Vascular Alterations in the Sprague-Dawley Rat Mandible During Intravenous Bisphosphonate Therapy

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Long-term use of intravenous bisphosphonates, such as zoledronic acid (zoledronate), has been linked to bisphosphonate-related osteonecrosis of the jaw (BRONJ). Invasive dental surgery seems to trigger the bone necrosis in most cases. To determine the effects of zoledronic acid on the vascular structure of the rat mandible. Extracted of the mandibular first molar in rats that received 2 IV injections of zoledronate (20 μg/kg), 4 weeks apart. Zoledronate-treated rats (n = 18) were then compared to a control group of untreated rats (n = 18). At the fourth, eighth, and 12th week after molar extraction, 8 rat mandibles from each group were perfused with 35% radiopaque triphenylbismuth in methyl methacrylate via carotid artery perfusion. Mandibles were harvested and examined by micro-CT to assess the spatial and dimensional changes of the vasculature as a result of zoledronate treatment. The micro-CT analysis showed that zoledronic acid-treated rats had blood vessels that were thicker, less connected, and less ordered than control rats that were not exposed to zoledronic acid. This study demonstrated that treatment with zoledronic acid in rats is associated with vascular changes in alveolar bone. Further studies are underway to explore whether these vascular changes contribute to the pathogenesis of BRONJ.

Key Words: bisphosphonate, BRONJ, vascular parameters, rat model, mandible

Introduction

Bisphosphonates can effectively inhibit bone resorption by decreasing osteoclastic activity. The popularity of this class of drugs has grown to treat disorders such as hypercalcemia of malignancy, skeletal-related events associated with bone metastases, multiple myeloma, osteoporosis, Paget's disease, and osteogenesis imperfecta.[1–5] However, the long-term use of this class of drugs, especially those that are intravenously administered such as zoledronic acid, can cause bisphosphonate-related osteonecrosis of the jaw (BRONJ). This devastating adverse condition causes painful intraoral exposure of the bone in the maxilla and mandible, which can be further complicated by infections.[1] BRONJ responds poorly to therapy and treatment primarily focuses on palliative care. Due to morbidity, the fundamental management of BRONJ should concentrate on prevention. Various serum protein biomarkers designed to detect the likelihood of the onset of BRONJ are under investigation.[6–8] Measuring serum levels of C-terminal cross-linking telopeptide is the only commercially available method to test susceptibility to BRONJ. However, many professionals question the accuracy of this test and its use remains controversial.[9–12]

Osteonecrosis can originate from the disruption of blood supply to an area of bone, which, in turn, produces ischemia and bone death and necrosis.[13] Whether the pathogenesis of BRONJ involves a bisphosphonate-induced ischemia remains controversial.[14] Nonetheless, vascular changes may be early manifestations of tissue necrosis in BRONJ. However, information on the vascular changes that occur with bisphosphonate treatment appears very limited. For this reason, gaining an understanding of the progressive vascular changes manifesting with BRONJ is a critical step toward the comprehension and prevention of this disease.

Materials and Methods

This study employs the rat osteonecrotic animal model published previously.[15] Marino and co-investigators utilized

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Sprague-Dawley rats that demonstrate clinical changes consistent with osteonecrosis after 4 and 8 weeks of low-dose intravenous zoledronic acid treatment.[15] The animal protocol for this study was approved by the Committee on Animal Use in Research and Education and the Institutional Animal Care and Use Committee of Georgia Regents University (formerly the Medical College of Georgia).

Forty-eight female retired breeder Sprague-Dawley rats (Harlan Sprague-Dawley, Inc, Indianapolis, Ind) weighing ~300 g each, were utilized for this study. Retired breeder Sprague-Dawley rats were chosen for this experiment for 2 reasons: (1) Retired female breeder rats are larger in size which facilitates intraoral procedures and aids in carotid perfusion and (2) the high metabolic rate of rats may allow for the expedient induction of BRONJ. An experimental and control group were established and randomly allocated with 24 rats each. Each group was subdivided into 4, 8, and 12-week groups (n = 8 per subgroup). The rats were housed individually and supplied with water and a standard rat chow diet *ad libitum*.

