Original Research
Oral Pathology

Treatment of bisphosphonate-related osteonecrosis using platelet-rich plasma: microtomographic, microscopic, and immunohistochemical analyses

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Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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https://doi.org/10.1590/1807-3107bor-2019.vol33.0050

Submitted: 2018, Jul 28
Accepted for publication: November 11, 2018
Last revision: May 22, 2019

Abstract: The present study aimed to investigate the use of platelet-rich plasma (PRP) on tooth extraction sites in rats treated with bisphosphonates. Thirty Albinus Wistar male rats were administered 0.035 mg/kg zoledronic acid intravenously for 8 weeks, divided into four administrations with a 2-week interval between each application, after which their upper right central incisors were extracted to induce the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ). The samples were divided into the following two groups: Group 1 (G1) underwent marginal resection of BRONJ followed by the use of PRP, while Group 2 (G2) underwent resection of BRONJ but without the use of PRP. The treatment groups were evaluated after 14, 28, and 42 days. Clinical, microtomographic, microscopic, and immunohistochemical (IHC) evaluations were performed. Microtomography results revealed no significant difference between the groups ($p < 0.05$) in any time period. Histomorphometric analysis showed increased bone formation over time for both groups ($p < 0.001$). G1 demonstrated a greater amount of new bone formation than G2 at 28 and 42 days ($p < 0.001$), with G1 presenting greater vascularization and a slightly higher VEGF expression. For both groups, RANKL/OPG expression levels were sufficient as a parameter for indicating the rate of bone remodeling in a previously treated area of osteonecrosis groups. Taken together, our findings indicated that the use of PRP improves the resolution process of BRONJ.

Keywords: Disphosphonates; Platelet-Rich Plasma; Wound Healing.

Introduction

In the literature, strategies on how to manage the treatment of bisphosphonate-related osteonecrosis of the jaw (BRONJ) are still widely debated, and an established protocol with predictable results remains to be established. Considering the failure of the conservative approach, the surgical approach has been widely recommended for handling more advanced cases; however, this entails greater consideration of the bone and symptoms associated with major infections (Stages 2

and 3) caused by the risk of bacteremia and sepsis in immunocompromised patients, as well as the reduced quality of life.[1,2]

Given this scenario, a variety of surgical treatment options have been used to improve resolution rates of BRONJ cases. Of these therapies, treatment via bone resection combined with the use of growth factors has yielded promising results in the search for a satisfactory cure for this complication.[3,4] Platelet-rich plasma (PRP) is an autologous source of growth factors that is obtained via centrifugation to yield very high concentrations of human platelets containing various growth factors (derived growth factor, transforming growth factor β, epidermal growth factor, and vascular endothelial growth factor), which promote the rapid healing of wounds.[5]

Given the absence of well-defined protocols for BRONJ treatment and the scarcity of detailed experimental studies using surgical therapy in combination with PRP, the present study aimed to analyze the surgical therapeutic method proposed by using imaging, microscopic, and immunohistochemical analyses.

Methodology

The present study was approved by the Ethical Committee on Animal Use (CEUA/USC 12/14) and conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), as well as the Brazilian Society of Laboratory Animal Science (COBEA).

Study design

Thirty (30) Albinus Wistar male rats, with a mean weight of 180 g, underwent surgical procedures for the extraction of upper right incisors following administration of zoledronic acid (Novartis Pharma Stein AG, Stein, Switzerland). The animals were divided into two groups according to the treatment type. The Experimental group (G1) received autogenous platelet-rich plasma (PRP) following marginal bone resection and curettage of the alveolus, while the Control group (G2) underwent marginal bone resection and curettage of the alveolus without PRP.

Zoledronic acid administration and tooth extraction

All animals received 0.1 ml of 0.035 mg/kg IV zoledronic acid in the tail vein every 15 days for 8 weeks.[6] For IV administration, the animals were neither sedated nor anesthetized. The administration time was approximately 1 min. No adverse effects were observed during the administrations. After the fourth dose, tooth extractions were performed in accordance with previously described method.[7,8] At the beginning of the surgical procedures, the animals were sedated by intraperitoneal administration of 1% ketamine (0.20 ml/kg) and 2% xylazine (0.30 ml/kg) (Francotar, Virbac Ltda., São Paulo, Brazil). Following the surgery, all animals were intramuscularly medicated with 1 mg/kg Metamizole sodium per day.

Clinical evaluation

Considering the chronology of alveolar repair in rats, the sockets were clinically evaluated on day 7 after tooth extraction, and the following diagnostic criteria for BRONJ was considered: presence of bone exposure and/or signs of inflammation and infection, such as suppuration, soft tissue swelling, and the presence of fistulae.[9] The animals were not anesthetized for this procedure.

