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ORIGINAL ARTICLE

Aminobisphosphonate-treated ewes as a model of osteonecrosis of the jaw and of dental implant failure

Mario R. Davison[1] | **Leonardo Lyardet**[1] | **Mariana Preliasco**[1] | **Graciela Yaful**[2] | **Perla Torres**[2] | **Marina S. Bonanno**[3] | **Gretel G. Pellegrini**[3] | **Susana N. Zeni**[3]

[1]School of Dentistry, Rio Negro National University, Río Negro, Argentina

[2]Veterinary Sciences, Rio Negro National University, Río Negro, Argentina

[3]Metabolic Bone Diseases Laboratory, Immunology, Genetic and Metabolism Institute (INIGEM), School of Pharmacy and Biochemistry, Clinical Hospital "José de San Martín", National Council for Scientific and Technologic Research (CONICET)/Buenos Aires University (UBA), Buenos Aires, Argentina

Correspondence
Susana Noemi Zeni, Researcher of CONICET, Av. Córdoba 2351 8vo Piso (1120), Buenos Aires, Argentina.
Email: snzeni@hotmail.com

Abstract

Background: Bisphosphonate (BP)-related osteonecrosis of the jaw (BRONJ) and dental implant failure are two negative side effects of chronic aminoBP treatment.

Methods: Eleven ovariectomized (OVX) ewes and four ewes subjected to sham surgery (SHAM) were treated as follows: OVX ($n = 5$): OVX plus saline solution; zoledronic acid-treated group (ZOL) ($n = 6$): OVX plus ZOL; SHAM ($n = 4$): SHAM plus saline solution. Extraction of the first upper molar was performed at 1 year, dental implant placement at 2 years, and sacrifice at 28 months.

Results: Implants remained in place in SHAM and OVX ewes but were lost in all ZOL ewes. ZOL sheep (2/6) showed inflammation and necrotic bone at mandibular region. No differences in serum calcium (Ca), inorganic phosphate (Pi) or 25-hydroxyvitamin D were observed, whereas bone alkaline phosphatase levels decreased in the three studied groups ($P < 0.05$). The significantly lowest levels of carboxy-terminal telopeptide of type I collagen were observed in ZOL ($P < 0.05$), and showed no differences between SHAM and OVX. OVX showed the lowest and ZOL the highest Ca and Pi contents in femur and maxilla ($P < 0.05$). Bone volume (BV/TV%) and iliac crest were similar at baseline and at month 4. At the end of the study, BV/TV%, proximal femur and hemi-mandible bone mineral content and bone mineral density, and trabeculae number were similar in SHAM and ZOL, and lower in OVX ($P < 0.05$).

Conclusion: All ZOL-treated ewes on a schedule similar to that used in cancer patients showed extensive suppression of bone remodeling and implant failure. Some of the ZOL ewes developed BRONJ.

KEYWORDS

bisphosphonate-associated osteonecrosis of the jaw, dental implants, metabolic bone diseases, osteoporosis, zoledronic acid

1 | INTRODUCTION

Bisphosphonates (BPs) are widely used as the first treatment of choice in skeletal disorders, which are characterized by increased resorption.[1] Depending on their affinity for bone, BPs are primarily incorporated into sites of active bone remodeling suppressing osteoclast activity and survival.[1] The mandible, especially the alveolar bone region, has a high

basal turnover rate because of the masticatory action of the teeth.[2] Therefore, it may be more susceptible to accumulating BPs than other bones.[3]

The pharmacological potency of BPs differs based on structural alterations of the so called R-2 side chain, which determines their efficiency as bone resorption inhibitors.[1] Zoledronic acid (ZOL) is the most potent BP to date. ZOL has the greatest affinity for osseous matrix, and once incorporated into bone, it can be retained in the skeleton longer than 10 years.[4]

Besides their effects on bone cells, BPs exert some non-skeletal effects, particularly on immune[5] and vascular cells.[6] Antiresorptive and antiangiogenic effects of BPs have been considered main risk factors implicated in the development of two negative side effects of chronic aminoBP treatment, and which are of major concern to dentists: BPs-related osteonecrosis of the jaw (BRONJ)[6] and dental implant failure.[7]

Most cases of BRONJ were initially reported in patients receiving high doses of intravenous BPs for treatment/prevention of cancer-related malignancies.[8] However, an increasing number of BRONJ has been documented in patients receiving the drug for post-menopausal osteoporosis treatment.[9,10]

The cumulative dose and potency of BPs appear to be the most important risk factors involved in early dental implant failure or the subsequent loss of functionality of integrated implants under osteoporotic conditions.[11] Nevertheless, published studies are controversial. Although some studies suggest that dental implants can osseointegrate and remain functionally stable in BP-treated patients, others suggest that long-term BP-therapy could be a potential risk of implant failure.[11,12]

The exact role of BPs in the pathophysiology of both oral conditions has not been fully elucidated. Studies on the etiology of human BRONJ and the rate of BP-associated dental implant failure are complex given numerous confounding factors, which are usually irrelevant in animal models.[13] Experimental models provide more uniform experimental material. Although some BRONJ experimental models have been reported, most also involved exposure to other confounding variables, such as glucocorticoid administration.[14] Such variables may be additional risk factors for the development of both negative side effects associated with BP treatment.[14–16]