Zoledronic acid administration and molar extraction

At the beginning of the study (week 4), rats in the experimental group received zoledronic acid (20 μg/kg diluted to 100 μg/mL in PBS); rats in the control group received an equivalent dose of PBS. This was accomplished by first anesthetizing each rat with 0.5–0.7 mL/kg "rat anesthesia" (a cocktail comprised of ketamine [100 mg/mL], xylazine [20 mg/mL], and acepromazine [10 mg/mL]) given intramuscularly. Complete anesthesia was verified by pinching the tail of the rat and assessing for a reactive response. Subsequently, the respective agent was intravenously administered through the rat tail vein.

Four weeks later (week 0), each rat was anesthetized, and a second dose of zoledronic acid was administered to the test group and PBS to the control group in conjunction with the extraction of the mandibular right first molar followed by wound preparation of the extraction site. Successive wound preparation of the extraction site was accomplished through the use of a series of dental round burs ranging in size from 0.5 to 2.0 mm with continuous water irrigation. This resulted in a 2.0-mm cylindrical defect so that the dimensions of the final wound in all animals were nearly uniform and identical. After extraction and preparation of the site, the area was swabbed with 0.12% chlorhexidine.

Vascular perfusion

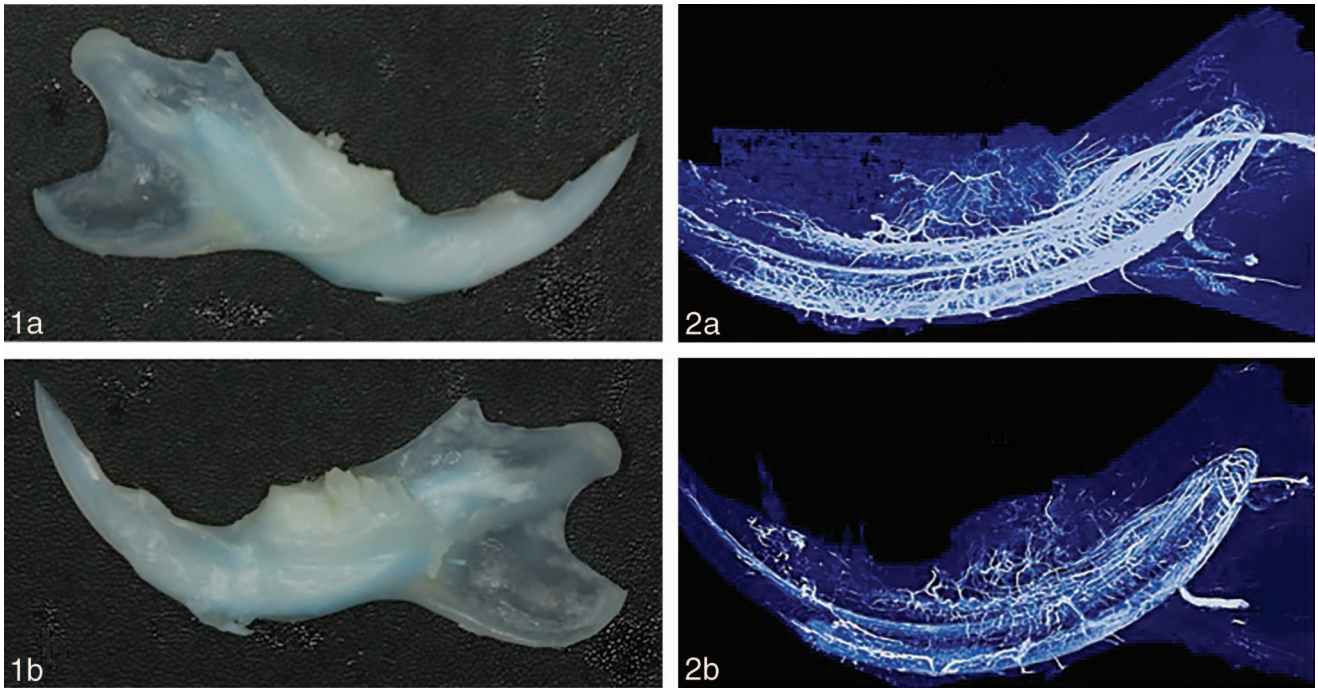
In order to visualize changes in the vascular structure of the mandible, 8 rats from each group were examined and sacrificed at 4, 8, and 12 weeks from the *second* dose of zoledronic acid or PBS. This procedure was carried out by initially administering an intramuscular injection of rat anesthesia. An incision that was approximately 2 to 3 inches long was made in the tracheal region. The right common carotid artery and right jugular vein were gently exposed, taking extra care that neither of these 2 structures were inadvertently incised or severed. The right common carotid artery was isolated from the rest of the anatomical structures by placing a thin, plasticized paper sheet underneath it, thereby acting as a trough. Untied 5-0 silk sutures (Ethicon, Somerville, NJ) were placed superior and inferior to the planned perfusion point on the right common carotid artery. The suture inferior to the perfusion point was then tied to direct perfusion cranially. A 24 gauge ¾" intravenous catheter (Jelco, Smiths Medical, Lancashire, UK) was carefully inserted and threaded into the right common carotid artery at the planned perfusion point. The suture superior to the perfusion point was then carefully tied to secure the threaded catheter. The catheter was then attached to a 4-way stopcock with 20-inch standard bore extension tubing (Smiths Medical, Dublin, Ohio). A small nick was placed on the jugular vein to function as an exit point for excess fluid.

