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ORIGINAL ARTICLE

# Effects of local application of alendronate on early healing of extraction socket in dogs

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## Abstract

**Objective** The aim of the present study was to assess the effects of alendronate (ALN) on bone remodeling following tooth extraction in a dog model.

**Material and methods** For the study, fifteen male Beagles dogs of approximately 12 months of age were used. Mesial roots of four mandibular premolars were endodontically treated, and the distal roots were removed. ALN concentrations of 0.5, 1, and 2 mg/mL were topically applied for 15 min, while a sterile saline was used as a negative control. After the healing period of 1, 2, and 8 weeks, the samples were analyzed by micro-CT and histology.

**Results** Treatment with ALN increased vertical distance between the lingual and the buccal crestal bones. While the ALN-treated sockets had preserved more lingual bone areas, control sockets showed better preservation of the buccal bone areas. ALN treatment resulted in more osteoid formation within the extraction sockets compared with the control. Higher bone volume was found in ALN groups than in the control at 2-week and 8-week healing periods, reaching the significant difference only for the extraction sockets pooled for the ALN treatment.

**Conclusions** Although ALN treatment could not prevent buccal bone resorption following tooth extraction in dogs, it proved beneficial for the preservation of the lingual bone and formation of new bone within the socket. There was no clear relation between the ALN dosages and the alterations within the extraction sockets.

**Clinical relevance** ALN affects bone remodeling of the extraction socket. The optimal concentration remains to be determined in future studies.

**Keywords** Dog model · Alendronate · Extraction socket · Bone remodeling

## Introduction

Tooth extraction triggers a cascade of biological events within the alveolus. The initial blood coagulum is gradually replaced with granulation tissue, which provides a provisional matrix for the woven bone formation [1, 2]. The healing process is typically accompanied by significant anatomical changes of the edentulous alveolar ridge [3, 4]. These changes represent a determining factor for the induction of the resorption process within the extraction socket, destroying the bundle bone, Sharpey's fibres, and root cementum [5]. As the buccal soft tissue starts to occupy the position of the former buccal bone plate, the space for bone regeneration is reduced leading to the buccolingual bone shrinkage. Bone substitute materials were shown to stabilize the buccal soft tissue from invagination during the healing process [6–8]. Although some beneficial effects in terms of horizontal ridge reduction were observed,

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none of the ridge preservation techniques could entirely prevent the resorption of the buccal bone [9, 10]. Moreover, no statistically significant differences were found between allografts, xenografts, and alloplastic materials compared with spontaneous healing in terms of new bone and connective tissue percentages within the extraction socket [11].

Resorption of damaged bone by osteoclasts is an essential step in the healing of tooth extraction sockets [12, 13]. Consequently, suppression of osteoclast activity by bisphosphonates (BPs) is likely to cause retention of dead bone within the persisting extraction sockets [14]. Repeated subcutaneous application of BP delayed removal of interdental alveolar bone and reduced vertical bone loss after tooth extraction in rats [15–17]. Furthermore, oral application of BP after wisdom tooth extraction in humans reduced bone resorption [18]. Tooth extraction in cancer patients treated with BP, however, significantly contributed to the development of osteonecrosis of the jaw [19]. Antiangiogenic properties of BP and suppression of bone turnover were shown to impede the integrity of oral mucosa [20] and increase the risk of infection [21, 22]. A positive correlation between the intravenous way of application, increased dosage, and duration of the BP treatment and the incidence of the osteonecrosis of the jaw was established [23]. Local treatment may be thus advantageous compared with a systemic application, as the amount of BP at the surgical site is easier to control (at least initially) [24, 25]. A dose-dependent effect on bone formation has been previously demonstrated for the local application of alendronate (ALN) combined with autogenous bone [26, 27], β-tricalcium phosphate [28], or implant placement [29, 30]. To the best of our knowledge, the effect of BP on the healing of extraction socket without using a carrier system has not been studied. To this aim, the healing of the extraction socket should be investigated using delineated, well-characterized, clinically relevant animal models [1, 2, 31].