Surgical treatment

The surgical treatment was performed 7 days after the extraction. The 30 animals were divided into two groups. The Experimental group (G1), which was subjected to marginal resection of the region of necrotic bone or to sequestrectomy and socket curettage, followed by insertion of PRP into the viable bone site. The Control group (G2) was subjected to the same treatment, but without PRP. The animals were sedated by intraperitoneal administration of 1% ketamine (0.20 ml/kg) and 2% xylazine (0.30 ml/kg) (Francotar, Virbac Ltda., São Paulo, Brazil). Platelet-rich plasma was prepared following the standardized protocol reported by Anitua et al.[10] After anesthesia, 1.0 ml of blood was extracted through an intracardiac puncture using an insulin syringe and a 25 × 7 gauge needle. The platelet concentration for each specimen was approximately 0.5 × 10 per microliter. Suturing was performed using

4.0 polyglactin 910 (Vicryl, Johnson and Johnson, São Paulo, Brazil), covering the entire affected region. In the immediate postoperative period, rats in both groups individually received a single 0.1 ml/kg intramuscular dose of the veterinary antibiotic for prophylaxis of local infection or bacteremia. For analgesia control, a single dose of 0.02-0.5 mg/kg Buprenorphine was administered subcutaneously and 1 mg/kg a day of Metamizole sodium for 3 days was administered intramuscularly. After 14, 28, and 42 days, five animals from each group were euthanized; the maxillae were removed and then immersed in 10% buffered formalin.

MicroCT evaluation

All specimens were submitted to microCT (CTAn Bruker, SkyScan, Belgium), following the surgical procedure (55Kvp; 72mA and high resolution). After axial reconstruction, qualitative analyses and bone histomorphometry were performed using the CTan software (Skyscan, Belgium). A volume of interest ("VOI") was applied on the treated region in equal amounts (in the extension parameters and area of the examination) for all specimens. The volume ratio (VR)/total bone volume (TV), trabeculae number (TN), and trabeculae thickness (TT) were calculated. Each "VOI" had the same geometry and was applied in 200 "slices" of each specimen, selecting the central region of the socket.

Microscopic evaluation

The specimens were fixed for up to 48 h before washing in tap water for 24 h. Afterwards, the specimens were demineralized by immersion in 4% EDTA solution for 20 to 30 days. When demineralization was achieved, the right and left maxillae were separated, and the right side containing the socket was subjected to histotechnical procedures by staining with hematoxylin and eosin (HE). Microscopic analysis was performed considering new bone formation, vascularization, connective tissue and inflammatory infiltrate. Ten fields at 20× magnification of each portion of the socket (apical, middle, and cervical) were captured using a light photomicroscope (Nikon Eclipse 80i trinocular optical microscope, Nikon Instruments, Inc.), resulting in a total of 30 images per socket per animal. The images were quantitatively analyzed using Image-Pro® Plus version 5.1.2 for Windows XP, (Media Cybernetics, Inc., Rockville, USA.). "GRID" was used for histomorphometry analysis of the variables. The volume density of each structure (bone, connective tissue, blood vessel, and inflammatory infiltrate) was calculated. Subsequently, the mean and standard deviation of the percentages obtained per group were calculated.

Immunohistochemistry

Immunohistochemical (IHC) analysis was performed to evaluate vascularization and bone remodeling between the two groups. The following primary antibodies were used: VEGF (A-20): sc-152, CD31: Pecam-1 Antibody (M-20); RANK-L and OPG (Santa Cruz Biotechnology®, Dallas, USA.). For analysis of the proteins relating to bone formation, maturation, and remodeling, 3-μm sections of each specimen were deparaffinized in xylene and rehydrated in a graded series of ethanol to distilled water, immersed in 0.01 M citrate-buffer at pH 6.0, and subsequently heated in a steamer for 30 min. Histological slices were treated with proteinase K for 30 min at room temperature. Endogenous peroxidase was blocked with 2% hydrogen peroxide for 10 min and then washed with PBS (phosphate buffer solution). Afterwards, the samples were incubated with the primary polyclonal antibodies anti-RANKL, anti-OPG, anti-VEGF, and anti-CD31 overnight at 4°C and then washed thrice for 30 min each wash. The slices were incubated with biotinylated secondary antibody for 30 min, washed in PBS, and then incubated with streptavidin-peroxidase conjugate (LSAB, Dakocytomation, Carpinteria, USA) for 30 min. The samples were then stained with 3.30-diaminobenzidine tetra-hydrochloride (Sigma Aldrich, St. Louis, USA) and counterstained with Harris hematoxylin. For negative control, incubation with the primary antibody was omitted. Staining signals for each antibody were determined by semi-quantitative analysis, considering the scores ranging from "-" to "+++" ("-": absent, "+": mild, "++": moderate, and "+++": intense) and was performed using two evaluations and a double-blind system.[10]

Statistical assessment

Data obtained from the histomorphometric assessment (microscopic and MicroCT) were organized into a table (Microsoft Office Excel, Redmond, USA) and analyzed using SigmaPlot software (SigmaPlot, San Jose, USA) version 12.3. For each histomorphometric assessment, data were analyzed in relation to the normal distribution using Shapiro-Wilkes and Equal Variance tests, followed by 2-way ANOVA (Group and Period) and the Tukey test to detect differences between the groups (SigmaPlot 12.0, San Jose, USA). $p < 0.05$ was considered statistically significant.

Results

Gross examination

Seven days after extraction, 12 out of 30 animals showed bone exposure, with five animals presenting suppuration and two animals with bone sequestration.

