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ORIGINAL ARTICLE

Mate tea (*Ilex paraguariensis*) improves bone formation in the alveolar socket healing after tooth extraction in rats

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Abstract

Objectives The objective of this study was to investigate the effects of mate tea (MT) [*Ilex paraguariensis*] on alveolar socket healing after tooth extraction.

Materials and methods Sixteen male rats were divided into MT and control groups. MT was administered by intragastric gavage at a dose of 20 mg/kg/day for 28 days before and 28 days after right maxillary incisor extraction. The control group received an equal volume of water. Histopathological and histometric analysis of the neoformed bone area and osteocyte density were performed, as well as immunohistochemical analysis of osteocalcin (OCN), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), tartrate-resistant acid phosphatase (TRAP), and manganese superoxide dismutase (MnSOD) in the alveolar socket. Calcium, phosphorus, alkaline phosphatase (ALP) activity, total antioxidant capacity (TAC), and malondialdehyde (MDA) were measured in plasma, whereas TRAP activity was determined in serum.

Results Histometry evidenced an increase in bone area ($P < 0.0001$) and osteocyte density ($P < 0.0001$). MT increased immunolabeling of MnSOD ($P < 0.001$), OCN ($P < 0.0001$), RANKL ($P < 0.001$), OPG ($P < 0.0001$), and TRAP ($P < 0.001$). Calcium and phosphorus concentrations did not differ between the groups. In addition, MT enhanced ALP ($P < 0.05$) and TRAP ($P < 0.0001$) activities. MT increased the TAC ($P < 0.001$), whereas it reduced MDA concentrations ($P < 0.0001$).

Conclusions MT increases bone area and osteocyte density in the alveolar socket healing on day 28 after tooth extraction.

Clinical relevance Regular MT ingestion improves the antioxidant defenses and bone formation, which is beneficial for alveolar socket bone healing after tooth extraction.

Keywords *Ilex paraguariensis* · Tooth extraction · Wound healing · Antioxidant

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Introduction

Alveolar socket bone healing after tooth extraction is a complex process, which involves tissue repair and regeneration. It encompasses chemotaxis of different cells into the wound, differentiation of undifferentiated mesenchymal cells to osteoprogenitor cells, proliferation of the committed bone-forming cells, synthesis of extracellular matrix, mineralization of osteoid, maturation, and remodeling of bones [1]. Prolonged wound healing may impair or delay rehabilitation of the extraction site. In addition, studies have investigated the control of residual alveolar ridge by decreasing alveolar bone resorption or accelerating new bone formation [2–4]. Identifying and understanding the mechanisms, by which osteogenic therapeutic stimuli contribute to the repair of the

dental alveolus and the process of osseointegration, is of critical importance. Acceleration of alveolar socket healing could be a good strategy to improve residual alveolar ridge preservation.

Remodeling and healing of the bone tissues depend on maintaining a delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts [5]. In this context, markers of bone turnover have been proven useful to understand the mechanisms controlling alveolar socket bone healing. For example, alkaline phosphatase (ALP), an enzyme involved with calcification, is used as a biochemical marker of bone formation during alveolar wound healing [6], whereas osteocalcin (OCN) is a late and specific marker of bone formation, synthesized by osteoblasts and becomes incorporated into the bone matrix via binding to hydroxyapatite in a calcium-dependent process [7]. RANKL/OPG/RANK signaling pathway is one of the mechanisms that also plays an important role in bone remodeling. RANKL (receptor activator of nuclear factor kappa-B) binding to RANK (receptor activator of nuclear factor kappa-B), expressed by hematopoietic progenitors and osteoclasts, triggers a series of cell signaling pathways that results in differentiation, maturation, and activation of osteoclasts. Consequently, osteoclasts become positive for tartrate-resistant acid phosphatase (TRAP), considered a marker of this cell or its activity [8]. In turn, osteoprotegerin (OPG) inhibits osteoclastogenesis or osteoclast activation by binding to RANKL and blocking its interaction with RANK [9, 10].

In wound-healing process, production of reactive oxygen species (ROS) is a necessary defense mechanism against bacterial pathogens [11, 12]. However, exposure to excessive ROS can increase oxidative stress and impair wound healing [13]. Studies have shown that malondialdehyde (MDA), an indicator of oxidative damage to cells and tissues caused by membrane lipid peroxidation, increases during fracture healing in rats [14] and after tooth extraction [15]. These studies have suggested that the use of antioxidants can counteract oxidative stress after tooth extraction [15] and accelerate cutaneous wound healing in streptozotocin-induced diabetic rats [16]. In this context, the use of antioxidants may be beneficial after oral surgeries [17].

Natural products have become the focus of studies on alveolar bone resorption and repair. In Chinese medicine, *Drynaria fortunei* aqueous extract has been shown to exhibit therapeutic effects on experimental alveolar bone resorption in rats by increasing bone mineral density, reducing osteoclast number to practically zero, but without causing significant changes in serum ALP activity, calcium, and OCN levels [3]. In a model of chronic inflammation-induced bone loss in female rats, green tea (*Camellia sinensis*) combined with alfacalcidol showed bone protective effects by increasing bone mass reducing serum TRAP and urinary 8-hydroxydeoxyguanosine (an oxidative stress biomarker) levels [4]. In addition, systemic administration of copaiba oil, a species indigenous to the Amazon region, leads to better alveolar bone healing by increasing bone neoformation and decreasing the number of inflammatory cells [18]. In contrast, healing of molar tooth extraction sockets of rats treated with bisphosphonate and regular green tea intake presented no relevant differences in comparison to those treated with bisphosphonate alone [19]. Although some natural compounds have been proven to exert effects in bone metabolism, there are still many other compounds that are pending to have their biological properties and mechanisms of action discovered and characterized.