The purpose of this study was to assess the effects of local application of ALN on the healing of the mandibular premolar extraction sockets in dogs. We hypothesized that ALN would have a dose-dependent effect on the vertical and horizontal socket dimensions.

## Material and methods

### Animals and surgery

The study was designed as a randomized, controlled experimental study. Fifteen male Beagle dogs of approximately 12 months of age were used. The dogs received standard feed and water ad libitum. The study protocol followed the guidelines of the European Union Council Directive on February 1, 2013 (R.D.53/2013), approved by the Ethics Committee of the Rof Codina Foundation, Lugo, Spain (AELU001/17/INVMED(02)OUTROS(04)/AGC/01).

The animals were preanesthetized with medetomidine (20 mg/kg/i.m., Esteve, Barcelona, Spain) and morphine (0.4 mg/kg/i.m., Morfina Braun 2%®, B. Braun Medical, Barcelona, Spain). The anesthesia was initiated by propofol (2 mg/kg/i.v., Propovet®, Abbott Laboratories, Kent, UK) and maintained by inhalation of an $O_2$ and 2.5–4% isoflurane mixture (Isobavet®, Schering-Plough, Madrid, Spain). Lidocaine with adrenalin (Anesvet®, Ovejero, Leon, Spain) was used for local anesthesia to reduce peri-operative pain and bleeding. Sulcular incisions were made in the premolar regions of the mandible, and the full thickness flaps were elevated (Fig. 1a). The canal of the mesial roots of the four mandibular premolars ($_3P_3$, $_4P_4$) was reamed and filled with gutta-percha. The premolars were then hemisected and the distal roots removed using elevators (Fig. 1b).

A solution of ALN was prepared by dissolving sodium-ALN trihydrate powder (Calbiochem, Darmstadt, Germany) in saline (150 mM NaCl) for 1 h under sonication and sterilization using 0.2 μm/Millipore filter. Three concentrations of ALN of 0.5 (ALN_0.5), 1 (ALN_1), and 2 mg/mL (ALN_2) were applied on the fresh extraction socket of the distal roots for 15 min (Fig. 1c) and then rinsed with sterile saline. The fourth extraction socket was rinsed with sterile saline for 15 min and served as a negative control. The buccal and lingual flaps at four sites were replaced to close the entrance of the sockets and held together with interrupted sutures (Fig. 1d). After the surgical intervention, atipamezole (50 μg/kg/i.m., Esteve, Barcelona, Spain) was administered to revert the effects of the medetomidine. Four treatment modalities per each animal were allocated according to a systematic random protocol (www.randomization.com). Dogs were divided into 3 groups and sacrificed 1, 2, and 8 weeks after interventions.

The animals were controlled daily for health status using the standardized score sheets. Postoperative pain was controlled with morphine (0.3 mg/kg/i.m./6 h, Morfina Braun 2%®, B. Braun Medical, Barcelona, Spain) for 24 h and meloxicam (0.1 mg/kg/s.i.d/p.o., Metacam®, Boehringer Ingelheim, Barcelona, Spain) during the next 3 days. Antibiotics (amoxicillin 22 mg/kg/s.i.d./s.c., Amoxil retard®, Syva, Leon, Spain) were administrated for 7 days. The dogs were fed with a soft-pellet diet for 1 week until removal of the sutures. During the first two postoperative weeks, the oral mucosa and the teeth were disinfected three times a week by using gauzes soaked in 0.12% chlorhexidine solution (Perio-Aid Tratamiento®, Dentaid, Barcelona, Spain). Subsequently, a toothbrush and a 0.2% chlorhexidine gel (Chlorhexidine Bioadhesive Gel, Lacer, Barcelona, Spain) were used for plaque control.