The vasculature was perfused with 4 series of fluids at a steady perfusion rate of 1 mL for every 4 seconds. First, a syringe containing 50 mL heparinized PBS (500 units, 0.08 mL of 1000 units/mL) was attached to the opposite end of the extension tubing and steadily perfused through the area. Second, a syringe containing 50 mL formalin solution, neutral buffered, 10% (Sigma-Aldrich, St Louis, Mo) was perfused into the vasculature. Third, this rat was perfused with 50 mL PBS. Finally, the extension tubing was carefully removed from the catheter and a syringe containing 18 mL blue Mercox II methyl methacrylate (Ladd Research, Williston, Vt) mixed with 35% triphenylbismuth (Alfa Aesar, Ward Hill, Mass) was attached to the catheter and perfused into the animal. The successful perfusion of the methyl methacrylate through the vasculature was confirmed by noting the blue color of the rat's eyes, skin, tongue, and nose. The rat was set aside for 5–6 minutes to allow the methyl methacrylate to polymerize within the vascular system. The rat was then decapitated and placed into a 50°C saline bath for 1 hour. The soft tissues were then thoroughly removed from the mandible and separated into hemimandibles in which the right hemi-mandible was set aside for analysis and fixed in formalin overnight. Subsequently, the right hemi-mandible was placed in a decalcifying solution, (Formical-2000, Decal Chemical Group, Tallman, NY), for 7 days. This decalcifying solution was replaced with fresh solution on a daily basis. The blue methyl methacrylate was clearly visible in the decalcified mandibles (Figure 1).