Yerba mate infusion, namely mate tea (MT), is a typical and inexpensive beverage consumed in Southeast Brazil, Argentina, and Uruguay. MT is prepared by infusion of *Ilex paraguariensis* [20]. It is rich in phenolic compounds, such as chlorogenic acid and its isomers (mostly phenolic acid); gallic, *p*-coumaric, caffeic, and ferulic acids; rutin; epicatechin; gallocatechin; and various saponins [20–24], which are considered the major bioactive compounds responsible for MT effects on bone health [25–27]. In Brazil, MT is popularly consumed for its antioxidant effects [28]. In vivo, in vitro, and ex vivo studies have shown that MT affects the oxidative status and lipid metabolism, besides its anti-inflammatory and immunomodulatory effects [24, 29–33]. Brun et al. [34] showed that although MT is rich in caffeine, it did not negatively affect bone health and was safe to the consumers. Additionally, Conforti et al. [27] showed that there was an association between prolonged consumption of MT, at least 1 l of MT daily during 4 or more years, and higher bone mineral density in postmenopausal women. Recently, our group showed that MT minimized bone loss in the femur neck of perimenopause rats and this effect was at least partly due to reduction of MDA and modulation of MnSOD [26], an isoenzyme that has been shown to be important for bone tissues to maintain the differentiation and function of osteoclasts [35] and osteoblasts [36, 37]. However, no studies have investigated the effects of MT on alveolar bone healing.

With respect to the growing importance of complementary or alternative medicine, and regarding that increased consumption of MT has been shown to promote positive effects on the remodeling of femur neck of perimenopause rats, we hypothesized that MT could exert positive effects on the socket healing after tooth extraction. Therefore, this study aimed to investigate the action of MT on alveolar socket healing in male rats on day 28 after tooth extraction, through histopathological and histometric analysis of the neoformed bone tissue, as well as by immunohistochemical analysis directed to enzymatic antioxidant defenses (MnSOD), biochemical markers of bone formation (ALP and OCN), and reabsorption (RANKL, OPG, and TRAP).

Methods

Preparation of MT

MT was prepared by dissolving instant powder (Leão Jr.®, Curitiba, PR, Brazil; batch 7891098000088) in pure water at a concentration of 0.05 g/mL at room temperature, as described by Pereira et al. [24]. Total polyphenol content was determined by Folin-Ciocalteau method [38], using gallic acid as a standard. TAC was evaluated by the ferric reducing/antioxidant power (FRAP) assay, as previously described [39]. A series of $Fe_2(SO_4)_3$ solutions was used for construction of a calibration curve. The reducing power was expressed in Fe^{2+} antioxidant capacity/g MT powder. The TAC of MT was 534.67 Fe^{2+} antioxidant capacity/g MT powder, whereas the total polyphenol content was equivalent to 113.37 ± 0.34 mg gallic acid/g powder.

Animals and treatment

Animal use and experimental procedures were approved by the Ethics Committee on Animal Use of School of Dentistry, Araçatuba, São Paulo, Brazil (Authorization Protocol No. 2013-01572). Sixteen male *Rattus norvegicus Albinus* Wistar rats (3 months old) were housed in a temperature-controlled room (22 ± 2 °C) under a 12/12 h light/dark cycles, with free access to food and water. To avoid variation in antioxidant intake, the same batch of standard rodent chow (Purina-Labina®, Brazil) was used from the acclimatization period until the end of the experiment. The animals were stratified by body weight and then randomly assigned at baseline into two groups, as follows: MT and control groups. The rats in the MT group received MT in pure water at a dose of 20 mg/kg/day, by intragastric gavage (0.5 mL) to guarantee total ingestion, for 28 days prior to tooth extraction. After 28 days of treatment, the teeth were extracted. The rats of MT group continued to receive MT aqueous solution for additional 28 days before euthanasia. The control rats received an equal volume of water by intragastric gavage during all the experiment. The treatment with MT prior to dent extraction is justified by its positive effect on the TAC in plasma, which was demonstrated by our study [24].

Tooth extraction

The rats were anesthetized using xylazine (10 mg/kg, *i.p.*; Cristalia, Brazil) and ketamine (50 mg/kg, *i.p.*; Cristalia, Brazil), and after polyvinylpyrrolidone iodide local prophylaxis, the right maxillary incisor was luxated using a tapered instrument and extracted with a small forceps. The movement of extraction was smooth and followed the curvature of the rat incisor, so that root fracture would not occur. An atraumatic surgical technique was used, which allowed tooth extraction without postoperative complications [40]. The dental sockets were sutured with silk thread. A single dose of a polyvalent veterinary antibiotic was administered immediately after surgery as systemic prophylaxis.

Sample collection

On postoperative day 28 (after 55 days of MT treatment), the animals were anesthetized, as previously described above, and euthanised by cardiac puncture exsanguination. Blood aliquots were transferred to heparinised tubes to obtain plasma by centrifugation (1000×*g*, 4 °C, 10 min). Other aliquots were placed in a tube to obtain serum after standing for 1 h at room temperature, followed by centrifugation (1000×*g*, 4 °C, 10 min). Plasma and serum samples were stored (0.2 mL aliquots) at − 80 °C until assay. For histological and immunohistochemical analyses, the right maxillary incisors were resected *en bloc* from all rats and fixed in 4% (*v*/*v*) buffered formaldehyde (pH 7.4) for 24 h at room temperature. Then, they were decalcified in 10% (*w*/*v*) ethylenediaminetetraacetic acid, which was changed weekly for 8 weeks. Decalcified samples were processed in a conventional manner, embedded in paraffin, and submitted to microtomy (6 μm thick). Semi-serial histological sections were obtained considering the long-axis of the dental sockets. Upon reaching the central region of the dental alveolus, seven histological sections were collected. The first and seventh histological sections were used for histopathological and histometric analysis; each of the other histological sections were used for immunohistochemical analysis.