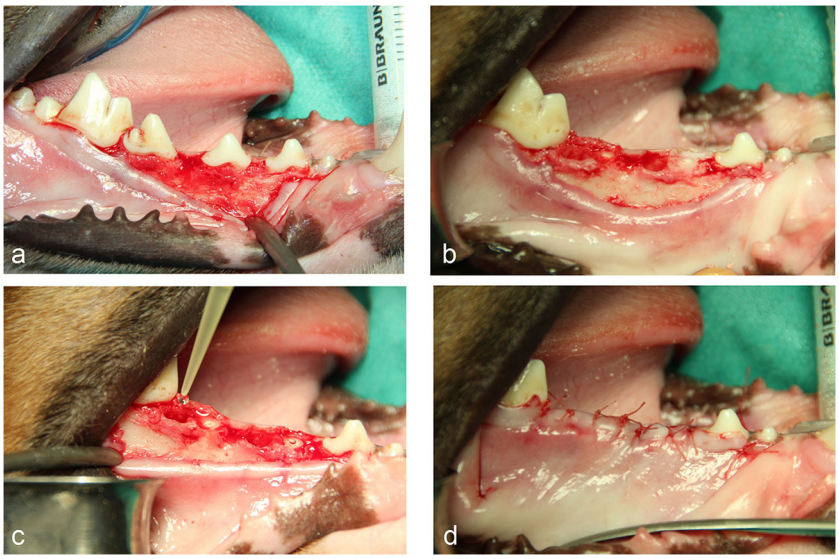



**Fig. 1** Intraoperative view of the treated area. A buccal mucoperiosteal flap is elevated following the mid-crestal incision (**a**). Two premolars on each side of the maxilla are hemisected and the distal roots extracted (**b**). The extraction sockets are treated with three concentrations of alendronate or sterile saline (**c**). Following 15 min, all sockets are rinsed and the wound closed with interruptive sutures (**d**)

## Sacrifice and retrieval of specimens

Five dogs were sacrificed after healing periods of 1, 2, and 8 weeks. Dogs were sedated with medetomidine (30 μg/kg/i.m., Esteve, Barcelona, Spain) and sacrificed with an overdose of sodium pentobarbital (60 mg/kg/i.v., Dolethal, Vetoquinol, France). Clinical evaluation was performed, and the mandibles were retrieved by sharp dissection. The mandibles were block-sectioned using a diamond saw (Exact® Apparatebeau; Norderstedt, Hamburg, Germany). Sample size calculation was not performed, as this was an exploratory study.