MicroCT evaluation

Based on microtomographic examination, all specimens showed areas of osteolysis and fracture or loss of socket integrity in some regions of the bone adjacent to the socket. When the new bone formation within the socket is considered, qualitative analysis revealed an increase in new bone formation over time which is expected in the process of alveolar repair of a healthy socket. At 42 days, increased new bone formation was observed compared to days 28 and 14 (Figure 1). Considering the results of the histomorphometric analysis, no significant difference was found between the groups ($p < 0.05$) (Table 1).

Histological analysis

Group 1 (with PRP)

On day 14, discrete bone formation (BF) was more abundant on the walls and the apical third of the socket than in the central areas. Bone trabeculae were

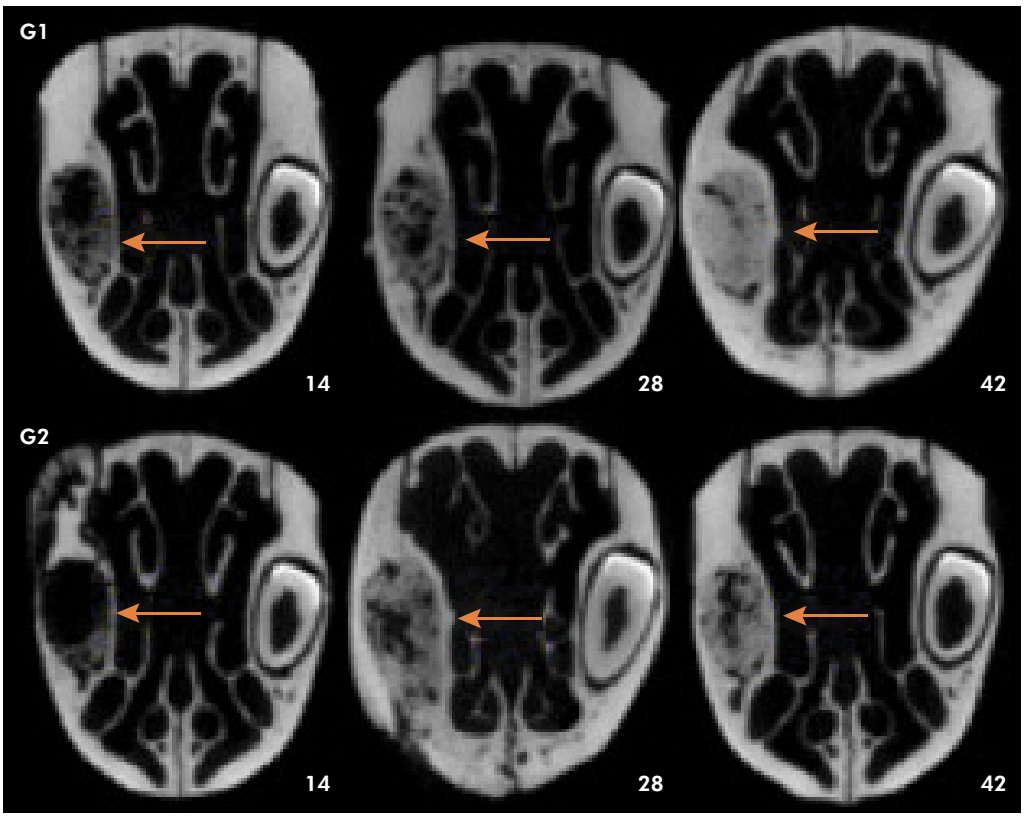

Figure 1. Photomicrographs of group 1 (G1), treated with PRP, and group 2 (G2), without PRP, showing bone neoformation within the alveolus (arrow) on days 14, 28, and 42.

Table 1. Results of statistical tests, histomorphometric analysis between groups, periods and analyzes.

Evaluation	p- value	Statistic test
G1		
14 days x 28 days x 42 days		
VO/VT(%)	0,001896*	ANOVA
ET	0,0009345*	ANOVA
NT	0,01854*	ANOVA
G2		
14 dias x 42 dias		
VO/VT(%)	0,2159	Mann-Whitney
ET	0,20097	t Student
NT	0,11812	t Student
G1 x G2		
VO/VT(%)		
14 days	0,8852	Mann-Whitney
28 days	-	-
42 days	0,8597	Mann-Whitney
ET		
14 days	0,42937	t Student
28 days	-	-
42 days	0,12959	t Student
NT		
14 days	0,12959	t Student
28 days	-	-
42 days	0,34559	t Student

*statistically significant difference.

observed to be surrounded by a highly vascularized connective tissue. The presence of blood vessels was observed across the whole socket, and was particularly conspicuous in the middle and cervical thirds. Discrete mononuclear inflammatory infiltrate was observed. Greater formation of new bone was observed on day 28. The presence of blood vessels was observed in the entire socket, notably in the middle (M) and apical (A) thirds (Figure 2). Discrete inflammatory mononuclear infiltrate was observed. On day 42, bone remodeling was noted, marked by basophilic reversal lines. Blood vessels were present in all socket thirds (Figure 2).