Sheep are large, docile animals; they are easy to handle and house, and are relatively inexpensive compared to other large animals. Sheep experimental models are well accepted for bone research because sheep are quite similar to humans in weight, sheer size, and skeletal biomechanical characteristics.[17] Moreover, their large size allows for the collection of substantial blood and urine samples, and multiple iliac crest biopsies.[18] Several other factors render sheep useful for oral studies.[19] They have molar and premolar teeth with a periodontium similar to humans, they experience age-related skeletal problems, including tooth loss, and they develop periodontitis with similar oral pathogenic bacteria to those in the human oral environment.[19,20] In addition, sheep jaws are large enough to permit major dental surgical procedures, such as dental implantation.[21]

The experimental model presented here involved inducing the three main risk factors of BRONJ and dental implant failure: bone loss by estrogen withdrawal, long-term intravenous administration of high-dose BPs, and dental procedures. We hypothesized that in our experimental model using ZOL as monotherapy, all oral changes observed throughout the study would be exclusively because of the effect of BP treatment. Based on the above, the objective of the present experimental study was to explore the occurrence of BRONJ, and to evaluate the effect of BP accumulation on dental implant success in ovariectomized (OVX) ewes.

2 | MATERIALS AND METHODS

2.1 | Animals

Fifteen healthy virgin adult *Corriedale* ewes, aged 3 to 4 years, 35 to 40 kg body weight (BW), and normal dentition were obtained from the Medium Valley region of Rio Negro (Argentina).

The animals were housed, treated, and then euthanized by veterinarians authorized by the "*National Service for Health and Agro-Food Quality*" (SENASA: "Servicio Nacional de Sanidad y Calidad Agroalimentaria") to handle animals.

All procedures were performed in compliance with the Clinical Hospital "José de San Martín," Buenos Aires University ethics guidelines. The animals were housed at the experimental farm of Rio Negro National University, and were fed a daily ration of standard dry sheep feed with hay, wheatgrass, and grasses to meet nutritional recommendations. Water was supplied *ad libitum* throughout the experiment.

2.2 | Drugs

Drug administration schedule and dose were in keeping with a published regimen for treating myeloma patients.[22] Fasting ewes were administered 4 mg ZOL/month* (equivalent to 66 μg/kg for a BW of 60 kg) by jugular injection.

In order to rule out confounding factors and evaluate exclusively the effect of aminoBPs accumulation on the jaw, no other drugs known to suppress the immune system (such as glucocorticoids) or inhibit angiogenesis (such as bevacizumab) were administered.

*Zoledronic Acid Trihydrate, Code 300520, Bacht 109640, Gador SA, Buenos Aires, Argentina.

When necessary, 2% lidocaine hydrochloride* was used as supplementary local anesthesia for dental procedures.

General anesthesia was induced by intramuscular injection of ketamine and xylazine (100 and 5 mg/kg, respectively).† Antibiotics (ampicillin 12.5 mg/kg)‡ were administered subcutaneously postoperatively.

2.3 | Experimental design

Eleven animals were subjected to bilateral OVX, and the four remaining animals were subjected to sham surgery (SHAM) by a veterinarian surgeon. All ewes were subjected to left flank laparotomy to gain access to the reproductive tract. OVX involved removal of both ovaries, and the SHAM surgery involved locating the ovaries and ligating the oviducts to prevent accidental pregnancy during the study.

Surgery was performed under general anesthesia and under cardiac and respiratory monitoring following standard protocols for this type of procedure.[23] Two days post-surgery, OVX ewes were divided into two subsets and treated as follows:

1. - OVX (n = 5): OVX ewes receiving saline solution,
2. - ZOL (n = 6): OVX ewes treated with ZOL (4 mg/month), for 28 months to obtain a high cumulative dose of ZOL in the bone.
3. - SHAM (n = 4): SHAM ewes receiving saline solution and serving as controls.

Figure 1 shows the experimental design.

Fasting blood samples were taken from the jugular vein prior to ZOL infusion once a month for 6 months. After this time, blood samples were obtained at 3-month intervals up to the end of the study. Thirty minutes after collecting the samples, the blood was centrifuged and the serum was stored at −20°C for biochemical analysis at the Metabolic Bone Diseases Laboratory, INIGEM, School of Pharmacy and Biochemistry, Clinical Hospital, CONICET-UBA.

After 12 months of treatment, the animals were anesthetized in order to obtain a bone biopsy of iliac crest and to perform the dental procedure. A cylinder of bone (diameter 7.5 mm) was obtained using a commercial needle§ at a site located 1.5 cm from the edge of the left iliac crest and 2.5 cm from the lateral edge of the pelvic brim. The 1rst right upper molar was extracted using dental levers; the extraction wound was closed with absorbable suture material.¶

After 2 years of treatment, all ewes underwent dental implantation using a threaded titanium implants (diameter: 3.75 mm and length: 8.5 mm).# Inadequate interdental space did not allow proper implant placement in the extraction site; therefore, a bone defect (1.6 × 2 mm) was created close to the extraction site using a fissure bur.‖ The extracted bone material was weighed** and digested in a glass tube containing a mixture of HCl-HNO3 (1:1) for calcium (Ca) and inorganic phosphate (Pi) determination.

The animals were euthanized at T = 2.4 years. The left hemi-maxillae, hemi-mandibles, and femurs were excised and cleaned of soft tissue. Immediately after excision, femurs were assessed by densitometry,†† whereas hemi-mandibles were analyzed using both densitometry†† and computed tomography.‡‡ Following, the removed bones were washed with saline and immersed in ethanol 60% (v/v) for 72 hours. The alcohol was replaced at 24 hours intervals. The samples were then dried in an oven at 100°C for 24 hours and weighed.** Dried bones were ashed at 600°C overnight, cooled in a desiccator, weighed and stored for Ca and Pi determination.

