transferred to heparinized plastic tubes and held at 4°C until centrifugation at 800g × g (4°C, 15 min) and stored in aliquots at -80 °C. Plasma concentrations of TNF-α and IL-6 were determined using the enzyme-linked immunosorbent assay (ELISA) method using kits following the manufacturer's instructions. The commercial kits were used (TNF-α, Biovedor, Heidelberg, Germany and Invitrogen Corporation, Camarilio, CA, USA; IL-6, BD Biosciences, San Diego, USA).

Analysis of Bone microtomography (micro-CT)

After euthanasia, the hemi-mandibles (right side) were disarticulated and prepared, removing all remaining soft tissue and immediately fixed in saline (9%) and kept at 4°C until the day of the scan. Bone microtomography was performed using a Skyscan 1272 computerized microtomograph (Skyscan, Aartselaar, Belgium), used for nondestructive three-dimensional evaluation of the bone architecture. The scanning parameters were 8 μm in size, 70 kV and 200 μA of source configuration, 0.5mm aluminum filter, 180 ° rotation, and 0.6 ° pitch.

After scanning, the reconstruction of each sample was carried out using NRECON software v. 1.6.10.2 (NRECON, Skyscan, Belgium), from the apex of the crown including the alveolar bone process and CTAn v.1.15.4.0 (Skyscan) software was used for three-dimensional analysis (3D). The regions of interest (ROI) in the sagittal axis were analyzed, composed of 80 slices that were interpolated by a geometric figure in the form of a rectangle allowing the 3D calculation of the volumetric indices performed in the furcation region of the first molar of the mandible (Figure 1A).

The volumetric parameters analyzed were: bone volume percentage = bone volume by tissue volume [BV/TV (%)], ratio between bone surface and bone volume [BS/BV (1/mm)]; trabecular thickness [Tb.Th (mm)]; trabecular number per millimeter of tissue [Tb.N (mm-1)]; trabecular separation [Tb.Sp (mm)] and total porosity [Po Tot (%)].

Linear parameter analyses were performed to quantify alveolar bone loss extracted from a two-dimensional representative image using the software DATA VIEWER v. 1.5.1.2 (Skyscan, Belgium) (Figures 1B and C), analyzing: Distance from the furcation ceiling to the interradicular bone crest of the first molar (M1); Point of contact between molar 1 (M1) and molar 2 (M2) to the interproximal alveolar bone crest (PC-IBC); Distance from the cementum-enamel junction (CEJ) to the bone crest by vestibular (CEJ-BCV); Distance from the cementum-enamel junction to the bone crest by Lingual (CEJ-BCL).

Immunohistochemistry

The hemi-mandibles (left side) were removed and fixed in 10% buffered formalin for 72 hours, washed in running water for 12 h, and placed in a ethylenediaminetetraacetic acid (EDTA) (EDTA, Sigma Chemical, St. Louis, MO, USA) solution buffered 10% until decalcified (about 60 days) and then embedded in paraffin. Longitudinal cuts of 5 μm thickness were obtained using a