Histopathological and histometric analysis

A certified histologist (E.E.), calibrated and blinded to the treatments, performed both histopathological and histometric analysis. Two histological sections from each animal were stained with hematoxylin and eosin. The following histopathological parameters were examined in all thirds of the alveolus (cervical, middle and apical): intensity of the local inflammatory response, extension of the inflammatory process, pattern of cellularity and structure of epithelial tissue, pattern of cellularity and structure of connective tissue, and pattern of cellularity and structure of bone tissue. Histometric analyses of the neoformed bone area were performed at the middle third of the rat alveolus, since it is the region in which the alveolar repair process occurs more regularly [40, 41]. Selected areas comprised the largest possible area to be analyzed in all sockets, without the involvement of cortical bone. Analyses were performed by light microscopy at a magnification of ×100. Images were obtained with a digital camera (DFC 450, Leica, Wetzlar, Germany) mounted on the microscope, and images were analyzed with Leica Qwin Color/RGB software. The osteocyte density (osteocyte/mm^2) was evaluated in

the same images used for the histometric analysis using the same software [42]. Subsequently, mean values of the measurements were statistically treated.

Immunohistochemistry

Immunohistochemistry was performed to measure the immunoreactivity of bone turnover markers OCN, OPG, RANKL, and TRAP, whereas immunolabeling of MnSOD was performed to measure the antioxidant enzymatic defenses. The sections were submitted to an indirect immunoperoxidase technique, and the following primary antibodies were used (Santa Cruz Laboratories, CA, USA, or Abcam, MA, USA); anti-MnSOD (1:200; ab13534), anti-OCN (1:180; SC-18319), anti-OPG (1:150; SC-8468), anti-RANKL (1:100; SC-7628), and anti-TRAP (1:100; SC-30833) were used. Immunohistochemical analysis was carried out according to the protocol described by Stringhetta-Garcia et al. [43].

Immunolabeling was defined as a brownish staining present in the cytosolic (OCN, OPG, RANKL, and TRAP) cell compartment. A certified histologist, calibrated and blinded to the treatments, attributed scores using a conventional optical microscope as described above for histometry. The scores used to determine TRAP immunolabeling pattern were adopted from Faria et al. [44], as follows: score 3, high pattern, > 8 immunoreactive (IR) cells/area; score 2, moderate pattern, 3 to 7 IR cells/area; score 1, low pattern, < 3 IR cells/area; and score 0, absence of immunolabeling. Only multinucleated TRAP-positive cells near the bone surface were quantified. The scores for OCN, OPG, and RANKL immunolabeling patterns were adopted from Stringhetta-Garcia et al. [43], as follows: score 3, high pattern, approximately 75% IR cells/area; score 2, moderate pattern, approximately 50% IR cells/area; score 1, low pattern, approximately 25% IR cells/area; and score 0, absence of immunolabeling. These immunolabeling scores were used for comparisons between the experimental groups.

Plasma calcium and phosphorus concentrations

Calcium and phosphorus concentrations were determined by a colourimetric method, using a commercial kit purchased from Labtest Diagnostic (Lagoa Santa, MG, Brazil) according to previously described methods [45, 46].

Plasma total ALP activity

The plasma bone formation marker alkaline phosphatase (ALP) was determined using a laboratory kit (Labtest Diagnostic). This colourimetric assay measured the time-dependent formation of thymolphthalein from thymolphthalein monophosphate, according to the protocol described by Roy [47].

Serum total TRAP activity

Serum total TRAP activity was determined using a colourimetric assay based on the protocol described by Granjeiro et al. [48] and Janckila et al. [49]. Briefly, an aliquot (25 μL) of serum was added to 0.5 mL of the reaction mixture, consisting of 10 mmol/L *p*-nitrophenyl phosphate (*p*-NPP), 100 mmol/L sodium acetate, pH 5.8, 50 mmol/L sodium tartrate, and 1 mmol/L *p*-hydroxy mercury benzoate (*p*-HMB); the latter acts by inhibiting low molecular weight acid phosphatases [50]. The reaction was initiated by addition of the substrate and was carried out in a water bath at 37 °C for 1 h. The reaction was terminated by adding 0.25 mL of 1 mol/L NaOH, and the absorbance was determined at 405 nm to measure the amount of *p*-nitrophenolate (*p*-NP) formed during the reaction. The amount of product, *p*-NP, was determined by reading the absorbance at 405 nm and using a molar extinction coefficient of 17,800 M^{-1} cm^{-1}. A control without the enzyme was included in each assay to adjust for non-enzymatic hydrolysis of *p*-NPP. One unit of enzyme activity was defined as the enzyme amount required to hydrolyse 1 μmol of *p*-NPP per min at 37 °C.

Total plasma antioxidant capacity

Total plasma antioxidant capacity was determined using the FRAP assay, as previously described by Benzie and Strain [39], based on the reduction of Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) to form Fe^{2+}-TPTZ complex with a maximum absorbance at 593 nm. Results were calculated using a standard curve constructed using different concentrations of $FeSO_4$ solutions and were expressed in mmol/L.

Plasma MDA concentration

Lipid peroxidation products were determined based on thiobarbituric acid reactive substance (TBARS) levels, which resulted in the production of MDA, as the main product of this reaction. Plasma TBARS was measured as previously described [51]. Briefly, aliquots were mixed with 15% (*w*/*v*) trichloroacetic acid plus 0.375% (*w*/*v*) thiobarbituric acid and heated at 100 °C for 15 min. Samples were cooled to room temperature, centrifuged at 1000×*g* for 15 min, and their absorbance was measured at 535 nm. The molar absorption coefficient was 1.56×10^5 M^{-1} cm^{-1}.