## Micro-CT analysis

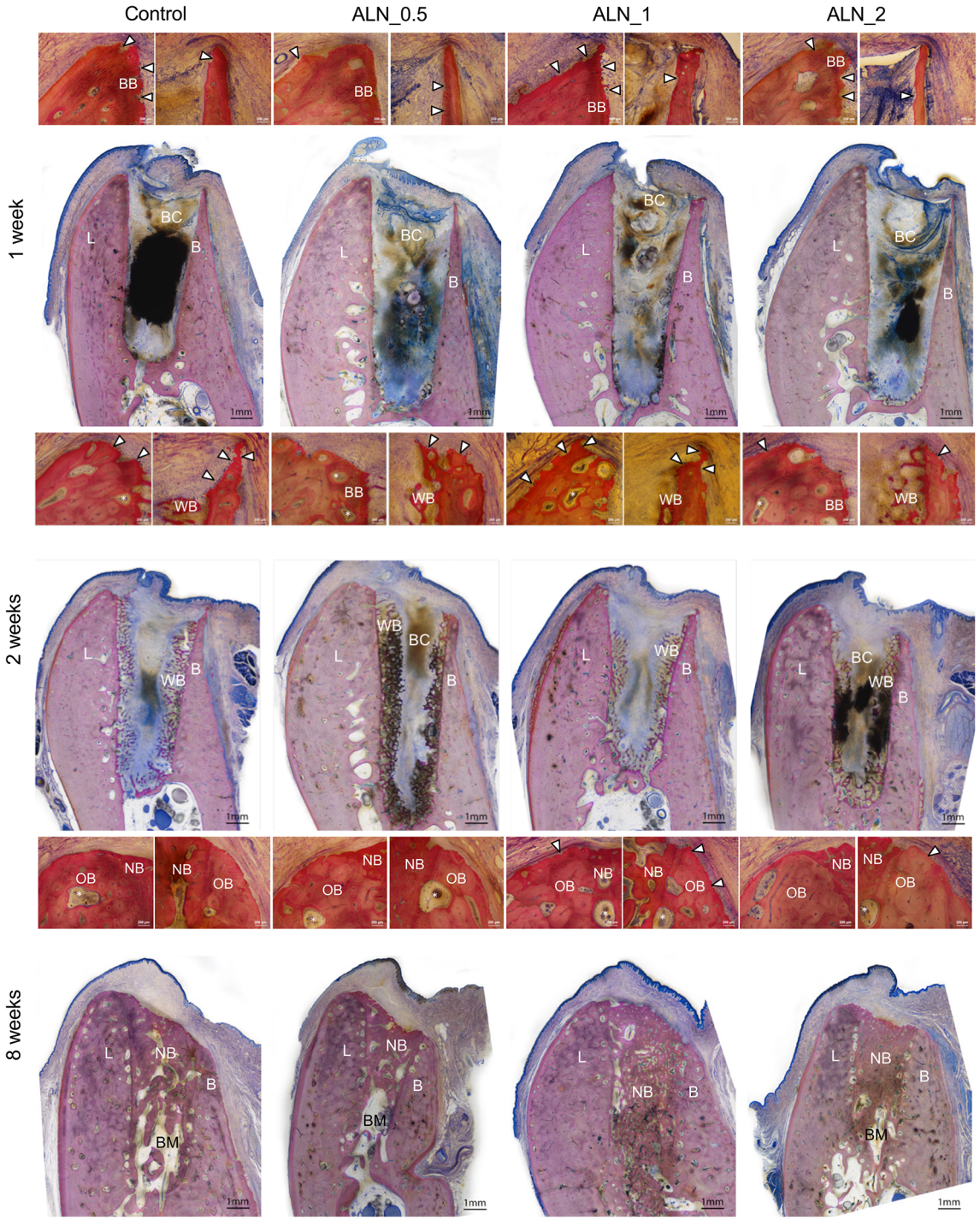
All premolar sites were radiographed in two projections at 25 kVP for 10 s. The scans were done with a desktop cone beam scanner (MicroCT 40®, Scanco Medical AG, Brüttisellen, Switzerland). The X-ray source (E) was set at 70 kVp with 114 μA at high resolution (1000 projections/180°), showing an image matrix of 2048 × 2048 pixels. The diameter of the sample holder was 30.7 mm, which allowed an increment (resolution) of 15 μm (=voxel size). Integration time was 3 s. For each site, the volume of interest (VOI) measuring 3 × 5 × 5 mm was selected manually. The VOI was positioned in the middle of the buccal bone (Supplementary Fig. 1). The micro-CT (μCT) slices were resampled and reconstructed in parallel to the line of the bone level of the neighboring teeth for each defect site using 3D structural analysis software (Amira 6®, Visualization Sciences Group, Düsseldorf, Germany). The bone volume (BV, $mm^3$), the ratio of the BV to the total volume (BV/TV, %), and bone mineral density (BMD, mg HA/$mm^3$) were measured.

Due to the small sample size, normal distribution was checked using the Kolmogorov-Smirnov test. As there was evidence of normality ($p = 0.200$), the differences between the four groups were compared with a one-way ANOVA. An independent Student's $t$ test was used to compare differences between two groups, with and without ALN. The $P$ value of 0.05 was considered significant in all tests. The analysis was performed using statistical software (SPSS for Windows®, Release 19.0, standard version; IBM SPSS, Chicago, IL, USA).