Group 2

On day 14, discrete (BF) was more abundantly observed on the walls and the apical third of socket than the central areas. Bone trabeculae were distributed throughout the socket and were highly vascularized. Discrete inflammatory mononuclear infiltrate was observed, along with mild polymorphonuclear infiltrate represented by neutrophils in the cervical third. On day 28, discrete newly formed bone tissue was observed in the cervical third, with the presence of granulation tissue in some specimens. On day 42, greater new bone formation was observed, except in the cervical third. The connective tissue found in the bone trabeculae was mature, with the presence of discrete mononuclear inflammatory infiltrate. Blood vessels were primarily observed in the apical and cervical thirds (Figure 2).

Based on histomorphometric analysis, both groups showed increased new bone formation over time ($p < 0.001$). G1 showed more pronounced new bone formation than G2 on days 28 and 42 ($p < 0.001$). In G1, the middle and cervical thirds of the socket revealed increased new bone formation than in G2 ($p < 0.001$). On days 28 and 42, G2 showed more connective tissue than G1 ($p < 0.001$). We observed no statistically significant difference in inflammatory infiltrate between the groups. G1 presented greater blood vessels than G2 in all time periods ($p < 0.001$).

Immunohistochemistry evaluation

VEGF – Group 1

On days 14 and 28 days, immunolabeling ranged from mild to moderate for the anti-VEGF antibody, especially in connective tissue fibroblast cells, mononuclear leukocytes, and focal regions of the bone tissue. On day 42, discrete immunolabeling was observed in the bone tissue, especially in osteocytes.

VEGF – Group 2

Immunolabeling was also detected on days 14, 28, and 42, especially in the connective tissue and osteocytes, but was less pronounced than that in G1 (Figure 3).

CD31 - Group 1

Absent to mild immunolabeling was observed at all time intervals. When present at 14 days, discrete

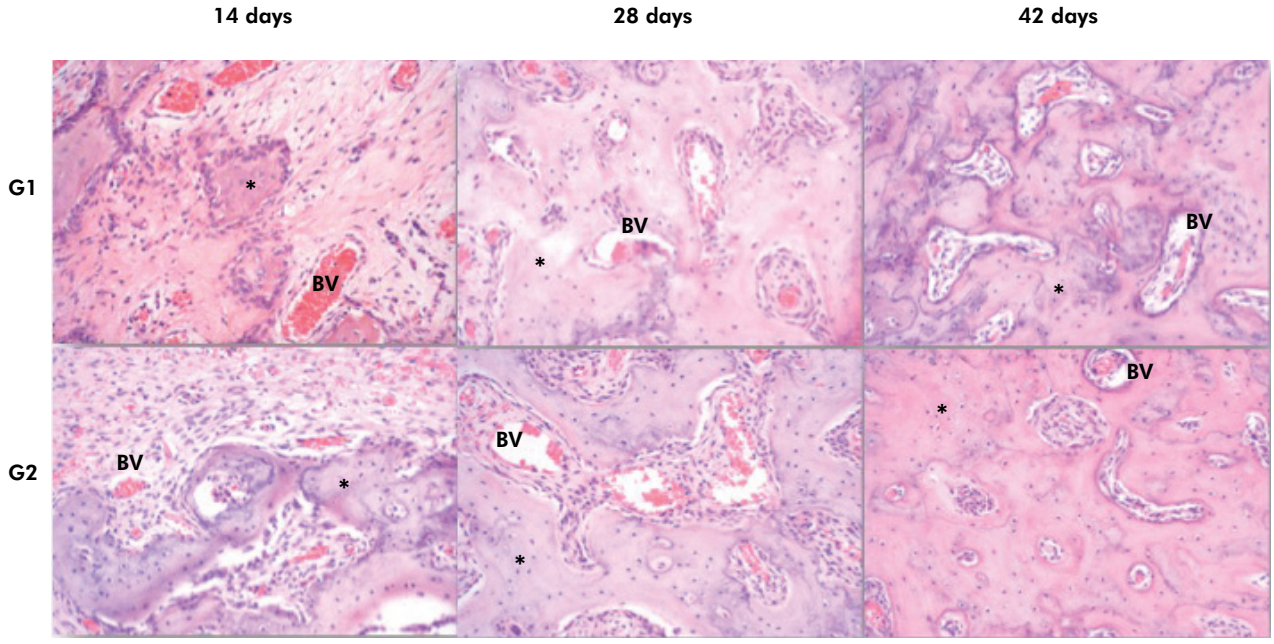

Figure 2. Photomicrographs of group 1 (G1), treated with PRP, and group 2 (G2), without PRP, showing blood vessels (BV) in neoformed bone trabeculae (*) on days 14, 28, and 42.