Statistical analysis

The normality of data was analyzed by the Kolmogorov–Smirnov test. Data were expressed as the means ± standard deviations (SD). Statistical comparisons were made using unpaired non-parametric Mann–Whitney *U* test or unpaired

parametric Student's t test. For all analyses, $P < 0.05$ was considered statistically significant. Statistical analyses were conducted using GraphPad Prism 3.02 (GraphPad Software, Inc.; La Jolla, CA, USA). Both groups included eight rats.

Results

The histopathological analysis on day 28 after the tooth extraction evidenced the reduction of inflammation ($P < 0.05$) and the volume occupied by the inflammatory infiltrate ($P < 0.05$) in the MT group compared with control group, which showed a small amount of inflammatory cells extending only in part of the connective tissue in the alveolar socket. In relation to the pattern of cellularity and structure of the epithelial tissue, we found that thin thickness epithelium migrated only at the edges of the open surgical wound in the control group, whereas in the MT group the epithelial tissue completely covered the extraction site ($P < 0.05$). On day 28, both groups showed a moderate amount of fibroblasts as well as collagen fibers, but the amount of collagen fibers was higher in the MT group ($P < 0.001$). In turn, the pattern of cellularity and structure of the bone tissue showed that, although the control and MT groups present specimens with bone trabeculae filling the dental alveolus until reaching the middle third, only MT groups also found specimens with bone trabeculae reaching the cervical third ($P < 0.05$). Parameters, scores, and distribution of the specimens according to the histopathological analysis are demonstrated in Table 1.

Histometric examination of the healing on day 28 in the alveolar middle third of rats in the control group (Fig. 1a, b) showed less bone formation with much larger areas of thin bone trabeculae and areas occupied by the connective tissue compared to the MT-treated group (Fig. 1c, d). In the MT group (Fig. 1c, d), there was a larger area of bone formation occupying most of the interior of the alveoli. On day 28 after tooth extraction, histometric evaluation (Fig. 1e) showed a significant increase in trabecular bone area ($P \leq 0.0001$) in the middle third of the alveoli of MT-treated animals (65.92 ± 7.66%), compared to that in the control group (37.8 ± 7.66%). In this region, MT-treated rats exhibited more bone matrix with higher osteocyte density (392.8 ± 57.17 cells/mm^2) [$P \leq 0.0001$], compared to that of the control group (232.5 ± 53.72 cells/mm^2) (Fig. 1f).

Immunohistochemistry of MnSOD, OCN, OPG, RANKL, and TRAP showed high specificity for these proteins, which was confirmed by the complete absence of immunolabeling in the control group. MnSOD, OCN, OPG, and RANKL were predominantly expressed in the cytosol of osteoblasts, whereas TRAP was predominantly expressed in multinucleated osteoclasts. The results of immunohistochemical analyses and the patterns of immunolabeling of all biomarkers are shown in the Figs. 2 and 3.

Immunolabeling of OCN, a marker of mature osteoblasts, was higher in the MT group ($P < 0.0001$) than in the control group (Fig. 2a–c). In addition, rats in the MT group showed higher immunolabeling of OPG and RANKL, the main local regulators of osteoclastogenesis and osteoclast activity, than that in the control group (RANKL, $P = 0.0082$, Fig. 3d–f; OPG, $P < 0.0001$, Fig. 3g–i). Similarly, MT-treated rats exhibited a larger number of TRAP-positive cells, the osteoclastic biomarker, than the control rats ($P = 0.0064$, Fig. 3a–c), which indicated increased bone resorption activity in these animals. Furthermore, MnSOD, an antioxidant enzyme upregulated during osteogenic differentiation, showed a more intense immunolabeling pattern in the MT group ($P < 0.001$) than in the control group (Fig. 4a–c).

The plasma concentrations of calcium (control, 8.6 ± 0.46 mg/dL; MT, 8.6 ± 0.4 mg/dL; Fig. 5a) and phosphorus (control, 7.82 ± 1.01 mg/dL; MT, 7.71 ± 0.96 mg/dL; Fig. 5b) did not significantly differ between both groups. However, the plasma concentrations of the bone formation marker, ALP (control, 17.82 ± 2.94; MT, 21.51 ± 2.26 U/L; Fig. 5c) [$P \leq 0.05$], and of the bone resorption marker, TRAP (control, 1.45 ± 0.16; MT, 1.79 ± 0.18 U/L; Fig. 5d) [$P < 0.0001$], significantly increased in the MT group. Despite tooth extraction, MT treatment increased TAC levels (control, 53.86 ± 16.53; MT, 91.92 ± 10.93 mmol Fe^{+2}/L; Fig. 5d) [$P < 0.001$]. Additionally, there was a 40% decrease in the plasma levels of MDA ($P \leq 0.0001$) in the MT group (Fig. 5e).

Discussion

In this study, we showed, for the first time, that MT promoted alveolar socket healing in rats after tooth extraction, as evidenced by the increased bone area in the middle third of alveolar socket observed in the histometric and histopathological examination. These local effects on alveolar socket healing were associated with higher levels of antioxidant defenses (MnSOD), increase of biochemical markers of bone formation (OCN) and reabsorption (RANKL, OPG and TRAP), which were systemically supported by the increase in ALP and TRAP activities in the blood, reduction of lipid oxidative damage, and increased TAC in plasma.