## Histological preparation and analysis

To prepare ground sections, undecalcified samples ($n = 3$) were fixed in 4% buffered formaldehyde, rinsed in running tap water, trimmed, dehydrated in ascending ethanol concentrations, and embedded in methyl methacrylate. The embedded tissue blocks were cut in the buccal-lingual plane into 1-mm-thick ground sections using a slow-speed diamond saw (Varicut® VC-50, Leco, Munich, Germany). After mounting on acrylic glass slabs, the sections were grounded and polished to a final thickness of 300 μm (Knuth-Rotor-3, Struers, Rodovre/Copenhagen, Denmark). Surfaces were stained with basic fuchsin and toluidine blue/McNeal. The most central sections from each root/extraction socket were used for descriptive and morphometric analyses. Digital photographs were taken under a light microscope (Nikon Eclipse E800; Nikon, Tokyo, Japan) equipped with a digital imaging system (NIS Elements; Nikon, Tokyo, Japan).

Morphometric analysis was performed using graphics software (Photoshop CC; Adobe, San Jose, CA, USA). The height of the alveolar bone was measured as previously described [2]. A line parallel to the long axis of the root (C) was positioned over the section of the extraction socket (Supplementary Fig. 2a). Two horizontal lines perpendicular to the C line were drawn to connect the most coronal portions of the buccal and lingual alveolar crest. The vertical distance




(VD) between the lines was measured and expressed in millimeter. Negative (−) values were given for higher VD values found at the extraction sites compared with the root sites.

The lingual and buccal bones on the root sites were divided into apical, middle, and coronal areas [31]. Correspondingly, the cross-sectional areas occupied by each portion on the root site on the buccal (BA, BM, and BC) and lingual bone (LA, LM, and LC) were measured with a polygon tool and expressed in square millimeter (Supplementary Fig. 2b). The outline of the alveolar process was projected over the extraction site, and the alteration in size was estimated by subtracting the value from the corresponding value at the tooth site. The percentages were calculated, giving less than 100% for values lower at extraction than at the corresponding root sites.

The composition of the newly formed tissue within the extraction socket was determined using a light microscope. Intersections were counted by employing an integrative

◀ **Fig. 2** Buccolingual stained ground sections illustrating the extraction socket following root extraction in control group and experimental groups (after application of ALN at the concentrations of 0.5 mg/mL, 1 mg/mL, and 2 mg/mL). The coronal boxed areas of the lingual and buccal bone are outlined and magnified. The marginal portion of the buccal bone (B) is apical to its lingual counterpart (L). The presence of bone resorption and Howship's lacunae in the crestal region of the buccal and lingual bone are indicated with arrowheads. Bundle bone (BB) is observed on the lingual wall of all samples. Large amounts of a provisional matrix are seen inside the socket. Remnants of the blood coagulum (BC) are located in the center of the extraction socket. Formation of woven bone from the original bone is occasionally visible at the apical portion of the extraction socket. At 2-week healing period, extraction sockets are covered with oral mucosa lined with keratinized epithelium. Numerous Howship's lacunae are observed on the outer and inner surface of the crestal region. Woven bone (WB) and BMUs (asterisks) are present in the newly formed bone. Bundle bone of the lingual wall is present in most of the sections and partially replaced by woven bone. Provisional matrix with the remnants of the blood coagulum is observed in the extraction socket. A large amount of woven bone is formed at the lateral and apical portions of the socket. At the 8-week healing period, the marginal portion of the buccal crest is more apical to its lingual crest. New, lamellar bone (NB) and BMUs can be seen in the crestal region. Howship's lacunae are sporadically present on the outer surface of the old cortical bone (OB). The entrance of the extraction socket is sealed by new bone. The extraction site is dominated by the newly formed lamellar bone in direct continuity with the old bone. The woven bone is in the process of remodeling. Bone marrow (BM) is present in the apical portion of the extraction socket

eyepiece with parallel sampling lines at a magnification of × 250 with a square grid (distance between test points = 40 μm). The area fractions of the osteoid, mineralized bone, total bone, soft connective tissue, blood clot, and bone/dentin fragments were determined.