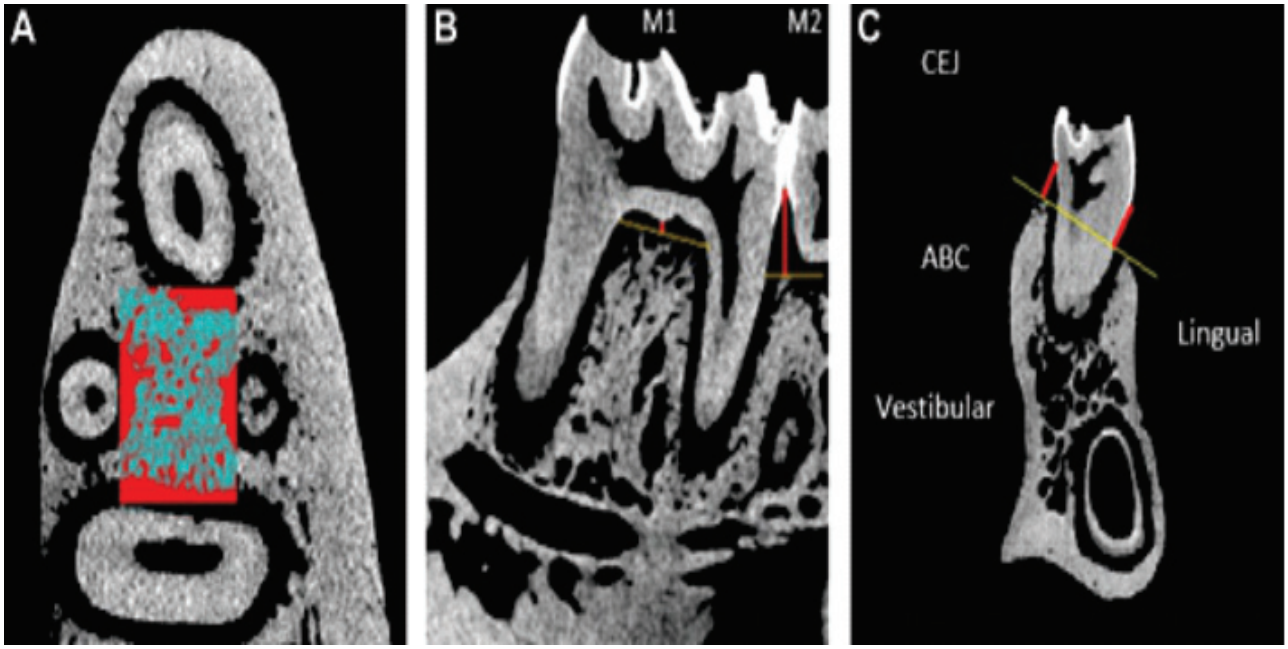

Figure 1. Representative image for volumetric and linear analyzes. A = region of interest (ROI) in the sagittal axis. B= transaxial axis of molar 1 showing linear measurements cementum enamel junction (CEJ) to alveolar bone crest (ABC), vestibular and lingual. C = coronal axis molar 1 (M1) and molar 2 (M2).

conventional microtome (Leica, RM2155, Microsystems GmbH, Wetzlar, Germany) and placed onto silanized slides (StarFrost® Polycat Knittel). Seven cuts were performed in the Furca region for immunohistochemistry.

For the immunohistochemical reactions, the histological sections were deparaffinized in xylene and hydrated in a decreasing series of ethanol (100º ° – 100º ° – 100º ° – 90º ° – 70º ° GL). Antigen retrieval was performed by immersing the histological slides in citrate buffer (Spring Bioscience, Pleasanton, CA, USA) in a pressurized chamber (Decloaking chamber®, Biocare Medical, Concord, CA, USA) at 95 °C for 20 min. At the end of each step of the immunohistochemical reaction, the histological slides were washed in 0.1 M PBS, pH 7.4. Subsequently, the slides were immersed in 3% hydrogen peroxide for 1 h and 1% bovine serum albumin for 12 h for endogenous peroxidase blockade and nonspecific site block, respectively. Slides containing the samples from each experimental group were divided into two batches and each batch was incubated with the following primary antibodies: anti-mouse OCN generated in goat (SC-18319, Santa Cruz Biotechnology® Inc, California, USA) and anti-mouse TRAP generated in goat (SC-30833, Santa Cruz Biotechnology® Inc, California, USA). The sections were incubated with biotinylated secondary antibody for 2 h and subsequently treated with streptavidin-conjugated with horseradish peroxidase (HRP) (Dako Universal HRP-Labeled Streptavidin-Biotin Kit®, Dako Laboratories, CA, and USA) for 1 hour. The revelation was performed using the chromogen 3, 3'-diaminobenzidine tetrahydrochloride (DAB Chromogen Kit®, Dako Laboratories, CA, and USA). Counterstaining was performed with Harris Hematoxylin and then dehydrated in ethanol, diaphanization in xylenes, and covering with mounting media (Permount, Fisher Scientific, San Diego, CA, USA), and glass coverslips. As a negative control, the specimens were subjected to the procedures described above by suppressing the use of the primary antibody.

Immunohistochemical analysis was performed by a certified histologist who was blinded to the treatments performed (E.E.). The region of interest was the center of the furcation area, and the coronary limit of this area was the alveolar bone crest. A semi-quantitative analysis was performed using histological sections of each animal, with an original increase of 200x. Immunostaining was assigned a score. The criterion adopted for the establishment of the scores was based on those established by Faria *et al.* (2008), where: SCORE 0 = absence of immunostaining; SCORE 1 = low immunolabeling pattern; SCORE 2 = Moderate immunostaining pattern; SCORE 3 = high standard of immunostaining.

Statistical analysis

Statistical analyses were performed by three-way ANOVA, followed by Tukey›s post hoc test, when analysis of variance suggested a significant difference between groups ($p<0.05$). Statistical analysis of immunohistochemistry was performed using the Kruskal-Wallis non-parametric statistical test followed by Dunn's post-test. The statistical program used was GraphPad Prism Version 7.0 (GraphPad Software, Inc., CA, and USA). The results are presented as mean ± SEM, and the level of significance was 5% (alpha = 5%).

Results

Evaluation of plasma concentrations of TNF-α and IL-6

The PD ($p<0.001$), PNX ($p<0.001$), and PNXPD ($p<0.05$) groups showed a significant increase in TNF-α plasma concentrations when compared to the other CN group. After the melatonin-treatment, there was a decrease in TNF-α plasma concentrations: PDMEL ($p<0.0001$) group compared with DP group; PNXMEL ($p<0.001$) group compared with PNX group; PNXPDMEL ($p<0.001$) group compared with PNX group (Table 1).