Commercial instant MT powder has been showed to exhibit various activities, mostly attributed to the high content of polyphenol compounds, such as antioxidant, antinflammatory and chemopreventive properties, inhibitor of atherosclerosis, and bone loss in perimenopause [29, 30, 52–54]. We previously confirmed the presence of cis-3-*O*-caffeoylquinic acid, 5-*O*-*p*-coumaroylquinic acid, 3-*O*-feruloylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in instant powder MT [24], which can be detected in plasma and various tissues (liver, kidneys, stomach and muscles)

Table 1 Parameters, scores, distribution of the specimens and median scores according to the histopathological analysis of the tissues at 28 days of the post-extraction dental repair process in the control group and treated with mate tea

Histological analysis			
Parameters and respective scores		Percentage of specimens	
		Experimental groups	
		Control (n = 8)	Mate tea (n = 8)
Intensity of the local inflammatory response			
(0) no inflammation		1	6
(1) small amount of inflammatory cells		6	2
(2) moderate amount of inflammatory cells		1	0
(3) large numbers of inflammatory cells		0	0
	Median	1	0*
	P value	P = 0.0319	
Extension of the inflammatory process			
(0) no inflammation		1	6
(1) extending only in part of the connective tissue		7	2
(2) extending throughout the connective tissue, without reaching the bone tissue		0	0
(3) extending throughout the connective tissue and bone tissue		0	0
	Median	1	0*
	P value	P = 0.0400	
Pattern of cellularity and structure of epithelial tissue			
(0) moderate-thickness epithelial tissue completely covering the extraction site		0	1
(1) thin-thickness epithelial tissue completely covering the extraction site		2	7
(2) thin-thickness epithelial tissue only at the edges of open surgical wound		6	0
(3) absence of epithelial tissue over the open surgical wound		0	0
	Median	2	1*
	P value	P = 0.0056	
Pattern of cellularity and structure of connective tissue			
(0) moderate amount of fibroblasts and large amount of collagen fibers		2	8
(1) moderate amount of fibroblasts as well as collagen fibers		6	0
(2) small amount of both fibroblasts and collagen fibers		0	0
(3) severe tissue disruption with areas of tissue necrosis		0	0
	Median	1	0**
	P value	P = 0.0007	
Pattern of cellularity and structure of bone tissue			
(0) bone trabeculae filling the dental alveolus until reaching the cervical third		0	3
(1) bone trabeculae filling the dental alveolus until reaching the middle third		5	5
(2) bone trabeculae filling the dental alveolus until reaching the apical third		3	0
(3) absence of bone trabeculae within the socket		0	0
	Median	1	1*
	P value	P = 0.0392	

The data were compared by Mann–Whitey tests (*$P < 0.05$; **$P < 0.001$)

shortly after the ingestion of MT [55]. These chlorogenic acids have been shown to be responsible for the antioxidant activity and some pharmacological properties of MT [24, 26, 55, 56].

However, there is no consensus in the literature about which dose or treatment period are most relevant for ensuring biological properties without the risk of toxic effects. For example, the consumption of MT (1 g/kg/day) for 4 weeks