### TRAP staining procedure for detection of osteoclasts

After fixation, samples ($n = 2$) were decalcified in 15% EDTA, pH = 8.0. The specimens were dehydrated through ascending ethanol baths, embedded in paraffin, cut into 5-μm sections, and stained for TRAP activity with the azo dye. The incubation medium consisted of 30 mg fast red violet LB salt, 5 mg naphthol AS-BI phosphate, and 50 mM L (þ) sodium tartrate 0.37 g in a 0.1 M acetate buffer pH 5.4 (Sigma, St. Louis, MO, USA). Samples were incubated for 45 min at 60 °C. The sections were faintly counterstained with methyl green.

## Results

### One week

The connective oral mucosa tissue covered the extraction site with modest signs of inflammation (Fig. 2). Bone resorption with Howship's lacunae was evident at the outer and inner portions of the crestal region of buccal and lingual bone walls. TRAP-positive multinuclear osteoclasts were present on the surface of the alveolar bone crest (Supplementary Fig. 3). The vertical distance between the lingual and buccal bones at extraction sockets compared with the tooth sites was reduced in the ALN-treated groups (Table 1). In controls, the distance between extraction and tooth sites was similar. Both, the buccal and the lingual bone walls contained a limited number of well-defined bone marrow spaces. Except for ALN_2, total buccal and lingual areas were well preserved as compared with the tooth sites. Apical, middle, and coronal portions of both buccal and lingual bony walls of the ALN sites were higher than the corresponding portions of the control sites (Table 2).

Immature granulation tissue harboring a large number of blood vessels, few fibroblasts, and inflammatory cells was found in the coronal portion of the sockets. The presence of a coagulum characterized central and marginal socket portions. Bundle bone was present in most of the sections except on the coronal part of the buccal bone. Direct apposition of the new bone was generally observed on the bundle bone. A severed periodontal ligament, including distinctly orientated collagen fibers and fibroblasts, sporadically resided lateral to the bundle bone. Active osteoclasts were frequently observed next to the blood vessels (Supplementary Fig. 4). Finger-like projections of newly formed woven bone occasionally extended from the apical part of the socket walls in this area into the provisional matrix. Osteoblasts lined most of the surface of the trabeculae of newly formed bone. However, no differences were observed in osteoid, mineralized bone, and total bone within the sockets between the groups (Table 3).

### Two weeks

The oral mucosa of the extracted socket was devoid of inflammatory cells but included the lamina propria (Fig. 2). Bone resorption with Howship's lacunae and the presence of osteoclasts were evident at the outer portions of the crestal region of both bony walls (Supplementary Fig. 5) and occasionally on the inner portions of both bony walls. Compared with tooth sites, vertical distance between lingual and buccal bone height increased in the ALN_0.5 group, decreased in the control group, and remained the same in the ALN_1 and ALN_2 groups (Table 1). Basal multicellular units (BMUs) were observed in the woven bone of the crestal region of the lingual sites. ALN_0.5 had the highest values at all three buccal portions and LC, whereas the control group had the highest values for LA and LM (Table 2).

The central and marginal portions of the socket contained cell-rich and highly vascularized provisional matrix. No periodontal ligament tissue could be identified lateral to the socket walls. Provisional matrix tissue resided in the central and marginal compartments of the extraction sites and the coagulum in

**Table 1** Vertical distance between lingual and buccal bone height on the root and extraction socket sites. All values are expressed in mm. Means ± SD