The results demonstrated that there was no significant difference in plasma concentrations of IL-6 between the groups (Table 1).

Table 1. Plasma concentrations of TNF-α and IL-6. Control rats (CN), periodontal disease (PD), pinealectomized (PNX) pinealectomized with periodontal disease (PNXPD) and rats treated with melatonin control (CNMEL) with Periodontal Disease (PDMEL) Pinealectomized (PNXMEL) and Pinealectomized with Periodontal Disease (PNXPDMEL). Values are presented as mean ± SEM.

	PARAMETERS	
GROUPS	TNF- α (pg/ml)	IL-6 (pg/ml)
CN	4.20 ± 0.17	24.29 ± 0.64
PD	6.12 ± 0.48 *	24.00 ± 1.14
PNX	6.06 ± 0.28 *	22.69 ± 0.39
PNXPD	5.80 ± 0.18 †	23.00 ± 0.38
CNMEL	4.03 ± 0.30	23.71 ± 0.39
PDMEL	3.69 ± 0.39 ‡	23.59 ± 0.90
PNXMEL	4.02 ± 0.27 §	23.66 ± 1.01
PNXPDMEL	3.89 ± 0.34 ¦	23.30 ± 0.26

* $p < 0.001$ for the comparison with CN
† $p < 0.05$ for the comparison with CN
‡ $p < 0.0001$ for the comparison with PD
§ $p < 0.001$ for the comparison with PNX
¦ $p < 0.001$ for the comparison with PNXPD

Analysis by Micro-CT

The PD and PNXPD groups presented a greater loss of alveolar bone than the other groups. It is emphasized that melatonin-treated groups prevented alveolar bone loss (Figures 2, 3, and 4).

There was a significant difference ($p<0.05$) between the following groups: CN vs. PD and PNXPD; PD vs. PDMEL; PNXPD vs. PNXPDMEL in all volumetric parameters (BV/TV, BS/BV, Tb.Th, Tb.N, Tb.Sp, and Po.Tot) and linear parameters (Furca, interproximal alveolar bone crest, CEJ-BCV, and CEJ-BCL) (Figures 3 and 4, respectively).

Quantification of immunohistochemistry

Immunostaining for OCN in CN, PNX, CNMEL, PDMEL, PNXMEL, and PNXPDMEL groups prevailing high standard immunostaining (median score = 3), whereas the PD and PNXPD groups prevailed low standard immunostaining (median score = 1). It is noteworthy that immunostaining for OCN showed a significant difference ($p<0.05$) between the following groups: CN vs. PD and PNXPD; PD vs. PDMEL; PNXPD vs. PNXPDMEL (Figure 5.1).

Regarding immunostaining for TRAP the CN, PNX, CNMEL, PDMEL, PNXMEL, and PNXPDMEL groups showed low immunostaining (median score = 1), whereas the PD and PNXPD groups showed high-levels of immunostaining (median score = 3). It is noteworthy that the immunostaining for TRAP revealed significant differences ($p<0.05$) between the following groups: CN vs. PD and PNXPD; PD vs. PDMEL; PNXPD vs. PNXPDMEL (Figure 5.2).

Discussion

The present study has demonstrated that pinealectomized rats with or without PD showed a significant increase in TNF-α levels, but only the PD groups showed any alveolar bone loss. In contrast, the groups that received melatonin supplementation (5 mg/kg) demonstrated reduced plasma concentrations of TNF-α and the absence of alveolar bone resorption.

Damage to the tissues supporting the teeth is a characteristic feature of PD. Thus, high concentrations of TNF-α associated with immune and inflammatory responses may have contributed to alveolar bone loss in the PD animals in the present study.

Several studies have reported that PD increases TNF-α levels (Kara *et al.*, 2013; Hernández *et al.*, 2011; Colombo *et al.*, 2012). Our results demonstrated that melatonin replacement therapy may be efficient in reducing TNF-α levels in the PD groups (Table 1), corroborating data from previous investigations (Balaji *et al.*, 2020; Santos *et al.*, 2018).

Various studies have reported that TNF-α inhibits melatonin synthesis in pinealocytes by activating the transcription factor NF-kappa B (NF-ϰB) (Markus *et al.*, 2007; da Silveira Cruz-Machado *et al.*, 2012; Fernandes *et al.*, 2006; Pontes *et al.*, 2007). On the other hand, melatonin has anti-inflammatory effects that are effected by inhibiting the rolling and neutrophil adhesion (Markus *et al.*, 2007; Lotufo *et al.*, 2001) resulting in decreasing pro-inflammatory cytokine and chemokine levels. In addition, melatonin inhibits the binding of NF-ϰB to DNA, thus preventing the translocation of this transcription factor to the nucleus (Choi *et al.*, 2011; Chuang *et al.*, 1996; Negi *et al.*, 2011; Szczepanik, 2007), a central pathway in inflammatory responses. Such melatonin actions on TNF-α may have attenuated the inflammatory process and alveolar bone resorption in the treated animals.