Micro-CT analysis

Visualization and 3-dimensional analysis of the perfused demineralized right hemimandible was performed through the use of a SkyScan 1172 micro-CT system (SkyScan, Aartlesaar, Belgium) (Figure 2). The following blood vessel parameters were scanned and evaluated using micro-CT:

(1) The percent vessel volume measures the percentage of the volume-of-interest occupied by blood vessels.
(2) The surface-to-volume ratio measures the complexity of a three-dimensional structure.
(3) Vessel thickness is a measure of the average lumen size of the blood vessel.
(4) Vessel separation measures the average distance between vessels.
(5) The degree of anisotropy measures the 3-dimensional symmetry along a directional axis. The higher the degree of anisotropy, the more "polar" the vascular arrangement is.
(6) Fractal dimension measures the surface complexity of an object (or how an object fills space); an increase in fractal



Figures 1 and 2. Figure 1. Lateral (a) and medial (b) view of a decalcified right hemi-mandible. The bluish dye of the contrast material can be seen. **Figure 2.** Reconstructed micro-CT images from representative control (a) and zoledronic acid-treated (b) decalcified right hemimandibles after perfusion with methyl methacrylate containing triphenylbismuth. Vascular perfusion is diminished in the zoledronic acid-treated animals.

dimension is indicative of an increase in the branching of the blood vessels.

(7) Connectivity measures the degree of anastomosis or branching of a vascular structure.

(8) Euler number measures the degree of redundant connectivity between blood vessels.

Reconstruction of the scanned images was performed using a SkyScan NRecon program. The reconstructed dataset was loaded into SkyScan CT-analyzer software for measurement of the selected 3-dimensional morphometric parameters. Two regions of interest on the right hemimandible were evaluated: (1) The vicinity of the extraction socket of the right first mandibular molar and (2) the area posterior to this extraction socket (Figure 3).

Statistical analysis of the data

The independent variables were the treatment groups (experimental and control) and time. The dependent variables were the changes in the vasculature of the rat mandible at the various time points in the zoledronic acid-treated and untreated rats. Statistical comparisons were made by analysis of variance (ANOVA) looking for all treatment effects and possible interactions. Prior to ANOVA, all data were analyzed for normalcy of distribution. The normal distribution generally leads to a bell-shaped distribution and represents most variations in the measured blood vessel parameters of volume, surface-to-volume ratio, thickness, number, separation, degree of anisotropy, fractal dimension, Euler number, and connectivity. The level of statistical significance was set at $P \leq .05$. This was followed, where appropriate, by a *post-hoc* analysis.

Results

Four weeks after the second administration of zoledronic acid or PBS, molar extraction, and wound preparation of the site, eight rats in the control group and eight rats in the test group were evaluated by intraoral inspection for healing of the extraction socket. In the control group, it was found that all rats exhibited complete healing of the area associated with the extraction socket. However, three of the eight rats in the test group exhibited open wounds and signs of osteonecrosis in the bony area of the extraction socket.

Results of micro-CT analysis at different time points are outlined in Tables 1–3. From the analyzed vascular parameters, it was found that the following were statistically

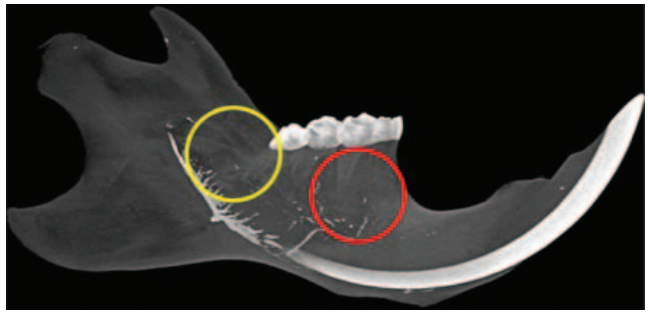


Figure 3. Micro-CT of an undecalcified right hemi-mandible taken from the initial pilot studies. The anterior region of interest, depicted by the red circle, was localized to the area of the first molar extraction socket while the posterior region of interest, depicted by the yellow circle, was localized to the area posterior to the extraction site.