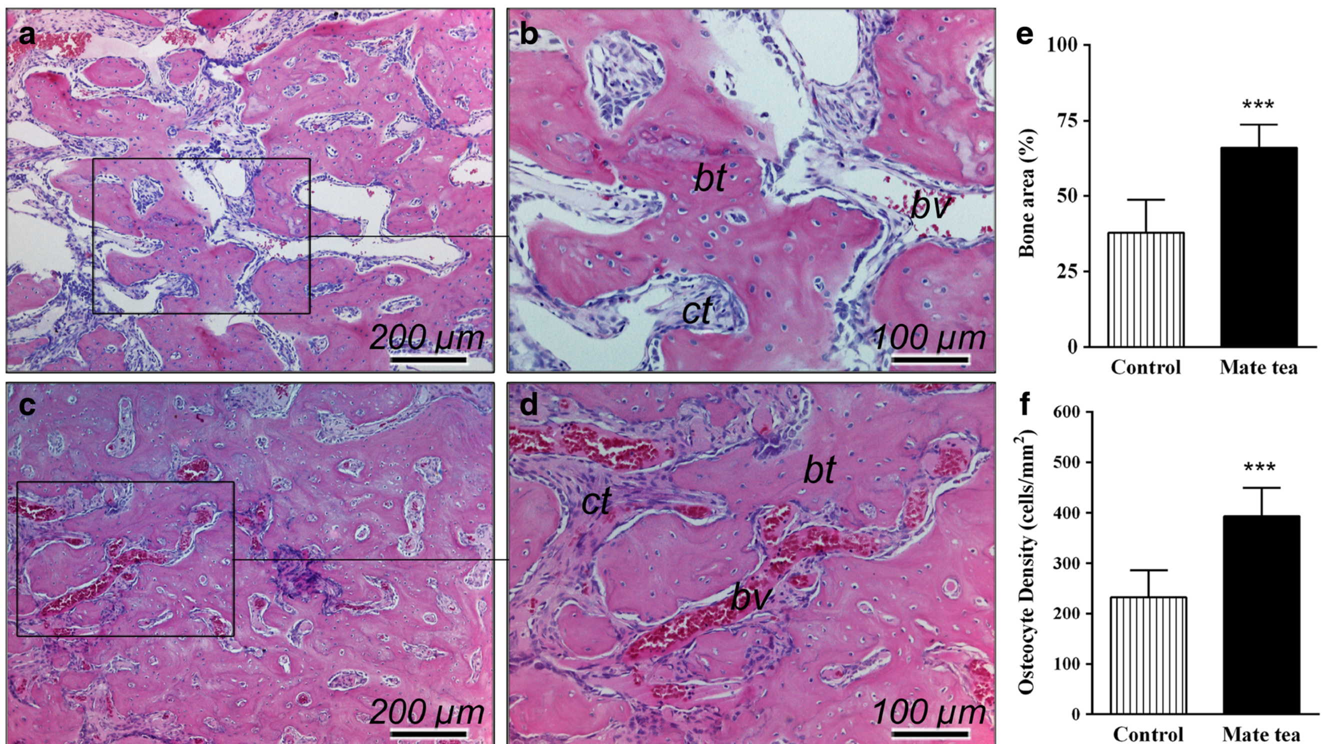

Fig. 1 Histological sections (6 μm thick) of the middle third of rat alveolus on day 28 of the alveolar bone healing. Rats received soluble MT powder at 20 mg/kg/day by intragastric gavage for 28 days prior and 28 days after tooth extraction. The control group (**a**, **b**) showed areas with thin bone trabeculae (BT) and connective tissue (CT), whereas the MT group (**c**, **d**) showed larger amount of bone matrix with more osteoblasts and osteocytes. Original magnification ×100 and ×200. Bone formation in the middle third of the alveoli on day 28 of alveolar socket healing (**e**) and osteocyte density (**f**). Histological sections were stained with hematoxylin and eosin. Data represent the means ± standard deviations (SD), $n = 8$ rats/group. The control and MT groups were compared using the unpaired t test (***$P < 0.0001$). P values < 0.05 were considered statistically significant

affected the production of interleukin (IL)-1α, IL-6, and tumor necrosis factor (TNF)-α by bone marrow cells, promoted weight loss, decreased the number of white blood cells, and significantly improved serum cholesterol levels in rats [32]. In addition, MT at 1 g/kg/day for 8 weeks showed potent anti-obesity effects in rats [52], while low doses (1 mg/kg/day for 8 weeks) also showed anti-obesity [57] and anti-inflammatory effects [58]. In turn, postmenopausal women, who drank at least 1 l (dose not specified) of MT daily during 4 or more years, presented an increased bone mineral density lumbar spine and femoral neck [27].

In the present study, we selected the dose of 20 mg/kg/day and time of treatment based on previous studies from our group. This dose is equivalent to the ingestion of

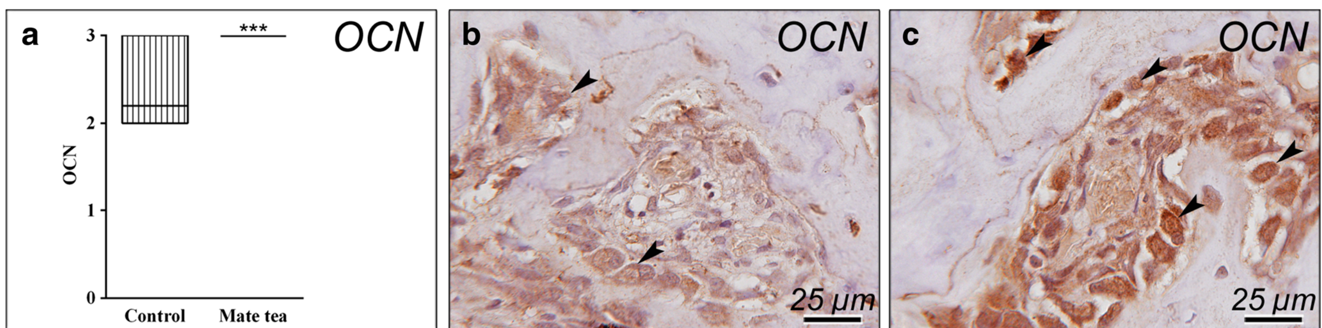

Fig. 2 Photomicrographs and graphics showing the immunoreactivity pattern of OCN (**a**–**c**) in the middle third of rat alveolus on day 28 of the alveolar socket healing. Rats received soluble MT powder at 20 mg/kg/day by intragastric gavage for 28 days prior and 28 days after tooth extraction. Data represent the means ± standard deviations (SD), $n = 8$ rats/group. The control and MT groups were compared using the nonparametric unpaired Mann–Whitney U test (***$P < 0.0001$). P values < 0.05 were considered statistically significant. Harris hematoxylin counterstaining for OCN. Scale bar = 25 μm. Original magnification, ×1000. OCN, osteocalcin

Fig. 3 Photomicrographs and graphics showing the immunoreactivity pattern of TRAP (**a**–**c**), RANKL (**d**–**f**), and OPG (**g**–**i**) in the middle third of rat alveolus on day 28 of the alveolar socket healing. Rats received soluble MT powder at 20 mg/kg/day by intragastric gavage for 28 days prior and 28 days after tooth extraction. Data represent the means ± standard deviations (SD), n = 8 rats/group. The control and MT groups were compared using the nonparametric unpaired Mann–Whitney U test (**P < 0.001; ***P < 0.0001). P values < 0.05 were considered statistically significant. Harris hematoxylin counterstaining for OPG, RANKL, and TRAP. Scale bar = 25 µm. Original magnification, ×1000. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B; and TRAP, tartrate-resistant acid phosphatase