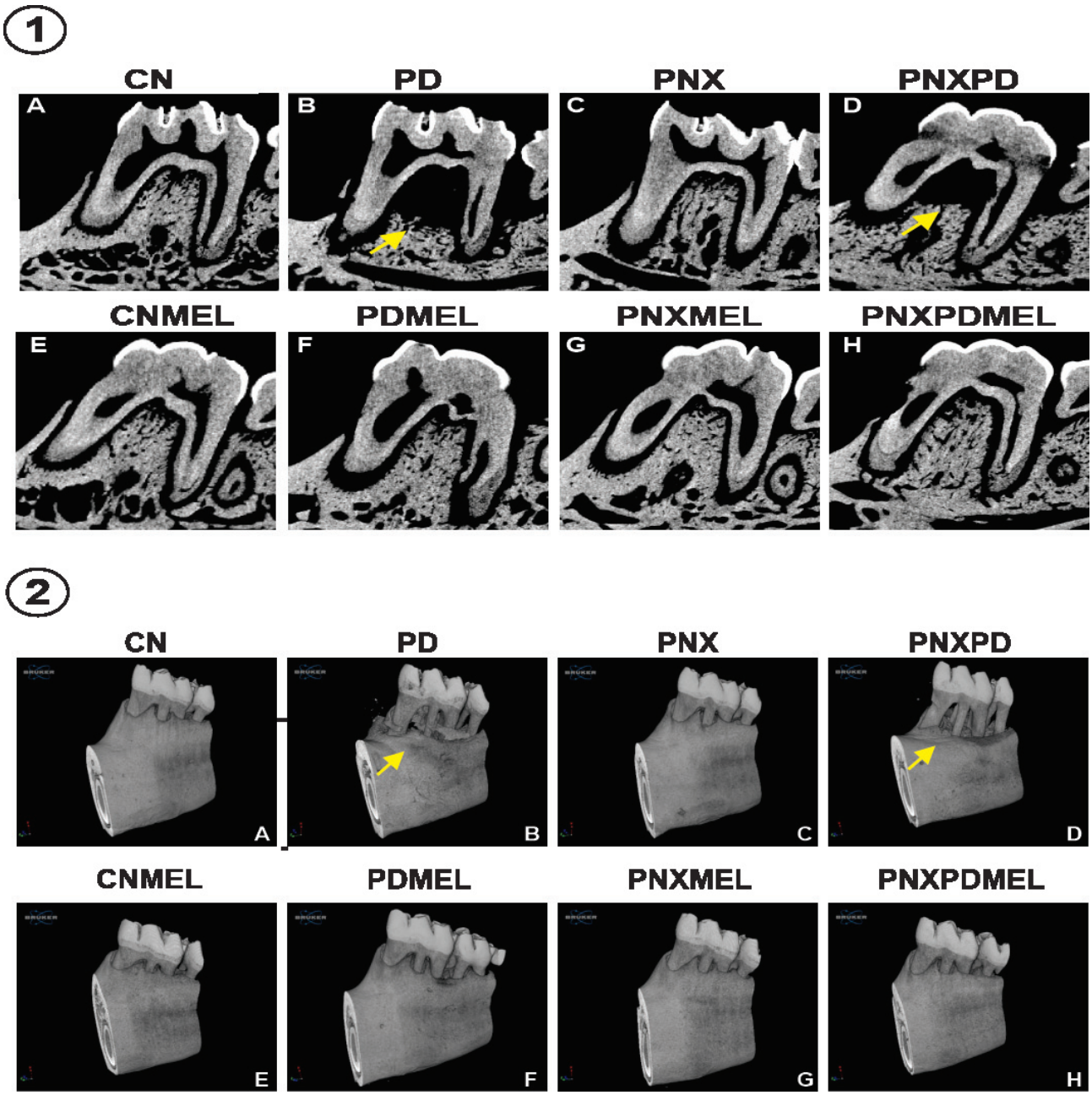

Figure 2. Bidirectional image (1) and representative 3D reconstruction (2) of the first molar of all study groups. A= Control (CN); B= Periodontal Disease (PD); C= Pinealectomized (PNX); D= Pinealectomized with Periodontal Disease (PNXPD); E= Control treated with melatonin (CNMEL); F= Periodontal Disease treated with melatonin (PDMEL); G= Pinealectomized treated with melatonin (PNXMEL); H= Pinealectomized with Periodontal Disease treated with melatonin (PNXPDMEL). Yellow arrows indicate the region of alveolar bone loss.

Santos *et al.* (2018) have demonstrated that rats with periodontal disease and pinealectomized have insulin resistance (according to the HOMA-IR index) accompanied by an increase in plasma TNF-α levels, and the treatment with melatonin improve these parameters.