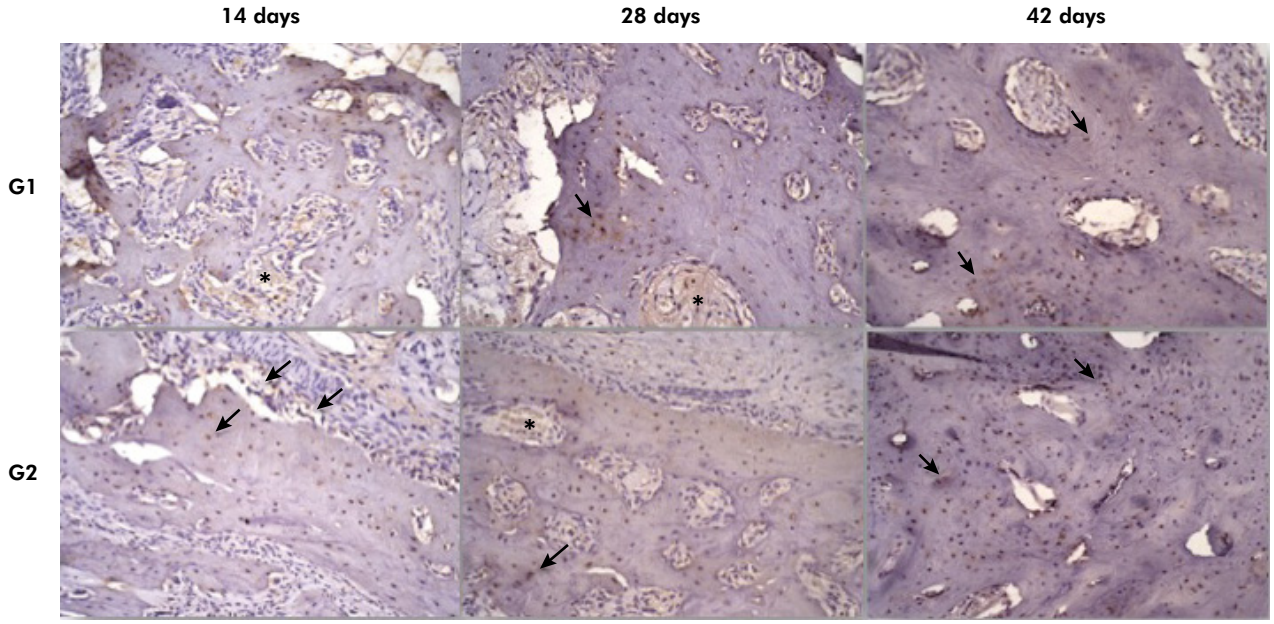

Figure 3. Photomicrographs of group 1 (G1), treated with PRP, and group 2 (G2), without PRP, showing the immunostaining results for the anti-VEGF antibody in osteocytes and osteoblasts (arrows) and connective tissue (*) on days 14, 28, and 42 (Magnification = 200×).

immunolabeling was observed in the connective tissue, especially in inflammatory cells, which were predominantly mononuclear. On days 28 and 42, discrete immunolabeling was observed in the endothelial cells of the blood vessels.

CD31 - Group 2

Similar to Group 1, absent to mild immunolabeling was present all time intervals in the same structures. On days 28 and 42, few specimens were positive for the CD31 antibody (Figure 4).

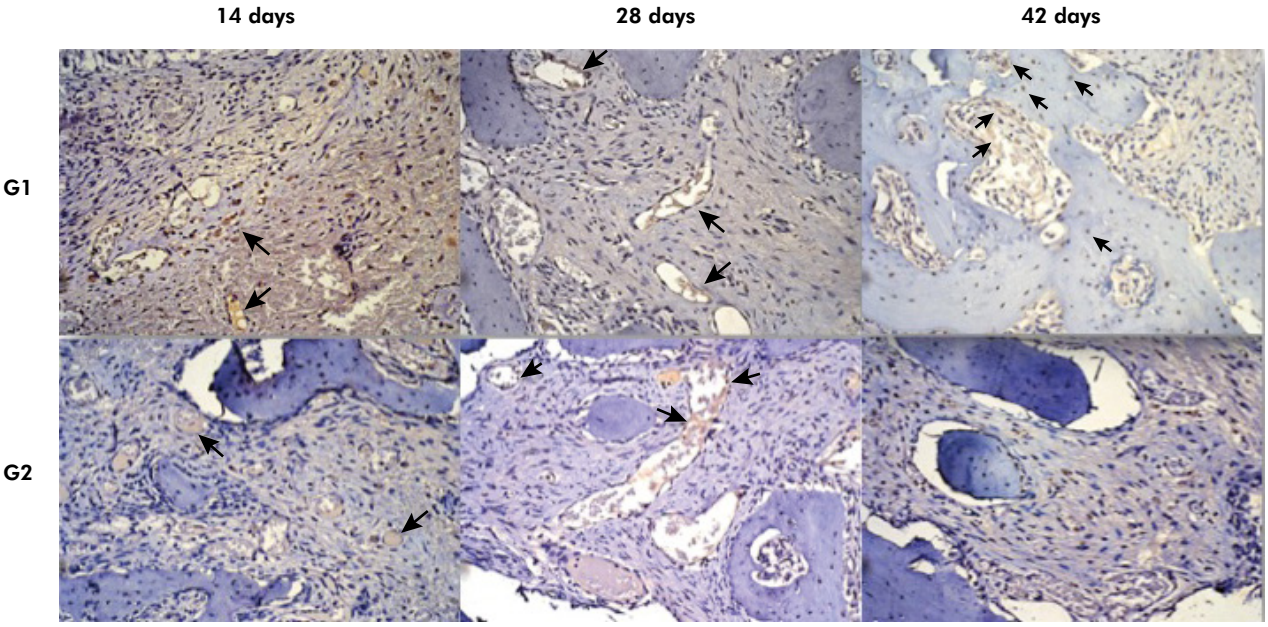

Figure 4. Photomicrographs of group 1 (G1), treated with PRP, and group 2 (G2), without PRP, showing the absence of signals corresponding to the anti-CD31 antibody on days 14, 28, and 42. Detailed in endothelial cells and mononuclear inflammatory cells (arrows) (Magnification = 200×).