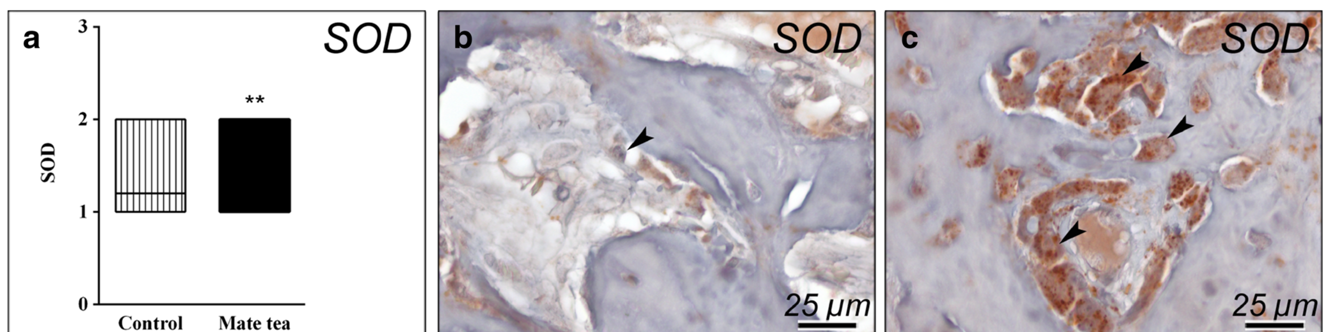

Fig. 4 Photomicrographs and graphics showing the immunoreactivity pattern of MnSOD (**a**–**c**) in the middle third of rat alveolus on day 28 of the alveolar socket healing. Rats received soluble MT powder at 20 mg/kg/day by intragastric gavage for 28 days prior and 28 days after tooth extraction. Data represent the means ± standard deviations (SD), n = 8 rats/group. The control and MT groups were compared using the nonparametric unpaired Mann–Whitney U test (***P < 0.0001). P values < 0.05 were considered statistically significant. Harris hematoxylin counterstaining for MnSOD. Scale bars = 25 µm. Original magnification, ×1000. MnSOD, manganese superoxide dismutase

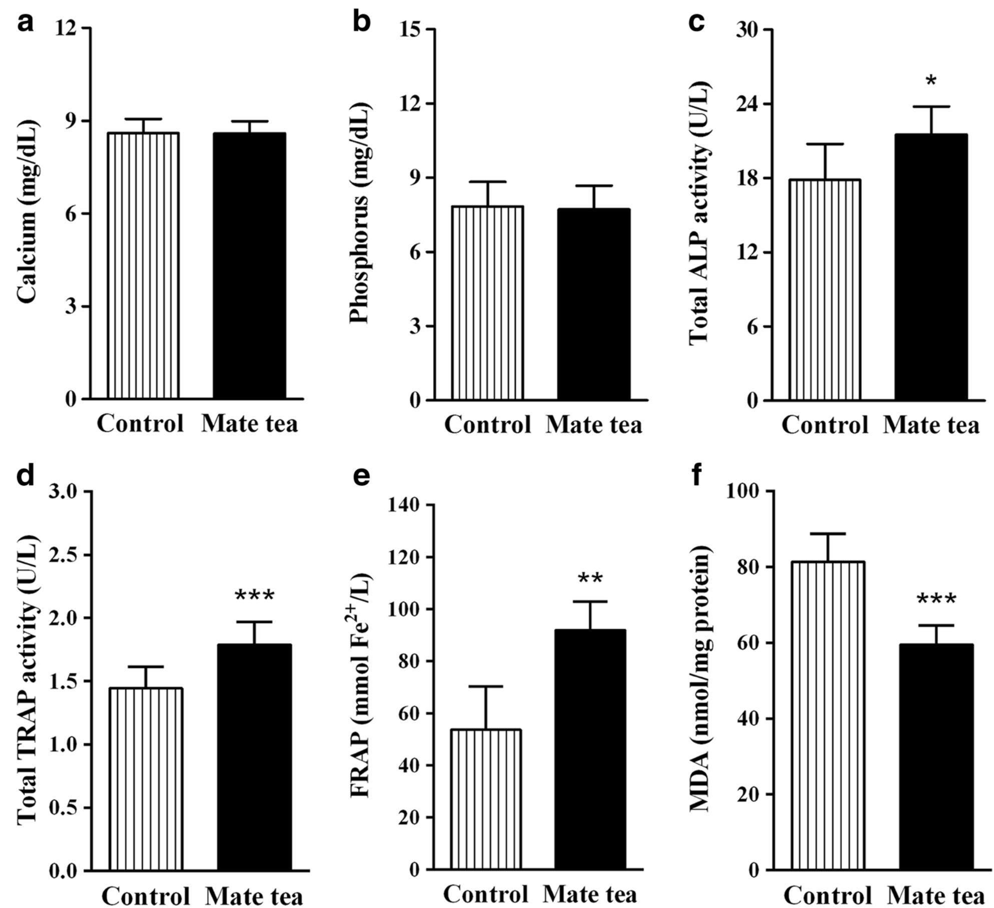

Fig. 5 Plasma concentrations of calcium (**a**), phosphorous (**b**), total ALP (**c**), and total TRAP (**d**) activities, FRAP (**e**), and MDA levels (**f**) on day 28 of the alveolar socket healing. Data represent the means ± standard deviations (SD), $n = 8$ rats/group. The control and MT groups were compared using the unpaired *t* test (*$P < 0.05$, **$P < 0.001$, and ***$P < 0.0001$). P values < 0.05 were considered statistically significant. ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; FRAP, ferric reducing/antioxidant power; and MDA, malondialdehyde

300 mL (1½ cup) of soluble MT per day by humans, prepared according to the manufacturer's instructions. Our group demonstrated that the daily intake of this dose for 4 weeks is sufficient to increase the TAC in plasma and modulate the activity of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase in different tissues, thus providing protection against damage from oxidative stress in perimenopause rats [24, 26]. This dose was shown to be safe since there were no changes in food intake, water consumption and body weight, which ensures that changes in oxidative stress markers did not result from changes in calorie intake or body mass reduction promoted by MT treatment [24, 26].

After tooth extraction, bone formation plays an important role in alveolar socket wound healing. MT ingestion improved bone formation during the alveolar wound healing due to increased bone area and osteocyte density associated with higher OCN immunolabeling and plasma ALP activity. Our group observed similar effects in the femur neck of perimenopause rats. After the treatment with the same dose of MT for 4 weeks, we found a higher area of trabecular bone and osteocyte density in the region of the femur neck, which was also systemically associated with increased plasma ALP activity [26]. In addition, in accordance with our results, previous bone metabolism-related studies conducted on male and female rats treated with ellagic acid [59] and oxytocin [6], higher OCN immunolabeling in the MT group indicates better bone mineralization, compared to the control group.