Table 1

Comparison of the 4-week mean results and its respective calculated *P* values between the test and control groups of the various vascular parameters in the anterior (a) and posterior regions of interest (b).*

	PVV	VS/VV	VTh	VN	VSep	DA	FD	TP	EN	Conn
(a)										
ZA										
Mean	3.69	4.28	1.15	0.03	8.68	1.75	1.77	96.31	81.86	89.00
SD	2.36	0.90	0.18	0.02	2.46	0.13	0.15	2.36	53.11	70.70
Control										
Mean	2.31	4.72	1.08	0.02	10.01	2.14	1.70	97.69	77.00	70.75
SD	1.51	0.57	0.15	0.01	2.79	0.50	0.15	1.51	44.45	37.21
***P* value**	**.21**	**.30**	**.42**	**.27**	**.35**	**.06**	**.35**	**.21**	**.86**	**.56**
(b)										
ZA										
Mean	2.76	6.10	0.92	0.03	8.18	1.49	1.55	97.24	176.14	233.17
SD	2.23	0.44	0.06	0.02	3.95	0.19	0.20	2.23	95.42	150.87
Control										
Mean	2.17	5.84	0.89	0.02	8.80	1.61	1.61	97.83	67.63	90.50
SD	1.29	0.87	0.10	0.01	2.38	0.20	0.15	1.29	40.42	48.37
***P* value**	**.55**	**.47**	**.46**	**.56**	**.73**	**.26**	**.55**	**.55**	**.02**	**.07**

*PVV indicates percent vessel volume; VS/VV, vessel surface/vessel volume; VTh, vascular thickness; VN, vessel number; VSep, vessel separation; DA, degree of anisotropy; FD, fractal dimension; TP, total porosity; EN, Euler Number; Conn, connectivity.

significant: Euler number at 4 weeks in the posterior ROI (test: 176.14 ± 95.42; control: 67.63 ± 40.42; $P < .02$); vascular thickness at twelve weeks in the anterior ROI (test: 0.74 ± 0.37; control: 1.07 ± 0.13; $P < .01$); and Euler number at 12 weeks in the anterior ROI (test: 250.80 ± 71.88; control: 139.88 ± 29.01; $P < .02$).

The following vascular parameters approached statistical significance: degree of anisotropy at 4 weeks in the anterior ROI (test: 1.75 ± 0.13; control: 2.14 ± 0.50; $P < .06$); connectivity at 4 weeks in the posterior ROI (test: 233.17 ± 150.87; control: 90.50 ± 48.37; $P < .07$); vascular thickness at 8 weeks in the posterior ROI (test: 1.06 ± 0.15; control: 0.74 ± 0.37; $P < .09$); degree of anisotropy at 8 weeks in the posterior ROI (test: 1.68 ± 0.08; control: 1.49 ± 0.22; $P < .09$); and connectivity at 2 weeks in the anterior ROI (test: 271.00 ± 175.17; control: 157.13 ± 43.60; $P < .06$).

At 4 weeks, the data indicates that the anterior vessels are more ordered in the control rats than in the zoledronic acid-treated rats (greater degree of anisotropy). In the posterior region, the vessels are more connected in three dimensions in the control rats than in the zoledronic acid treated rats (higher Euler number and higher connectivity).

At 8 weeks, the data demonstrates that the posterior vessel thickness is greater in the zoledronic acid-treated rats than in the control rats. Additionally, the posterior vessels are more ordered in the control rats than in the zoledronic acid-treated rats (degree of anisotropy).

Finally, at 12 weeks the anterior vessels were thicker and less connected in 3 dimensions (lower Euler number and lower

Table 2

Comparison of the 8-week mean results and its respective calculated *P* values between the test and control groups of the various vascular parameters in the anterior (a) and posterior regions of interest (b).*