At necropsy, iliac crests and right hemi-maxilla were removed. A 2 cm^2 sample of iliac crest and a 5 cm-thick sample sectioned at the implant centerline of the maxilla were immediately placed in formalin–PBS for histological analysis. Maxilla samples were radiographed‡‡ prior to formalin-PBS immersion.

2.4 | Methodology

2.4.1 | Clinical and anthropometric analysis

Animal behavior, macroscopic oral mucosal changes, presence of the implant, and BW were assessed weekly by the veterinarians in a blinded manner. Veterinarians also evaluated the degree of oral inflammation, classified as normal, medium, or severe, presence of ulcers, and presence of necrotic bone.

2.4.2 | Biochemical analysis

Serum calcium (sCa) was determined by atomic absorption spectrophotometry at 423 nm, using lanthanum chloride as interference suppressor. Serum phosphate (sPi) was determined by UV-visible colorimetric method, using a commercial kit.§§ Bone alkaline phosphatase (BAP) was determined

* Lidocaine hydrochloride 2%, Denver Farma, Buenos Aires, Argentina.

† Injectable Ketamine 50 and Acedan, respectively. Holliday Scout SA, Buenos Aires, Argentina.

‡ Ampicillin Fada, Fada Pharma, Buenos Aires, Argentina.

§ OBN 16075 Bone Biopsy 16G x 7,5cm, Argon Medical Devices, Dallas, USA.

¶ Vicryl (polyglactin 910) Absorbable Suture Ethicon, J&J medical devices companies, New Jersey, USA.

Efedea, Megagen Implant Ltd, Seoul, Korea.

‖ Odontit Lote Nº: S27567, Buenos Aires, Argentina.

** Sartorius analytical balance, Sartorius group, Germany.

†† Densitometer Lunar DPX Alfa, Small Animal Software. Lunar Radiation Corp., General Electric Company, Connecticut, USA.

‡‡ Tomograph Gendex CB-500, Cone Beam, Georgia, USA.

§§ Phosphorus, BioSystems Laboratories, Barcelona, Spain.

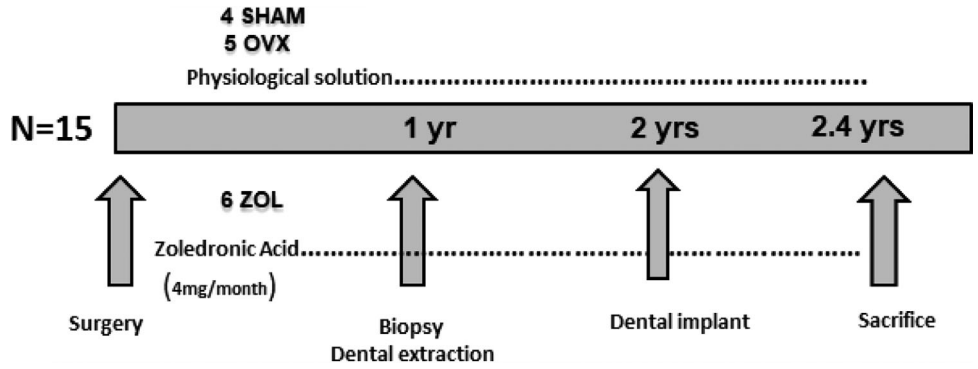

FIGURE 1 Experimental design

by a colorimetric method, using a commercial kit* to determine the activity of total BAP after bone isoform precipitation with wheat germ lectin. Cross-reactivity with liver isoform was <5%. Carboxy-terminal telopeptide of type I collagen (CTX) was measured by enzyme-linked immune-absorbent assay (ELISA).† Intra-assay coefficient of variation (CV) was 6%. Twenty-five hydroxyvitamin D (25OHD) was determined by competitive protein binding assay based on the use of radiolabeled ligands (ELISA).‡ Intra-assay CV was 8.6% to 12.5%, and inter-assay CV was 8.2% to 11.0%.[24]

2.4.3 | Bone studies

Excised mandibles were macroscopically evaluated, recorded, and photographed by the oral and maxillofacial surgeon.

Iliac crest and maxilla samples were fixed in formalin–PBS for 24 hours, decalcified in 10% EDTA for 45 days and embedded in paraffin. Bone sections (7 to 8 μm thick) were obtained and stained with hematoxylin-eosin for blinded microscopic evaluation. The sections were micro-photographed,§ and the digitalized images¶ were used to perform histological measurements. Bone volume (BV/TV%), defined as the percentage of cancellous bone within the total measured area, was determined.[25]

Ca and Pi content was determined in the material obtained from the bone defect created in the maxilla to place the implant (Ca and Pi content at month 24) and in a sample of the total bone ashes obtained from mandibles and femurs, at necropsy (Ca and Pi content at month 28).

Hemi-mandible and femur bone mineral density (BMD) and bone mineral content (BMC) were evaluated ex vivo by DXA using a specifically designed software for small animals.†† All bones were scanned using an identical scan procedure. Proximal femur was analyzed on a bone image on the screen, using a ROI for each segment. Software precision for total body BMD was assessed by measuring one piece of bone five times after repositioning between scans both on the same and on different days.[26] CVs were as follows: mandible and femur BMD = 0.8% and BMC = 3.0%; proximal femur BMD = 3.2%. All analyses were carried out in a blinded manner by the same technician, to minimize inter-observer variation.