According to some studies, periodontitis is able to increase serum concentrations of IL-6 in humans (Shimada *et al.*, 2010; Buhlin *et al.*, 2003). However, in the present study, there were no differences in IL-6 plasma levels between groups (Table 1), corroborating some data from the literature (Takahashi *et al.*, 1994; Santos *et al.* 2018). Furthermore, in elderly patients with PD, no difference was found in this parameter (IL-6 in the blood) (Murata *et al.*, 2001). Thus, it appears that there are divergences in the literature regarding the increase in inflammatory cytokines in periodontal diseases. This divergence of results may be, among other factors, due to the type of bacteria prevalent in dental biofilm (Matsushita *et al.*, 1999).

The host immune responses and action of the bacteria present in the biofilm generate considerable amounts of reactive oxygen species that accentuate the destruction of the tissues of the oral cavity (Gustafsson *et al.*, 1996; Kimura *et al.*, 1993), and it can promote an increase in osteoclastogenesis. Moreover, the production of cytokines, such as TNF-α, stimulates these alterations (Nakashima *et al.*, 2000; Liu *et al.*, 2010).

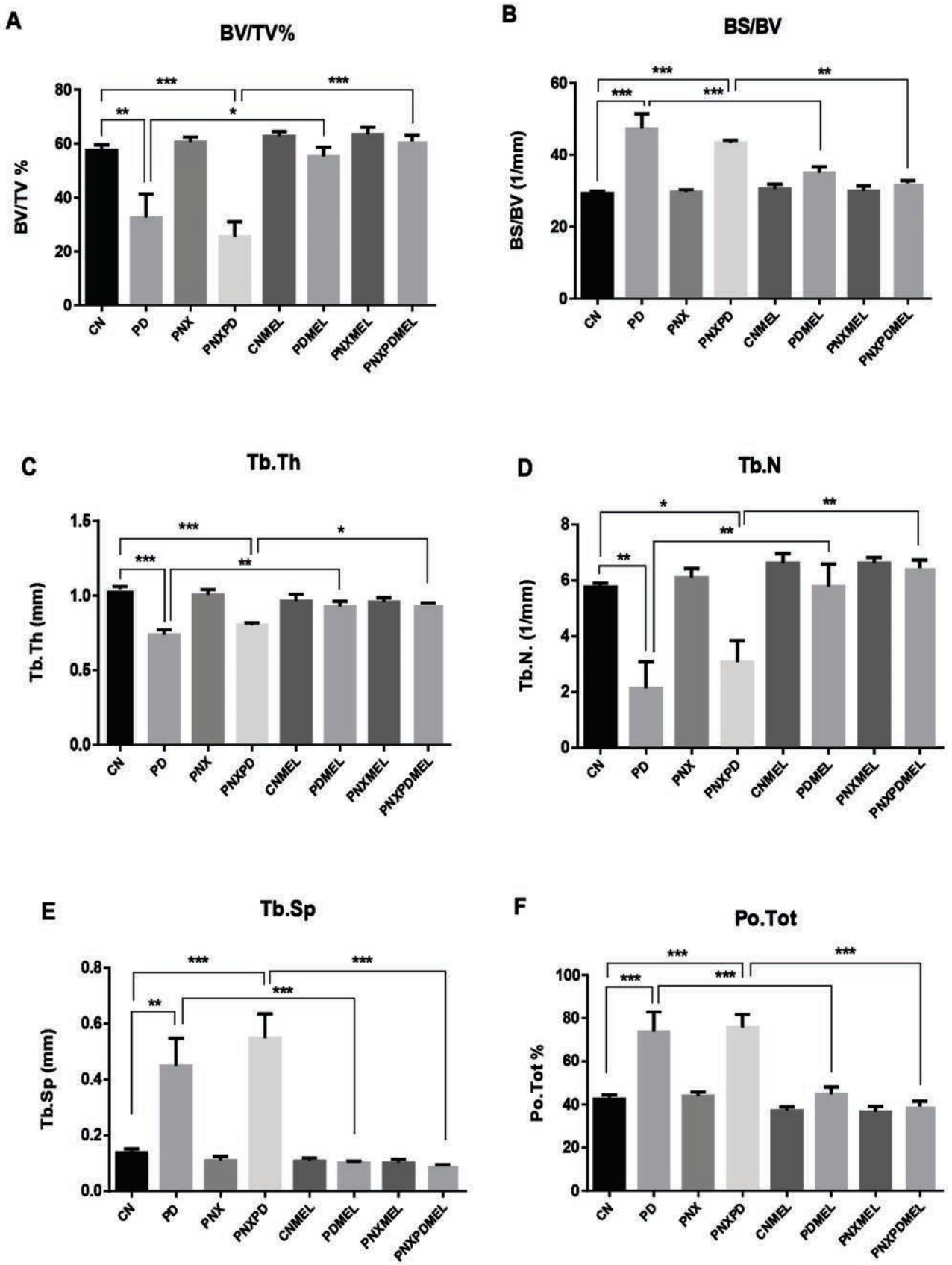

Figure 3. Volumetric measurement. Control rats (CN), periodontal disease (PD), pinealectomized (PNX) pinealectomized with periodontal disease (PNXPD) and control mice treated with melatonin (CNMEL) with Periodontal Disease (PDMEL) pinealectomized (PNXMEL) and Pinealectomized with Periodontal Disease (PNXPDMEL). (A) BV/TV (%) = bone volume by tissue volume; (B) BS/BV (1/mm) = ratio between bone surface and bone volume; (C) Tb.Th (mm) = trabecular thickness; (D) Tb.N (mm-1) = trabecular number per millimeter of tissue; (E) Tb.Sp (mm) = trabecular separation and (F) Po Tot (%) = porosity total. Values are presented as mean and ± SEM of each group (n = 06). * p< 0.05, ** p< 0.01, *** p< 0.001.