RANK-L - Group 1

On day 14, intense immunolabeling was observed in the connective tissue, especially in the stromal cells (fibroblasts and osteoblasts). In addition, moderate immunolabeling was observed in the bone tissue. On day 28, mild-to-moderate immunolabeling was observed in the connective tissue, and moderate immunolabeling was observed in the bone tissue (osteocytes). On day 42, moderate immunolabeling was observed in the osteocytes, and mild immunolabeling was observed in the connective tissue.

RANK-L - Group 2

On day 14, moderate-to-intense immunolabeling was observed in the connective tissue, especially in the stromal cells (fibroblasts and osteoblasts). In addition, moderate immunolabeling was detected in the bone tissue. On day 28, mild-to-moderate immunolabeling was observed in the connective tissue, and moderate immunolabeling was observed in the bone tissue (osteocytes). On day 42, moderate immunolabeling was observed in the osteocytes (Figure 5).

OPG - Group 1

On day 14, mild immunolabeling was observed in the connective tissue, and moderate immunolabeling was observed in the osteocytes. On day 28, mild to moderate immunolabeling was detected in the bone tissue, while moderate immunolabeling was observed in the stromal cells. On day 42, absent to mild immunolabeling was observed in the connective tissue and, when present, in osteocytes of bone tissue.

OPG - Group 2

On day 14, mild immunolabeling was observed in both bone tissue (osteocytes) and connective tissue. On day 28, mild immunolabeling was observed in the bone tissue, and on day 42, moderate immunolabeling was present in the bone tissue and connective tissue (Figure 6). The results of semiquantitative analysis are shown in Table 2.

Discussion

In the literature, the therapeutic treatment of BRONJ remains controversial, considering the absence of a definitive protocol with good predictability.

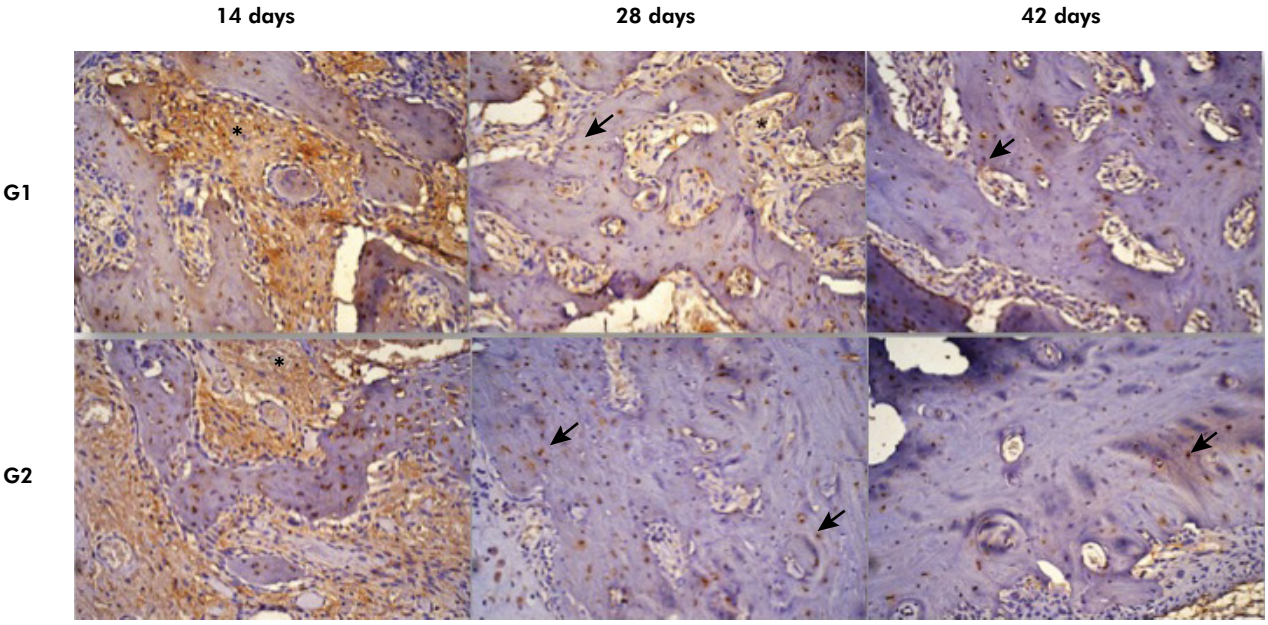

Figure 5. Photomicrographs of group 1 (G1), treated with PRP, and group 2 (G2), without PRP, demonstrates immunostaining for the anti-RANKL antibody at 14, 28 and 42 days, respectively. Detailed view of connective tissue and osteocytes marked by arrows. (Magnification = 200×).