| Healing period | Group | Root sites | Extraction socket | Difference |
|---|---|---|---|---|
| 1 week | Control | 0.62 ± 0.03 | 0.57 ± 0.26 | 0.04 ± 0.25 |
| | ALDN_0.5 | 1.02 ± 0.22 | 0.67 ± 0.25 | 0.35 ± 0.43 |
| | ALDN_1 | 0.78 ± 0.29 | 0.63 ± 0.30 | 0.15 ± 0.49 |
| | ALDN_2 | 0.87 ± 0.24 | 0.72 ± 0.36 | 0.15 ± 0.58 |
| 2 weeks | Control | 1.29 ± 0.50 | 0.53 ± 0.11 | 0.76 ± 0.55 |
| | ALDN_0.5 | 1.43 ± 1.72 | 2.18 ± 1.18 | − 0.76 ± 0.73 |
| | ALDN_1 | 0.77 ± 0.21 | 0.83 ± 0.16 | − 0.05 ± 0.07 |
| | ALDN_2 | 0.94 ± 0.41 | 0.89 ± 0.66 | 0.05 ± 0.97 |
| 8 weeks | Control | 1.28 ± 0.28 | 0.76 ± 0.05 | 0.51 ± 0.33 |
| | ALDN_0.5 | 1.48 ± 1.37 | 2.16 ± 0.74 | − 0.68 ± 0.63 |
| | ALDN_1 | 1.13 ± 0.06 | 1.83 ± 0.62 | − 0.70 ± 0.69 |
| | ALDN_2 | 0.93 ± 0.50 | 1.80 ± 0.30 | − 0.87 ± 0.47 |

the most central portion of the socket. Inactive osteoclasts were observed on the surface of the bundle bone and the newly formed bone (Supplementary Fig. 6). The apical and lateral portions of extraction sites were characterized by the presence of a multitude of trabeculae of newly formed bone. This new woven bone was in direct contact with the bundle bone, except in the coronal part of the buccal bone. Osteoblasts were present on the surface of the woven bone and included a primitive bone marrow that was consistently present in the vicinity of the vascular units. The newly formed bone also exhibited signs of remodeling. Similar to the 1-week observation period, no difference was found for any of the morphometric parameters between the groups (Table 3).

## Eight weeks

In both the control and experimental sites, the healed extraction sockets were covered with oral mucosa lined with a keratinized epithelium (Fig. 2). Signs of bone resorption and the presence of osteoclasts were occasionally found on the outer surface of the crestal bone. In comparison with 2 weeks, the vertical distance at the extraction sites in the ALN_1 and ALN_2 groups further increased, while it remained stable in the control group (Table 1). BMUs were observed in the woven bone immediately next to the pristine bone on both buccal and lingual sites. Apical, middle, and coronal portions of the buccal bone were better preserved in control sites, in contrast to the lingual bone portions which were better preserved in ALN-treated sites (Table 2).

A broad zone of mineralized tissue including woven and lamellar bones bridged the buccal and lingual bony walls of the extraction sockets. On the crestal margin of the extraction sockets, osteoclasts were present on the surface of the newly formed bone, indicating remodeling of woven bone. Bundle bone was only occasionally present on the inner surface of the pristine bony walls. In the central region, a large portion of the woven bone had been replaced with lamellar bone. The apical portion of the socket region was occupied by bone marrow, but also included mineralized trabeculae that consisted of woven bone and lamellar bone. Bone marrow was either primitive or mature. The ALN_1 and ALN_2 groups showed more osteoid and mineralized bone as compared with the ALN_0.5 and control groups (Table 3).

## μCT

The changes in BV and BV/TV demonstrated similar pattern in all groups (Table 4). Mean BV decreased from 1-week to 2-week healing period, being significant only for the control group ($P = 0.041$). At 8 weeks, mean BV significantly increased compared with 2 weeks in the control ($P = 0.007$), ALN_1 ($P = 0.028$), and ALN_2 ($P = 0.005$) groups. Higher BV/TV values at 8 weeks were also observed for control, ALN_0.5, ALN_1, and ALN_2 as compared with 1 week ($P = 0.030$, $P = 0.021$, $P = 0.009$, $P = 0.048$, respectively) and at 2 weeks ($P = 0.003$, $P = 0.035$, $P = 0.016$, $P = 0.012$, respectively). Mean BV and BV/TV values at 1 week were similar between the groups. At 2 weeks and 8 weeks, all ALN groups showed higher BV and BT/TV means than the control group without significant differences. When ALN groups were pooled together at 2 weeks, they had significantly more BV ($10.87 \pm 3.86$) than the control group ($8.39 \pm 1.03$; $P = 0.037$). This difference was not significant for BV/TV.