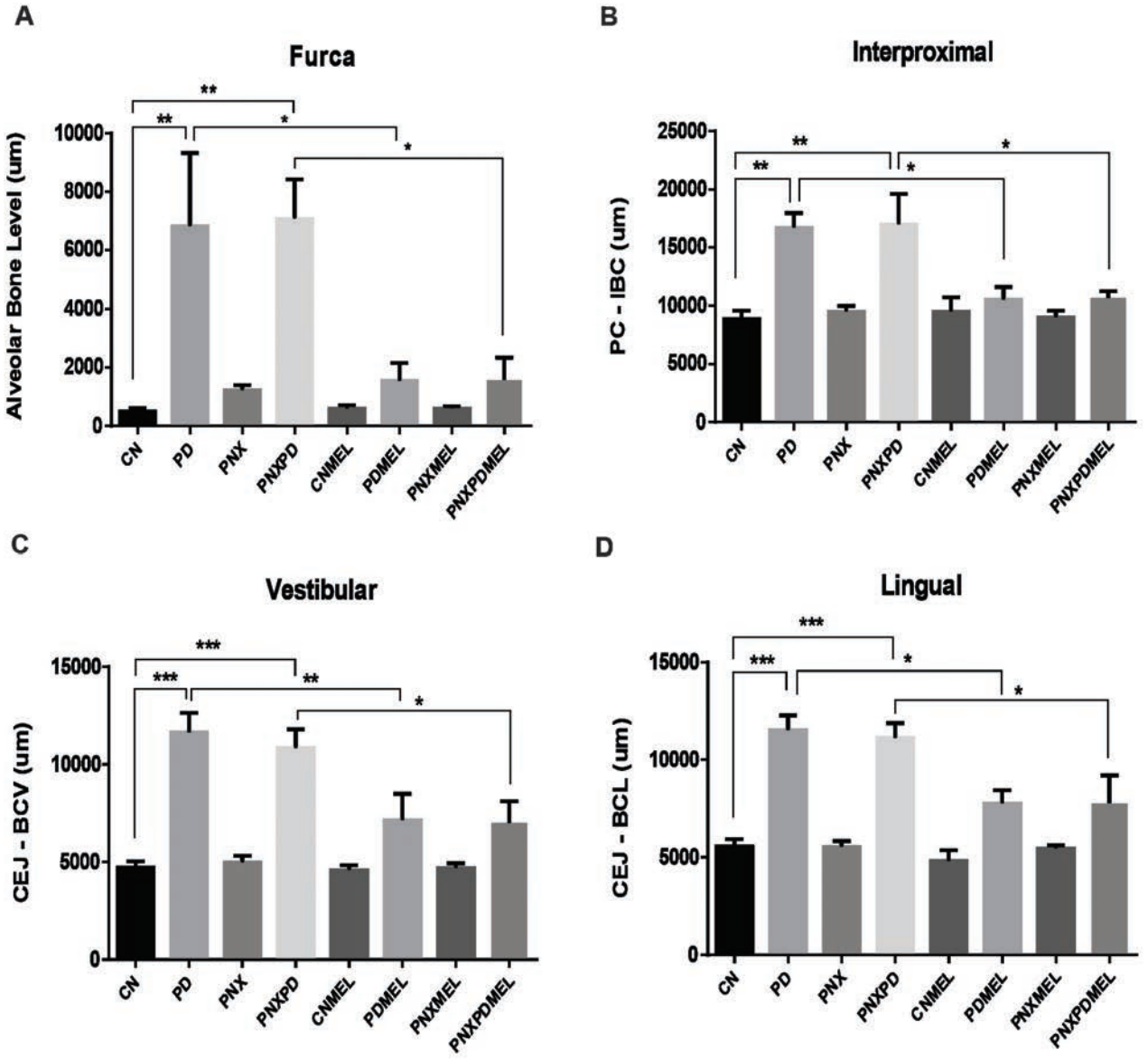

Figure 4. Linear measurements. Control (CN), Periodontal Disease (PD), Pinealectomized (PNX), Pinealectomized patients with Periodontal Disease (PNXPD), and Controlled Melatonin (CNMEL), Periodontal Disease (PDMEL), Pinealectomized (PNXMEL) and Pinealectomized with Periodontal Disease (PNXPDMEL). Values are presented as mean and ± SEM of each group (n = 06). (A) furcation region; (B) PC-COI = point of contact between 1st molar (M1) and 2nd molar (M2) to interproximal alveolar bone crest; (C) CEJ-BCV = distance from the cemento-enamel junction to the bone crest by vestibular and (D) CEJ-BCL = distance from the cemento-enamel junction to the bone crest by lingual. * p< 0.05, ** p < 0.01, *** p < 0.001.

The results of the present study showed that pinealectomy did not affect alveolar bone loss in the animals with PD (Figures 3 and 4). This may be due to the production of local melatonin by cells of the immune system, such as lymphocytes, acting as an autocrine and/or paracrine substance, coordinating the local immune response (Markus *et al.*, 2007; Carrillo-Vico *et al.*, 2005), attenuating the exacerbated inflammatory process caused by PD, and the absence of melatonin via the pineal gland with anti-inflammatory and antioxidant actions.

Several studies have verified changes in bone health in PNX animals (Egermann *et al.*, 2011; Ostrowska *et al.*, 2002; Turgut *et al.*, 2006). However, these changes occurred after a longer time than those used in the present study. Therefore, the absence of differences in the bone parameters analyzed in the present study may be due to the short period evaluated after pinealectomy (48 days, 20 days of recovery after surgery, and 28 days of the PD protocol).

It is well established that PD is associated with alveolar bone resorption (Colombo *et al.*, 2012; Arabacı *et al.*, 2015; Mattera *et al.*, 2019; Ricoldi *et al.*, 2017); however, few studies have investigated the effects of pinealectomy on alveolar bone. In all bone parameters analyzed, it was verified that the animals with PD showed accentuated alveolar bone resorption and that melatonin prevented these changes (Figures 3 and 4).