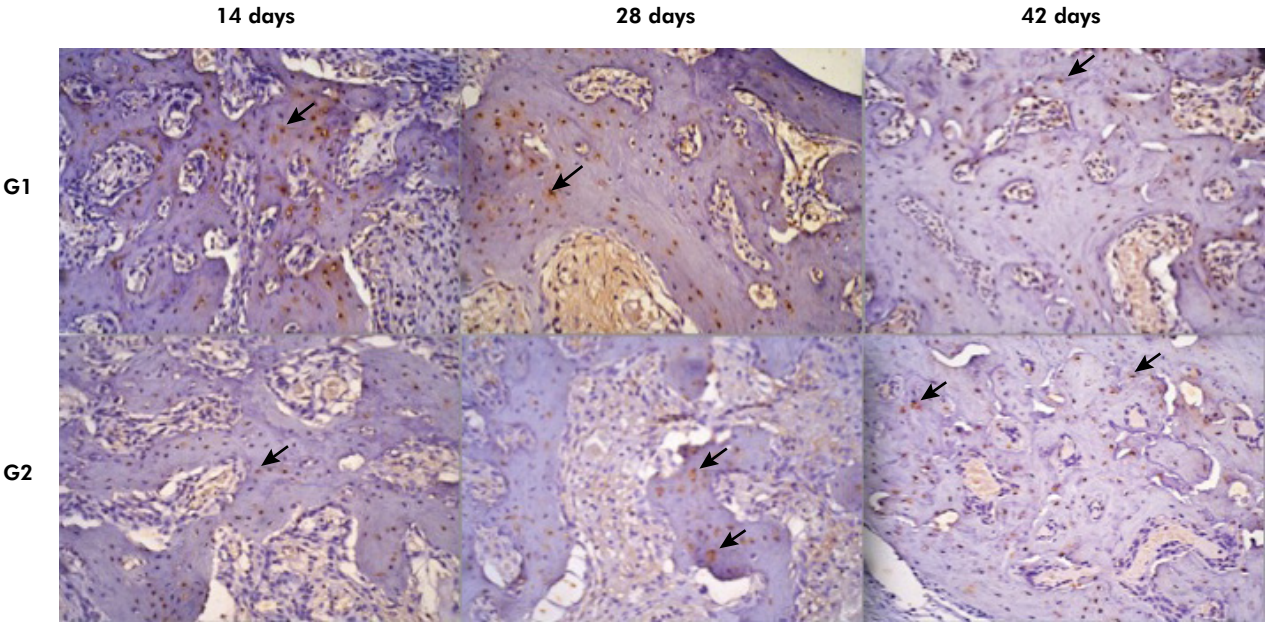

Figure 6. Photomicrographs of group 1 (G1), treated with PRP, and group 2 (G2), without PRP, showing the immunostaining results for the anti-OPG antibody on days 14, 28, and 42. Detailed view of osteocytes marked by arrows. (Magnification = 200×).

The first studies conducted to evaluate the surgical approaches for the treatment of BRONJ were published in 2006[12]. Since then, various other studies reported findings on the therapeutic treatment of BRONJ, with the aim of identifying the optimal surgical approach.[13,14,15,16,17,18,19,20,21,22] The current consensus for BRONJ treatment, as recommended by the AAOMS[2], dictates that the treatment strategies should always

Table 2. Frequency of results in semiquantitative analysis of each specimen. Absent immunolabeling (-); slight (+); moderate (++) and intense (+++).

Sample/Group	Days	VEGF	CD31	OPG	RANKL
1/G1	14	++	-	+	++
2/G1	14	+	-	+	++
3/G1	14	++	+	+	+++
4/G1	14	++	-	+	++
5/G1	14	+	+	++	++
6/G1	28	++	-	+	+
7/G1	28	+	+	++	+
8/G1	28	+	+	++	++
9/G1	28	++	-	+	++
10/G1	28	++	-	+	+
11/G1	42	+	+	++	++
12/G1	42	++	-	+	++
13/G1	42	+	-	+	++
14/G1	42	+	-	+	++
15/G1	42	+	-	+	+
16/G2	14	+	+	+	+
17/G2	14	++	-	+	++
18/G2	14	+	+	+	++
19/G2	14	+	-	+	++
20/G2	14	+	-	++	+++
21/G2	28	+	-	+	++
22/G2	28	+	+	+	+
23/G2	28	+	-	+	+
24/G2	28	+	-	+	++
25/G2	28	+	+	+	++
26/G2	42	+	+	+	++
27/G2	42	+	+	++	++
28/G2	42	+	-	+	+
29/G2	42	+	-	+	++
30/G2	42	+	-	+	++

be in accordance with the stage of the BRONJ disease. The healthcare provider should initially consider the most conservative approach and, when necessary, carry out surgical intervention when infections and comorbidities are present to effectively improve the patient's quality of life.