2.5 | Statistical analysis

Data were expressed as mean ± standard deviation (SD). Normality and variances homogeneity were evaluated using Shapiro Wilk test and Levene's test, respectively. ANOVA was used to detect differences in percentages and means, respectively. Analyses were performed with SPSS;# statistical significance was set at a P-value < 0.05.

3 | RESULTS

3.1 | Clinical examination and anthropometric analysis

Survival was 100% in SHAM and OVX groups and 77% (4/6) in ZOL group. Cause of death was renal insufficiency and diarrhea, and both deaths occurred during the last week of the study. The veterinarians recorded the deaths within 12 hours post-mortem. For that reason, we decided to include these two ewes in bone analysis and to exclude them from biochemical analysis at month 28. SHAM and OVX ewes successfully maintained their implants (Figure 2 A, D and E), whereas 100% of ZOL ewes had lost their implants by the end of the study (Figure 2 B, C and F).

Cyanosis degree of oral mucosa increased in the following order: SHAM < OVX < ZOL. Wound healing of the extraction site was uneventful in all three groups. Neither control

* Optimized Alkaline Phosphatase, Wiener Laboratories, Santa Fe, Argentina.

† Serum Crosslaps, Nordic Bioscience Diagnostics A/S, Denmark.

‡ 25-Hydroxyvitamin D 1,25 I RIA kit, DiaSorin Inc, Vercelli, Italy.

§ AxiosKop, Carl 9, Carl Zeiss Microscopy, Germany.

¶ Image Pro Plus 4.5, Image Processing Software, Media Cybernetics, Rockville, USA.

IBM SPSS Statistics, Version 20, IBM Corp., Chicago, IL.

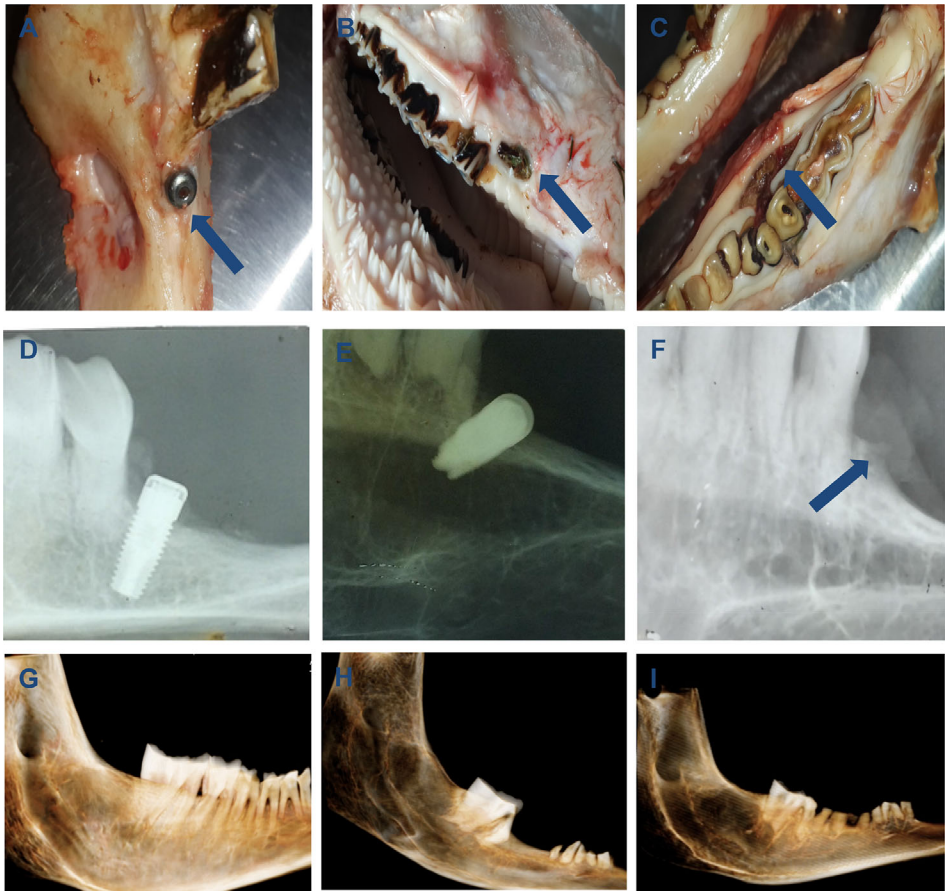

FIGURE 2 Mandible photographs, maxilla radiography and tomography of the three study groups at the end of the experimental period. The arrows in the oral cavity photographs show the successfully maintained mandible implant in SHAM and OVX groups (**A**) and the implant failure (**B**) and mandibular changes in ZOL treated ewes (**C**). These changes were characterized by severe inflammation with exposed and necrotic bone (similar to BRONJ) associated with *Actinomyces spp* infection. Radiograph of the upper right maxilla of SHAM (**D**) and OVX (**E**) groups shows no structural changes in the alveolar bone surrounding the implant. However, OVX ewes showed a reduced radiopacity area. ZOL (**F**) group evidenced implant loss and structural bone changes; the arrow shows bone sequestrum at the extraction site. Panoramic tomography of the lower left jaw of ewes showed similar findings to those of the radiographic evaluation in all 3 studied groups: SHAM (**G**), OVX (**H**), and ZOL (**I**)

nor OVX animals showed ulceration, exposed bone, or signs of inflammation at the extraction site or at other skeletal sites. However, 2/6 (33%) ZOL ewes exhibited inflammation and necrotic bone in the mandibular region (Figure 2 C). In addition, radiographic images corresponding to two ZOL ewes showed bone sequestra at the extraction site (Figure 2 F).