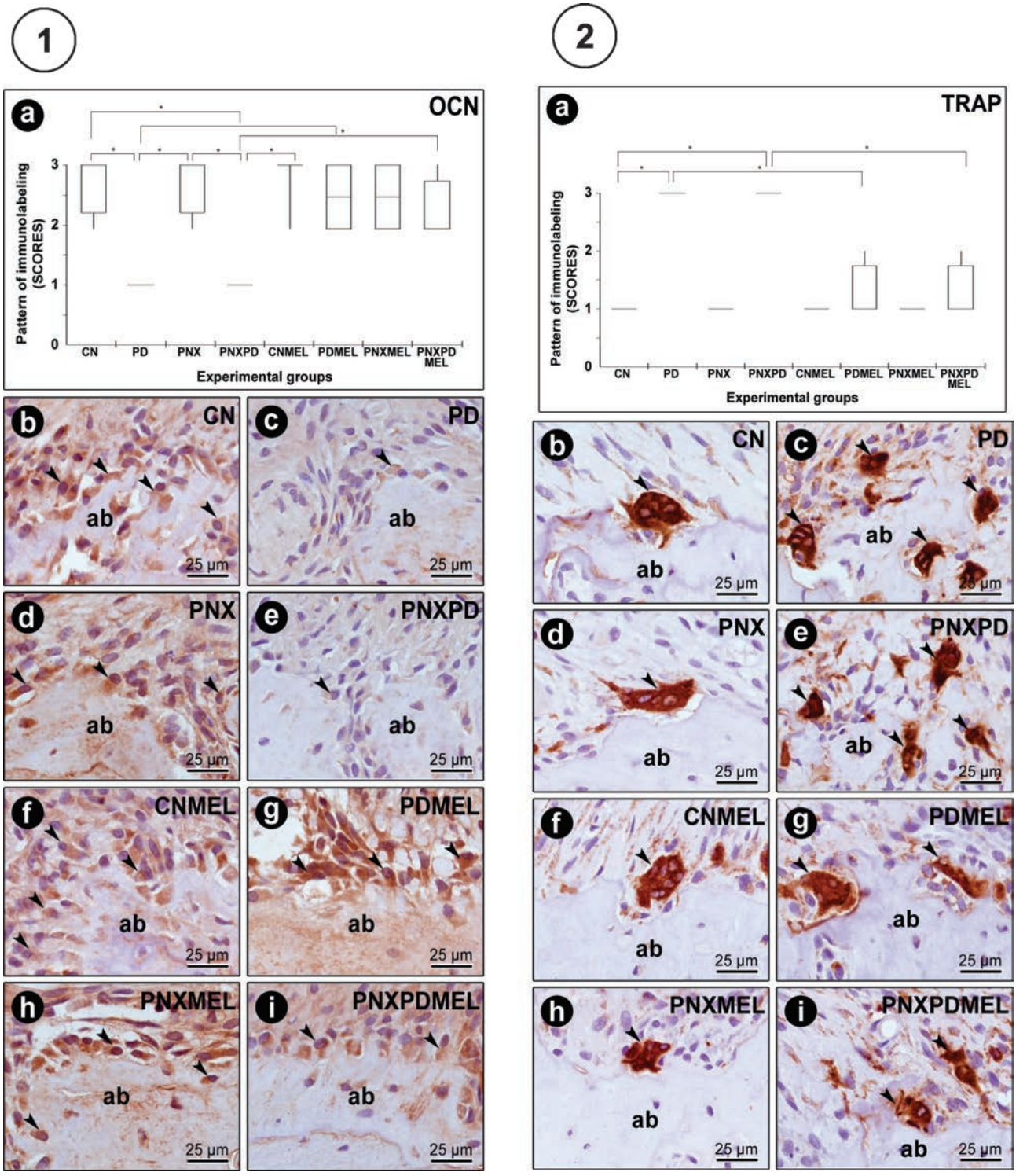

Figure 5 - Immunostaining pattern for OCN (1) and TRAP (2). (a) Graph showing the median and interquartile range of the scores assigned to the pattern of immunostaining for OCN and TRAP. Statistical test: Kruskal Wallis. * p< 0.05, ** p< 0.01, *** p< 0.001 (b-i) Photomicrographs evidencing the immunostaining pattern for OCN TRAP in the CN (b), PD (c), PNX (d), PNXPD (e), CNMEL (f), PDMEL (g), PNXMEL (h) and PNXPDMEL (i) groups. Symbols: arrows, immunoreactive cells. Original increase: 1000x. Scale bars: 25 µm. Contra-staining: Hematoxylin from Harris.

Studies have shown that OCN is a potential marker of bone formation (Bullon *et al.*, 2007; Lee *et al.*, 1999), indicating the activity of osteoblasts. In this study, the PD and PNXPD groups showed a low standard immunostaining OCN, indicating low activity of osteoblasts in these groups. In contrast, in these same groups, there was a high standard of immunostaining for TRAP, which is a marker of bone resorption, indicating osteoclast activity (Blumsohn *et al.*, 1997; Wheater *et al.*, 2013). This explains the alveolar bone loss in the PD and PNXPD groups. It is remarkable that in the melatonin treated groups, these immunolabels demonstrated a

pattern similar to that of the CN group, indicating that melatonin protected bone resorption in animals with PD favoring alveolar bone formation (Figure 5).

Several studies have shown that melatonin enhances bone formation through its stimulatory actions on the differentiation of osteoblasts (Roth *et al.*, 1999; Radio *et al.*, 2006; Sethi *et al.*, 2010; Park *et al.*, 2011), activating the melatonin 2 (MT2) receptors by increasing the activity of MAPKs and Wnt/β-catenin signaling pathways, which stimulate osteoblastogenesis by increasing the gene expression of Runt-related transcription factor 2 (Runx2), which induces osteogenic expression including osterix, bone morphogenetic protein-2 (Bmp-2), and OCN (Park *et al.