Recent systematic review studies have exposed the dearth of scientific evidence discussing the treatment of BRONJ, making it difficult to draw conclusions on this topic.[23] A recent study reviewed the following types of surgical approaches for BRONJ treatment: debridement or sequestrectomy and surgical resection and, in some cases, reconstruction of the affected areas. Clinical success was observed in the majority of cases who received surgical treatment; however, in addition to the lack of detail in the cases presented, the patient outcomes during the follow-up period varied greatly.[24]

The difficulty in identifying effective treatments for osteonecrosis explains the emergence of adjuvant therapy or the use of growth factors combined with resections in an attempt to increase the success rates of the surgical approach for this disorder. Regarding the surgical treatment proposed in this study, the use of platelet-rich plasma (PRP) in clinical settings has been reported since 2007.[1,25,26,27,28,29] The utilization of PRP aims to improve the healing process, given the existence of multiple growth factors that promote tissue vascularization and amidst the lack of vascularization in the pathogenesis of osteonecrosis.[2] The use of PRP was first described in 2007; in particular, PRP treatment followed by marginal bone resection in the surgical site of three clinical cases of BRONJ were unresponsive to conservative treatment.[13] All the cases would later be completely resolved. Subsequently, other studies reported using the same surgical procedures, also with satisfactory results.[26,29]

Another study demonstrated clinical experiences with the use of marginal bone resection and PRP in the surgical treatment of 25 patients suffering from BRONJ.[1] Eighty percent (80%) of the patients recovered, presenting full and complete healing of the oral mucosa and with no signs of exposed bone, as observed during the follow-up period. The authors concluded that bone resection combined with PRP was effective for the majority of patients in the advanced stages of BRONJ. Another clinical study evaluating the application of PRP reported a 100% success rate.[28]

A literature review evaluated the surgical treatment of 72 patients with BRONJ, out of which

15 patients received surgical treatment without PRP and 34 patients with PRP.[26] A comparison of the two groups showed that the success rate of surgery without PRP was lower compared to the group with PRP. Another systematic review comprising 18 studies[16] concluded that PRP could significantly improve the resolution of BRONJ. Clinical cure was observed in 91.6% of the 143 patients treated. However, the authors advise caution in the interpretation of the results, given the low degree of evidence in the studies and the small sample sizes. Moreover, the studies presented a number of limitations because of differences in the study focus and methodologies, specifically in terms of the type of bisphosphonates, clinical indication, treatment duration, trigger factors, type of study, length of follow-up, details of the PRP preparation, and the evaluation of the treatment success.

We found little histomorphometric evidence of repair in these studies when comparing areas of patients with BRONJ who were treated with PRP. The present study aimed to evaluate the effect of PRP on alveolar repair in terms of the microscopic, imaging, and immunohistochemical aspects. Given the limitations of a clinical study, the experimental model applied in this investigation was found to be adequate for the proposed analysis. As in previous studies,[11,30] the model of osteonecrosis induced by zoledronic acid was shown to be effective, considering that all the samples presented significant bone changes under computed microtomography. In this context, we retrieved only one experimental study on animals, which conducted microscopic evaluation of the effects of PRP in the treatment of BRONJ, wherein rats subjected to this therapy with zoledronic acid and received PRP presented better bone repair compared to the rats in the treatment group that did not receive PRP.[31]

Considering the results of the histomorphometric analysis in the computed microtomography examination, slightly greater new bone formation was observed in the group receiving PRP. Given that the computed microtomography examination only evaluated the bone structures, it can therefore be safely concluded that microscopic examination was fundamental to a detailed investigation of the repair process. Optical microscope examination revealed in greater detail significant new bone formation compared to the group without PRP. Importantly, these significant results were recorded on days 28 and 42, which are the most pertinent time periods for evaluating new bone formation.

The expression of anti-VEGF in bone tissue reflects the activity of the osteoblast cells in the induction of revascularization in repaired areas.[32] Osteocytes also produce VEGF, particularly when it is necessary to supply apoptotic osteocytes and during bone remodeling.[33] In this study, an analysis of the reaction of the samples of anti-CD31 antibody revealed a variation from absent-to-slight labeling and a notable nonspecific labeling. In addition, we observed no significant difference between the treated groups, such that we did not consider it appropriate to draw conclusions thereon. In the light of the results, the histomorphometric analysis can be considered an important approach for reliably comparing the two groups.

The use of PRP in the present study likely improved vascularization, as evidenced by histomorphometry results, which revealed more abundant blood vessels in the group that received PRP. In addition, the PRP group showed stronger immunolabeling signals for the anti-VEGF antibodies. We considered the results relating to vascularization significant enough to draw a conclusion, since vascularization is a crucial factor affecting the prognosis of BRONJ. The observed results are most likely caused by the presence of PRP in the surgical site based on prior evidence involving the use of PRP.

Qualitative evaluation revealed that bone remodeling activity was delimited by lines of reversal in both groups. The rate of bone remodeling was found to be normal within immunohistochemical parameters, as demonstrated by the equilibrium in the RANKL:OPG ratio of expression levels. Although the osteoclasts have not been quantitatively analyzed in this study, the analysis of the RANKL:OPG expression ratio was sufficient for determining the rate of bone remodeling in a previously treated area of osteonecrosis.

Conclusions

In the present study, the experimental group (G1) that received treatment with PRP presented improved bone repair compared to the control group (G2). The analysis took into account new bone formation and vascularization, which were both quantitatively evaluated.

Acknowledgments

Thanks are due to São Paulo Research Foundation (FAPESP - 2014/10693-6). The authors are grateful to Maira Cristina Rondina Couto from Sagrado Coração University, for histology and immunohistochemistry assistance and to the Endodontics Department, Universidade de São Paulo (FOB/USP), for the use of MicroCT.

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