Our study also showed intense OPG immunolabeling in the MT group, accompanied by an increase in RANKL and TRAP immunolabeling. In addition, MT-treated rats exhibited higher plasma TRAP activity on day 28 of the alveolar socket healing. With the exception of OPG immunolabeling, the results related to RANKL and TRAP immunolabeling and plasma TRAP activity were the opposite of those found in the study about the remodeling of the femoral neck of perimenopause rats after treatment with MT, compared with perimenopause rat nontreated [26]. These results suggest that the biologic effects of MT ingestion in bone tissue depend on the age, sex and bone conditions such as bone remodeling and repair. In consistence with our results, oxytocin, an anabolic bone hormone, increased TRAP-positive osteoclast formation by improvement of both proliferation and differentiation owing to the upregulation of RANKL [60]. Our results are also supported by the findings of Iida-Klein et al. [61], which showed an association between

the anabolic response of the skeleton to exogenous parathyroid hormone in mice and the increase in OCN, RANKL, and TRAP expression.

Our results also showed that the increased bone area in the middle third of the alveolar socket after MT treatment was related to a reduction of lipid oxidative damage and increase of enzymatic and non-enzymatic antioxidant defense. Cutando et al. [15] showed a significant increase in plasmatic MDA immediately after tooth extraction in dogs, which was counteracted by administration of melatonin into the alveolar sockets. In a model of chronic inflammation-induced bone loss in female rats, green tea combined with alfacalcidol showed bone protective effects by reduction of oxidative stress-induced damage and inflammation [4]. In this study, rats treated with MT for 8 weeks showed higher plasma TAC, despite extraction of the right maxillary incisors. This may explain the reduction of plasma MDA concentrations in the MT group. These findings might be attributed, in part, to the high concentrations of polyphenols present in MT, which act as non-enzymatic antioxidant defenses, reacting directly with reactive oxygen species and/or free radical intermediates. Furthermore, the more intense immunolabeling pattern of MnSOD in the MT group supported that the bioactive compounds in MT upregulated the enzymatic antioxidant defenses involved in the alveolar repair process. The ability of the bioactive compounds in MT or their metabolites to regulate MnSOD gene expression was previously reported by Matsumoto et al. [29]. Our results are consistent with Pereira et al. [24], which showed that MT ingestion (20 mg/kg/day) for 8 weeks increased the total SOD activity in the liver and erythrocytes in perimenopause rats. However, they diverge from the immunostaining pattern of MnSOD in the femur neck of perimenoupause rats treated with the same dose for 4 weeks [26]. These results reinforced that the benefits of MT ingestion in bone tissue depend on the age, sex, and bone conditions such as bone remodeling and repair.

Yerba mate is rich in phenolic compounds and various saponins [20–24]. The polyphenol content of *I. paraguariensis* is higher than that of green tea [62]. Since yerba mate contains bioactive compounds that affect the bone cells, it could contribute to the increase in bone mass in the middle third during the alveolar repair process. For example, chlorogenic acid has been proven effective in the treatment of hormonal femoral head necrosis by protection of osteoblasts, inhibition of necrosis, and reduction of apoptosis [63]. Sassa et al. [64] showed that ferulic acid promoted bone remodeling, which led to a predominantly osteoblastic phase, besides bone resorption by osteoclasts in an ovariectomised rat model of postmenopausal bone loss. In addition, *p*-coumaric acid, caffeic acid, and ferulic acid stimulated osteoblast cell (MG-63) proliferation [65]. Green tea catechins have been proven to exhibit positive effects on bone metabolism by a double process of promoting osteoblastic activity and inhibiting osteoclast differentiation [66]. Although the pure bioactive compounds were effective, they are extremely expensive and difficult to obtain, compared to yerba mate, which would hinder its application as a therapeutic option. Furthermore, it has been shown that the benefits of MT depend on the synergism among its bioactive compounds, such as phenolic acids and triterpenoid saponins [30].

In this study, alveolar socket healing was evalatued only on day 28 after tooth extraction, since studies indicate that this is the period in which tissue repair process is in its advanced stage [6, 41]. However, the evaluation of the early stage, for example, on postoperative days 7, 14, and 21, would provide information about the speed the alveolar socket healing process in the animals treated with MT, a very interesting information when one thinks about the rehabilitation of the area with osseointegrated implants. Another point worth mentioning is that, in this study, we use MT prepared according to the manufacturer's instructions, and not its bioactive forms, because our purpose was to evaluate the effect of the beverage in the way it is consumed popularly; therefore, this limits us to attribute the beneficial action to some of its specific components. It would be interesting to conduct subsequent studies to evaluate the effectiveness and safety of the other doses and of the pure bioactive compound, although it has been shown that the benefits of MT depend on the synergism among its bioactive compounds [29].

Conclusion

Taken together, the results of this study show that MT increases bone neoformation in the middle third of the alveolar socket on day 28 after tooth extraction. These local effects on alveolar socket healing were associated with higher levels of antioxidant defenses (MnSOD), increase of biochemical markers of bone formation (OCN) and reabsorption (RANKL, OPG and TRAP), which were systemically supported by the increase in ALP and TRAP activities in the blood, reduction of lipid oxidative damage, and increased TAC in plasma, which confirmed our hypothesis.

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Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval The study was conducted according to the national (CONCEA—National Association for Animals Experiments Control: http://concea.mct.gov.br) and institutional laws and it was approved by the Ethics Committee on Animal Use (CEUA), of the São Paulo State

University (Unesp), School of Dentistry, Araçatuba, São Paulo, Brazil (Authorization Protocol 2013-01572). All surgery was performed under ketamine anesthesia, and all efforts were made to minimize suffering.

Informed consent For this type of study, formal consent is not required.

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