	PVV	VS/VV	VTh	VN	VSep	DA	FD	TP	EN	Conn
(a)										
ZA										
Mean	4.42	4.14	1.20	0.04	6.78	1.66	1.75	95.58	108.00	115.00
SD	1.63	0.62	0.17	0.01	0.86	0.28	0.11	1.63	50.81	39.59
Control										
Mean	3.01	5.02	1.05	0.03	8.02	1.87	1.67	96.99	84.17	150.33
SD	2.27	1.03	0.17	0.02	1.70	0.28	0.16	2.27	77.20	101.24
***P* value**	**0.24**	**0.11**	**0.14**	**0.37**	**0.15**	**0.22**	**0.28**	**0.24**	**0.54**	**0.45**
(b)										
ZA										
Mean	4.87	4.97	1.06	0.11	5.67	1.68	1.74	95.13	204.00	254.29
SD	2.16	0.87	0.15	0.17	1.03	0.08	0.12	2.16	100.10	75.59
Control										
Mean	3.72	6.49	0.74	0.04	5.65	1.49	1.57	96.28	250.80	271.00
SD	2.55	2.18	0.37	0.02	1.50	0.22	0.26	2.55	71.88	175.17
***P* value**	**.41**	**.16**	**.09**	**.32**	**.98**	**.09**	**.20**	**.41**	**.37**	**.85**

*PVV indicates percent vessel volume; VS/VV, vessel surface/vessel volume; VTh, vascular thickness; VN, vessel number; VSep, vessel separation; DA, degree of anisotropy; FD, fractal dimension; TP, total porosity; EN, Euler Number; Conn, connectivity.

TABLE 3

Comparison of the 12-week mean results and its respective calculated *P* values between the test and control groups of the various vascular parameters in the anterior (a) and posterior regions of interest (b).*

	PVV	VS/VV	VTh	VN	VSep	DA	FD	TP	EN	Conn
ZA										
Mean	3.72	6.49	0.74	0.04	5.65	1.49	1.57	96.28	250.80	271.00
SD	2.55	2.18	0.37	0.02	1.50	0.22	0.26	2.55	71.88	175.17
Control										
Mean	3.73	4.97	1.07	0.03	6.62	1.80	1.67	96.27	139.88	157.13
SD	1.13	0.53	0.13	0.01	0.98	0.23	0.08	1.13	29.01	43.60
***P* value**	**.63**	**.45**	**.01**	**.49**	**.16**	**.16**	**.23**	**.63**	**.02**	**.06**
ZA										
Mean	3.16	6.20	0.87	0.05	5.35	1.42	1.61	95.78	289.75	249.63
SD	1.24	1.18	0.12	0.03	0.95	0.18	0.20	3.22	142.31	93.20
Control										
Mean	3.20	6.94	0.82	0.04	5.27	1.39	1.55	96.80	281.50	257.00
SD	1.67	1.38	0.14	0.01	0.32	0.18	0.16	1.67	102.43	50.42
***P* value**	**.95**	**.27**	**.51**	**.43**	**.82**	**.72**	**.49**	**.44**	**.90**	**.85**

*PVV indicates percent vessel volume; VS/VV, vessel surface/vessel volume; VTh, vascular thickness; VN, vessel number; VSep, vessel separation; DA, degree of anisotropy; FD, fractal dimension; TP, total porosity; EN, Euler Number; Conn, connectivity.

connectivity) in the zoledronic acid-treated rats when compared to the untreated rats.

Discussion

In this study, experimental rats received 2 intravenous administrations of 20 μg/kg zoledronic acid; the second dose was delivered 4 weeks after the first administration. This is a nonlethal dose known to produce effects on osteoclasts and bone turnover in previous rat studies.[16] Moreover, patients with osteoporosis are given 5 mg zoledronic acid once a year.[17] It was calculated that the 2 administrations of zoledronic acid (total: 40 μg/kg) in our experimental rats is equivalent to the human osteoporosis dose.