No significant differences in BW were observed among groups either at the beginning or at the end of the study. Maxilla and femur weight at the end of the study were lowest in OVX group ($P < 0.05$). No significant differences in femur weight were observed between SHAM and ZOL groups, although maxilla weight was significantly higher in ZOL ewes ($P < 0.05$) (Table 1).

3.2 | Biochemical analysis

Whereas serum determinations did not differ significantly among groups at the beginning, some parameters differed among groups at month 4 and at the end of the study (Table 2). In the three studied groups, sCa tended to decrease as compared to baseline values ($P = 0.062$), but did not differ significantly among groups. sPi levels increased significantly in OVX and ZOL groups at month 4 compared to baseline and were significantly higher in these groups than in the SHAM group ($P < 0.05$). At the end of the study, sPi levels of OVX and ZOL ewes were similar to the levels observed at the beginning of the study. No significant differences in sPi levels were observed among the three groups at the end of the study. In agreement with seasonal changes in radiation, all groups showed a slight decrease in 25OHD levels between baseline and month 4 (summer to autumn) ($P = 0.055$), and a significant increase between month 4 and the end of the study (spring to summer) ($P < 0.05$); no significant differences in 25OHD levels were observed among the three studied groups. BAP levels significantly decreased in all three groups throughout the study, and reached significantly lower values at the end of the study ($P < 0.05$), with no significant differences among groups (Table 2). CTX in OVX ewes was higher at month 4 as

TABLE 1 Body weight (BW), mandible and femur weight, perceptual content of calcium (Ca) and phosphate (Pi), and Ca/Pi ratio in maxilla and femur; femur and hemimandible bone mineral density (BMD) and bone mineral content (BMC) (data were expressed as mean ± SD)

Determination	SHAM	OVX	ZOL
Baseline body weight (Kg)	33.00 ± 2.00	34.00 ± 4.00	35.00 ± 2.00
Final body weight (Kg)	30.00 ± 6.00	31.00 ± 5.00	33.00 ± 4.00
Maxilla weight (g)	178.40 ± 4.00	142.80 ± 1.20†	185.10 ± 9.70*,†
Femur weight (g)	114.00 ± 5.50	106.40 ± 8.80†	119.60 ± 3.50*
Maxilla Ca% (g/100 g) at month 24	17.10 ± 1.20	15.60 ± 1.70†	16.80 ± 0.00*
Maxilla Pi% (g/100 g) at month 24	14.70 ± 0.60	13.20 ± 0.20†	13.80 ± 0.80*
Ca/Pi (in maxilla) at month 24	1.18 ± 0.04	1.14 ± 0.13	1.15 ± 0.06
Mandible Ca% (g/100 g) at month 28	17.30 ± 1.60	14.50 ± 0.70†	18.30 ± 5.50*,†
Mandible Pi% (g/100 g) at month 28	15.2 ± 2.4	11.7 ± 3.2†	14.1 ± 1.1*
Ca/Pi (in mandible) at month 28	1.49 ± 0.28	1.41 ± 0.34	1.65 ± 0.35
Femur Ca% (g/100 g) at month 28	14.00 ± 1.40	10.50 ± 3.10†	15.40 ± 2.6*
Femur Pi% (g/100 g) at month 28	9.20 ± 0.60	8.40 ± 0.80†	10.20 ± 0.50*
Ca/Pi (in femur) at month 28	1.28 ± 0.08	1.26 ± 0.16	1.22 ± 0.09
Total femur BMD	0.480 ± 0.026	0.370 ± 0.035†	0.457 ± 0.019*
Total femur BMC	36.990 ± 3.280	27.180 ± 2.600†	34.000 ± 2.010*
Proximal femur BMD	0.733 ± 0.052	0.587 ± 0.047†	0.830 ± 0.052*
Proximal femur BMC	10.320 ± 0.740	8.250 ± 0.620†	11.810 ± 0.970*
Hemimandible BMD	0.210 ± 0.020	0.166 ± 0.040†	0.255 ± 0.026*,†
Hemimandible BMC	32.110 ± 9.140	24.000 ± 6.080†	42.270 ± 3.200*,†

P-value determined by one-way ANOVA followed by Bonferroni post hoc test.
*$P < 0.05$ was considered significantly different compared to the OVX group levels.
†$P < 0.05$ was considered significantly different compared to the SHAM group levels.

compared to baseline and to the other studied groups ($P < 0.05$), and was significantly higher as compared to ZOL ($P < 0.05$) at the end of the study. CTX decreased progressively in ZOL, and was significantly lower at month 4 and month 28 compared to baseline ($P < 0.05$), but did not differ significantly from month 4 to the end of the study. At the end of the study, CTX was lowest in ZOL ewes ($P < 0.05$) and did not differ significantly between SHAM and OVX ewes (Figure 3).

3.3 | Bone analyses

At month 24, Ca and Pi percentage (g/100 g of tissue) in the material obtained from the bone defect created in the maxilla for implant placement was significantly lower in OVX than in SHAM and ZOL ewes ($P < 0.05$) (Table 1).

At the end of the study, Ca and Pi content (g/100 g of bone) of femurs and maxillae was significantly lower in the OVX ($P < 0.05$) than in the other two groups, whereas the highest values were observed in ZOL ewes. Regarding the ZOL and SHAM groups, only maxilla Ca content was significantly higher in ZOL than in control ewes ($P < 0.05$); the difference in femur Ca and Pi content, and in mandible, Pi content almost reached statistical significance (*P*-value between 0.055 and 0.063). As a result, the Ca/Pi ratio in the maxilla and femur did not differ significantly among groups at month 24 or at month 28 (Table 1).