*, 2011; Maria and Witt-Enderby, 2014), Evidence indicates that melatonin also inhibits osteoclast differentiation by suppressing the expression of the receptor activator of NFkB (RANKL) ligand (Koyama *et al.*, 2002; Maria and Witt-Enderby, 2014). Therefore, melatonin acts to favor bone formation, contributing to the homeostasis of this tissue.

Previous studies have shown that levels of salivary and gingival melatonin decrease in individuals with periodontitis compared to clinically healthy individuals, indicating that melatonin may play a protective role against periodontal disease (Cutando *et al.*, 2006; Srinath *et al.*, 2010). Hence, the decrease in salivary melatonin levels in periodontal diseases can occur in response to an increase in oxidative stress and bacterial attack, exhausting it in the oral cavity (Bertl *et al.*, 2013). Therefore, as evidenced in the present study, melatonin had a protective role in the oral cavity of PD animals.

Thus, melatonin promotes bone formation by promoting osteoblastic differentiation and acting as a scavenger of free radicals with antioxidant properties (Cengiz *et al.*, 2012; Galano *et al.*, 2011; Maria and Witt-Enderby, 2014). Therefore, this study demonstrated that melatonin significantly inhibits alveolar bone resorption in rats with PD, suggesting that melatonin may have therapeutic and protective functions in diseases of the oral cavity.

Conclusion

The present study demonstrated that the absence of treatment with melatonin in rats worsens the local and systemic effect of periodontal disease. Therefore, these results demonstrate that melatonin is a potent adjuvant treatment in alveolar bone resorption and reduced plasma level of TNF-α in rats with periodontal disease and pinealectomized.

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