Recent studies involved the use of much higher oncologic doses of zoledronates over extended periods of time.[18,19] However, these studies used active periodontitis as the triggering factor for BRONJ in rats. Recent epidemiologic studies, however, documented that dental extraction is the leading risk factor for BRONJ.[14]

Healing of the molar extraction sites was only evaluated in the fourth week test and control groups. It was found that 3 of the 8 rats in the test group exhibited signs of osteonecrosis while all rats in the control group fully healed without complications. This model utilized for the development of osteonecrosis was based on the work of Marino et al.[15]

Micro-CT analysis of the harvested rat mandibles indicated a statistically significant result for Euler number at 4 weeks in the anterior region ($P < .02$), Euler number at 12 weeks in the anterior region ($P < .02$), and vascular thickness at 12 weeks in the posterior region ($P < .01$). Several vascular parameters approached statistical significance: degree of anisotropy at 4 weeks in the anterior region ($P < .06$), connectivity at 4 weeks in the posterior region ($P < .07$), vascular thickness at 8 weeks in the posterior region ($P < .09$), degree of anisotropy at 8 weeks in the posterior region ($P < .09$), and connectivity at 12 weeks in the anterior region ($P < .06$).

These results indicate that, at the 4-week interval, untreated rats have anterior blood vessels that are more ordered and are more connected in three dimensions in the posterior region than in rats treated with zoledronic acid. At 8 weeks, untreated rats had posterior blood vessels that appeared thinner and more ordered than zoledronic acid-treated rats, although not statistically significant. Finally, at 12 weeks, the anterior blood vessels of untreated rats were statistically significantly thinner and statistically significantly more connected in 3 dimensions than in treated rats. From these results, it can be inferred that untreated rats have blood vessels that are thinner, more connected, and more ordered while zoledronic acid treated rats have blood vessels that are thicker, less connected, and less ordered.

In regards to the thickness of blood vessels, one has to take into account that it is the radiopaque methyl methacrylate that is being measured under micro-CT and not necessarily the blood vessel itself. As a result, the thickness indicates the size of a blood vessel. Therefore, untreated rats have smaller blood vessels while zoledronic acid treated rats have larger blood vessels. This result suggests that zoledronic acid-treated rats possess less of the finer and smaller vasculature that is necessary to increase surface area and provide nutrition to a given region of bone.

Untreated rats also possess blood vessels that were more ordered as indicated by its degree of anisotropy. The degree of anisotropy measures the 3-dimensional symmetry along a directional axis. Therefore, zoledronic acid-treated rats have a lower degree of 3-dimensional symmetry when compared to untreated rats.

Furthermore, zoledronic acid-treated rats have a lower degree of anastomosis or branching of blood vessels when viewed 2-dimensionally and 3-dimensionally as indicated by its connectivity and Euler number, respectively. Similar to thickness, this result may convey that zoledronic acid-treated rats have decreased branching of blood vessels, which is also necessary to increase its surface area to provide more nutrients to a given region.

Bone angiography has been used as a measurement of bone vitality below the mucosal surface as suggested by other

investigators.[20] Local ischemia can cause bone necrosis in the limbs.[13] Ischemia has also been implicated in the pathophysiology osteoradionecrosis of the jaw.[21] Regarding BRONJ, the role of tissue ischemia in the pathogenesis of bone necrosis has been controversial.[22] Some studies clearly demonstrated a direct effect of bisphosphonates on angiogenesis in cancer.[23] On the other hand, there has been no report of BRONJ with systemic anti-angiogenic therapy. Therefore, some investigators dispute any vascular involvement in the etiology of BRONJ in humans.[22] Results of the current study proved the presence of vascular changes in alveolar bone associated with only two doses of zoledronate. However, it remains unclear if these changes are due a negative effect of zoledronate on angiogenesis or simply a consequence of bone tissue necrosis.

Conclusion

The results in this study demonstrate that zoledronic acid-treated rats undergo multiple vascular changes, including increased vessel thickness, decreased connectivity and branching, and a decrease in the ordered pattern of blood vessels observed in control rats. Further studies are under way to explore whether these vascular changes are a cause or an effect of bone necrosis.

Abbreviations

ANOVA: analysis of variance

BRONJ: bisphosphonate-related osteonecrosis of the jaw

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