Total femur and proximal femur BMC and BMD were significantly lower in OVX as compared to SHAM and ZOL ewes ($P < 0.05$). Whereas total femur BMC and BMD did not differ significantly between the latter groups, proximal femur BMC and BMD tended to be higher in ZOL than in SHAM ewes ($P = 0.067$). Maxillary BMC and BMD were lowest in OVX ewes ($P < 0.05$), and were significantly higher in ZOL than in SHAM ewes ($P < 0.05$) (Table 1).

At the end of the study, all ZOL ewes had lost their implant (Figure 2 B and F) whereas the latter remained in place in SHAM (Figure 2 A and D) and OVX ewes (Figure 2 A and E).

No differences in iliac crest BV/TV% were observed among groups at month 12 (48 ± 6, 47 ± 10, and 49 ± 5 for SHAM, OVX, and ZOL groups, respectively). At the end of the study, iliac crest trabecular number was significantly lower in OVX (19.69 ± 3) than in SHAM (25.96 ± 5) and ZOL (27.07 ± 6) ewes ($P < 0.05$) (Figure 4 A, B and C).

4 | DISCUSSION

BP-treatment for osteoporosis and other bone pathologies is fairly frequent at present.[1] Nevertheless, two adverse effects have been reported in patients receiving BP therapy for

TABLE 2 Biochemical determination at baseline, 4 months post-surgery, and at the end of the study (data were expressed as mean ± SD)

Groups	Baseline	4 Months	28 Months
Serum calcium (mg/dL)			
SHAM	9.7 ± 0.1	9.5 ± 0.2	9.2 ± 0.3
OVX	9.1 ± 0.3	9.4 ± 0.2	8.9 ± 0.4
ZOL	9.2 ± 0.4	9.1 ± 0.3	8.9 ± 0.3
Serum inorganic phosphate (mg/dL)			
SHAM	4.1 ± 0.4	3.9 ± 0.2	4.3 ± 0.4
OVX	4.6 ± 0.3	5.6 ± 0.3*,†,‡	4.0 ± 0.4§
ZOL	4.7 ± 0.5	4.9 ± 0.2*,†	4.0 ± 0.2§
Bone alkaline phosphatase (UI/L)			
SHAM	66.0 ± 4.2	64.7 ± 9.2	51.5 ± 3.1*,§
OVX	65.0 ± 4.7	69.3 ± 5.1	58.3 ± 0.6*,§
ZOL	62.3 ± 3.6	58.4 ± 2.9	50.8 ± 3.4*,§
25-Hydroxyvitamin D (ng/mL)			
SHAM	20.5 ± 2.7	18.3 ± 3.1	27.4 ± 6.7*,§
OVX	22.6 ± 6.4	17.6 ± 1.8	29.5 ± 6.2*,§
ZOL	24.6 ± 6.9	18.5 ± 1.1	29.1 ± 2.8*,§

P-value determined by one-way ANOVA followed by Bonferroni post hoc test.
$^{*}P < 0.05$ was considered significantly different compared to baseline levels.
$^{\dagger}P < 0.05$ was considered significantly different compared to the SHAM group levels.
$^{\ddagger}P < 0.05$ was considered significantly different compared to the ZOL group levels.
$^{\S}P < 0.05$ was considered significantly different compared to the month 4 levels.

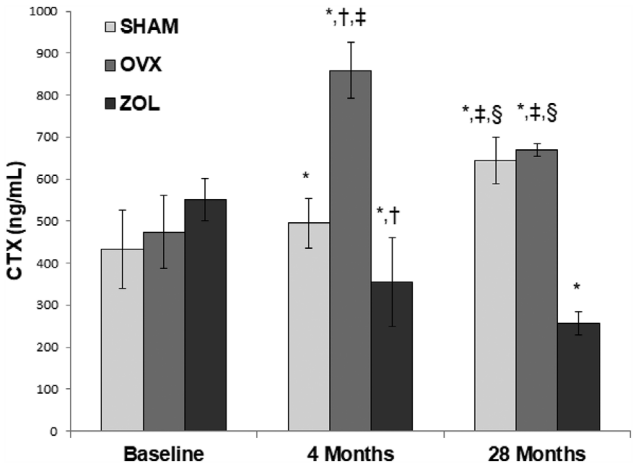

FIGURE 3 Carboxy-terminal telopeptide of collagen type I (CTX) levels at baseline, 4 months post-surgery, and at the end of the study. CTX is one of the most specific and sensitive biochemical resorption marker to evaluate changes in osteoclastic activity. CTX levels of ZOL group decreased significantly from month 4 to month 28. CTX levels in ZOL ewes were significantly lower compared with the others groups at the end of the study. Bars represent mean and error bars show SEM. *P*-value determined by one-way ANOVA followed by Bonferroni post hoc test. Significant differences between groups are shown as follows: $^{*}P < 0.05$ was considered significantly different compared to baseline levels, $^{\dagger}P < 0.05$ was considered significantly different compared to SHAM levels, $^{\ddagger}P < 0.05$ was considered significantly different compared to ZOL levels, $^{\S}P < 0.05$ was considered significantly different compared to levels at month 4

>3 years: BRONJ[8] and dental implant failure.[7] The exact pathophysiology of BRONJ has not been fully elucidated, and appears to be multifactorial.[27] The effect of BPs on bone turnover, angiogenesis, and immunity is well documented. In addition, alveolar bone is separated from the oral microflora by a thin layer of mucosal tissue. Therefore, tooth extraction, deep caries, trauma, and periodontal disease could allow pathogens to reach the bone surface and cause infection, which is commonly observed in BRONJ.[28] Moreover, clinical evidence suggests that long-term BP-treatment could impair osseointegration causing dental implant failure or loss of function of an osseointegrated implant.[7] Given that BPs significantly reduce bone turnover, it is not surprising that osseointegration may be compromised, or that subsequent loss of integration may occur after successful implant placement.[12] It should be taken into account that osseointegration has a phase of bone remodeling, involving renewal of bone and contact between the bone and the implant surface, both of which are affected by BP-treatment.

Animal models are important to understand several aspects of the pathological mechanism of both aforementioned side effects, as well as to establish prevention and management strategies. Most basic investigations in this field were conducted in rats.[29] Even though rodents are less expensive and easier to house, they also pose several disadvantages. Not only are rats too small to allow several oral surgical procedures, but also adult rats develop cementosis.[30,31] Moreover, rat cortical bone lacks Haversian systems and does not undergo intracortical turnover, which seems to play an important role in the onset of ONJ.[29] Ewes are large animals, and are a suitable experimental model for many reasons. They are genetically closer to humans than rodents; their metabolic rate is closer to that of man (0.22 vs. 0.21); they undergo Haversian bone remodeling, and like women, they have menarche and regular, frequent ovulatory cycles.[32] Bone metabolism in OVX sheep resembles that of women during early postmenopause,[33] and osteoblast and osteoclast products (e.g., osteocalcin, CTX)[18] are clearly defined. Regarding the oral cavity, the systemic bone loss following ovariectomy is accompanied by oral bone loss. Also, in addition to developing periodontitis, sheep are large enough to undergo dental interventions.[17–19,33]

Many models of BRONJ and implant failure have been evaluated in small (rodents)[14] and large (dogs, minipigs, and sheep) animals.[15,29,34] These studies showed the presence of necrotic regions in the oral cavity after BP treatment. To our knowledge, all studies but one[34] co-administered other drugs that might also have contributed to increasing the incidence of BRONJ and/or implant failure. Our experimental report evaluated specific, ZOL-associated oral changes using a similar regimen to that used for cancer patients.[22]

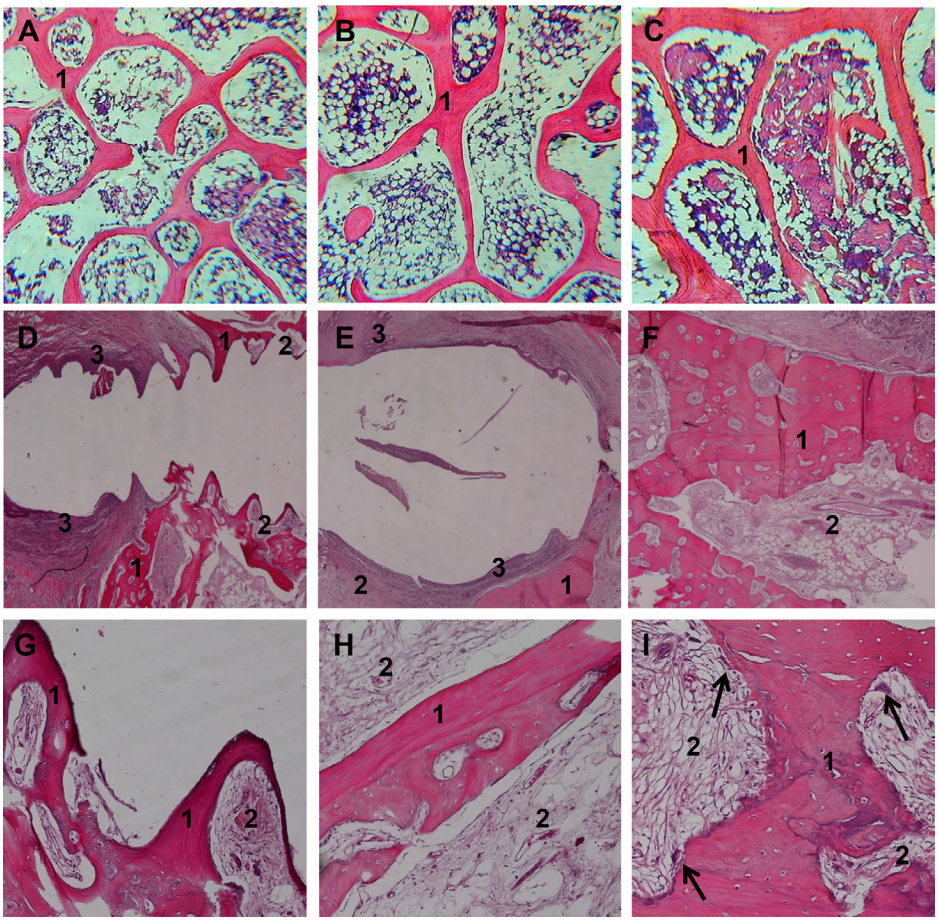

FIGURE 4 Histomorphological sections of iliac crest and mandible areas in SHAM (**A, D and G**), OVX (**B, E and H**) and ZOL (**C, F and I**) groups at the end of the study. IIiac crest photomicrographs were stained with Hematoxylin–Eosin, magnification 40×. T**rabeculae** bone tissues were stained in red (1). The lowest iliac crest trabecular number and thickness were observed in OVX group (**B**) whereas ZOL ewes (**C**) presented a higher trabecular thickness than SHAM ewes (**A**). Mandible photomicrographs were stained with hematoxylin–eosin, magnification 20× (**D, E and F**) and 100× (**G, H and I**). SHAM ewes (**D and G**) showed a well-defined space with indentations (similar to implant threads); (1) trabecular bone tissue, (2) connective tissue, and (3) epithelialized areas were observed. OVX ewes (**E and H**) showed the implant area limited by (3) stratified epithelium; (1) well-formed trabecular bone tissue and (2) connective tissue were observed. ZOL group (**F and I**) did not show implant area; (1) trabecular bone tissue of pagetoid appearance; (2) connective tissue were observed. The arrows show giant apoptotic osteoclasts

As mentioned above, mandible and alveolar bone are sites of high BP uptake and accumulation,[14] which results in a high concentration of the drug that is toxic to oral epithelium. In the present study, the changes observed in the oral cavity throughout the experiment suggest some degree of toxicity. Indeed, ZOL-treated ewes exhibited a delay in epithelialization of the extraction socket, as compared to the remaining groups, during the first month post-tooth extraction. However, epithelialization of the extraction-site was similar in all groups at the end of the study, likely because of the length of the experiment. In addition, gingival epithelium showed a great degree of inflammation as compared to the other two groups throughout the entire study, and more importantly, two of six ZOL-treated ewes showed signs of exposed oral bone similar to BRONJ, with no signs of necrosis at other skeletal sites. The finding that some animals developed BRONJ whereas others did not could be because of individual susceptibility or resistance to developing this pathology, or to genetic differences in bone homeostasis among ewes, as occurs in humans. ONJ may occur in either maxilla or mandible, but it is often seen in the mandible. In the present report, though dental extraction and implant placement were performed in the maxilla, BRONJ developed in the mandible. Of note, the mandible is directly exposed to the interior of oral cavity. Moreover, it is covered only with a thin oral mucosa, and it is, therefore, susceptible to injury from inflammation because of dental infection or even mastication. Hence, these invasive dental procedures may readily have spread the dental infection through the thin epithelium, which may have played a crucial role in the development of BRONJ in the jaw. In addition, implant failure was observed in all ZOL-treated ewes, whereas all implants remained in place in control and OVX-untreated animals.

It is known that turnover suppression increases the mean tissue age because regions of older bone are remodeled less frequently affecting the biomechanical properties of bone. Both the pathological conditions studied here are associated with excessive and prolonged suppression of bone turnover caused by chronic BP treatment. This effect was confirmed in the present report by biochemical, histological, and densitometric studies. The present results showed substantially lower CTX levels in BP-treated ewes. Of note, CTX is considered one of the most specific and sensitive biochemical bone markers to evaluate changes in osteoclastic bone resorption.[35] Histological studies of the iliac crest showed that trabecular number and thickness were far higher in ZOL-treated ewes than in untreated-OVX and control animals. Lastly, both maxillary and femur bone mass values were several time higher in BP-treated ewes.

Renal toxicity and diarrhea have been described as side effects of treatment with ZOL. Renal toxicity has been identified as a complication of treatment with 4 mg of ZOL, and although the causes of renal deterioration are multifactorial, acute tubular necrosis has been described as a potential mechanism.[36] Given that ZOL is predominantly excreted unchanged via the kidneys, cellular effects similar to those documented in the osteoclast may account for the potential mechanisms of the nephropathies observed after prolonged infusion times of ZOL.[37] This known ZOL-associated toxic tubular injury cannot be ruled out as one of the causes of death by renal failure in one of the ewes in the present study.

One of the cardiovascular side effects of high-dose ZOL treatment is the increased risk of atrial fibrillation events. The potential mechanism has been attributed to altered intracellular ion concentration and pro-inflammatory, pro-fibrotic, and anti-angiogenic properties of BPs.[38] In addition, according to the World Health Organization (WHO) causality scale, ZOL is a probable cause of acute severe diarrhea and subsequent hypokalemia.[39] Such electrolyte imbalance is linked to atrial fibrillation, which increases the risk of heart failure, cardiac mortality, and total mortality. We suggest that the diarrhea observed in the second ewe may have led to the aforementioned adverse cardiovascular effect and death.

5 | CONCLUSION

Under the experimental conditions used here, changes in bone metabolism observed in ZOL-treated ewes were similar to findings reported in the literature in patients receiving BPs. Indeed, all ZOL ewes showed extensively suppressed bone remodeling and dental implant failure, and some developed BRONJ. Although further studies are necessary, the results of the present report show the usefulness of this animal model to evaluate BP-associated oral implant problems and the different factors involved in the pathogenesis of BRONJ.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTION

Dent. Mario Davison: doctoral fellow; everything related to the dental procedures, analysis of the results, and participation in the article discussion; Dent. Leonardo Lyardet: data entry and statistical analysis, and bone excision; Dent. Mariana Preliasco: clinical observation of oral mucosa and dental implantation; Vet. Graciela Yaful: animal surgery and euthanasia; Vet. Perla Torres: animal control, drug administration, and euthanasia; Biotech Marina S Bonanno: Biochemical and densitometric analysis; PhD. Gretel G Pellegrini: bone imaging and histological measurements; Prof. PhD. Susana N Zeni: Design, organization and check of the study, writing of the article including analysis of the results and discussion.

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