Mean MBD increased over time in all groups (Table 4). This difference was significant from 1 week to 8 weeks in the control ($P = 0.002$), ALN_0.5 ($P = 0.002$), ALN_1 ($P = 0.012$), and ALN_2 ($P = 0.015$) groups. In the control group, mean BMD at 8 weeks also reached significance compared

**Table 2** Mean relative alteration of the surface area ($mm^2$) of the buccal and lingual bony walls of the extraction sockets as compared with the corresponding tooth sites. All values are expressed as percentages. Means ± SD

| Healing period | Group | Lingual | | | Buccal | | | Lingual total | Buccal total |
|---|---|---|---|---|---|---|---|---|---|
| | | Apical | Middle | Coronal | Apical | Middle | Coronal | | |
| 1 week | Control | 85.81 ± 18.55 | 95.92 ± 28.83 | 59.11 ± 16.67 | 73.94 ± 6.39 | 64.02 ± 8.62 | 43.83 ± 14.62 | 83.10 ± 21.01 | 67.14 ± 5.20 |
| | ALDN_0.5 | 113.43 ± 28.27 | 118.98 ± 18.45 | 101.02 ± 31.37 | 112.19 ± 20.69 | 100.77 ± 30.27 | 77.85 ± 33.05 | 111.98 ± 24.11 | 105.10 ± 24.73 |
| | ALDN_1 | 117.16 ± 22.32 | 136.48 ± 18.97 | 146.63 ± 18.84 | 95.71 ± 16.25 | 105.64 ± 18.81 | 118.49 ± 32.53 | 130.35 ± 19.22 | 101.61 ± 17.49 |
| | ALDN_2 | 112.81 ± 8.56 | 119.73 ± 3.35 | 75.66 ± 45.08 | 87.11 ± 39.19 | 70.58 ± 51.01 | 39.98 ± 37.04 | 105.19 ± 16.16 | 77.37 ± 43.40 |
| 2 weeks | Control | 131.11 ± 15.35 | 123.53 ± 21.23 | 89.17 ± 55.29 | 90.72 ± 23.06 | 86.61 ± 45.04 | 76.78 ± 56.47 | 117.21 ± 23.58 | 88.36 ± 32.27 |
| | ALDN_0.5 | 95.76 ± 18.83 | 101.22 ± 25.57 | 105.89 ± 59.45 | 123.15 ± 11.39 | 140.44 ± 72.26 | 102.37 ± 89.65 | 102.56 ± 30.23 | 122.46 ± 40.06 |
| | ALDN_1 | 88.92 ± 24.66 | 100.29 ± 26.34 | 97.06 ± 23.23 | 100.11 ± 30.87 | 76.90 ± 19.45 | 64.34 ± 25.22 | 94.49 ± 25.17 | 86.88 ± 23.21 |
| | ALDN_2 | 101.91 ± 34.30 | 102.42 ± 34.40 | 71.33 ± 22.05 | 101.38 ± 49.36 | 72.60 ± 35.82 | 55.55 ± 68.84 | 93.88 ± 29.23 | 85.56 ± 44.51 |
| 8 weeks | Control | 58.22 ± 6.99 | 58.71 ± 7.96 | 31.13 ± 0.52 | 136.82 ± 13.34 | 120.38 ± 13.18 | 48.96 ± 40.78 | 53.06 ± 6.64 | 121.10 ± 17.54 |
| | ALDN_0.5 | 105.43 ± 13.64 | 106.99 ± 16.58 | 54.37 ± 5.70 | 72.59 ± 18.87 | 55.01 ± 10.31 | 0.00 ± 0.00 | 91.84 ± 15.92 | 61.02 ± 18.47 |
| | ALDN_1 | 124.88 ± 39.03 | 114.42 ± 20.42 | 69.90 ± 29.52 | 121.44 ± 12.86 | 88.10 ± 32.09 | 35.19 ± 47.91 | 107.28 ± 25.52 | 100.52 ± 14.36 |
| | ALDN_2 | 115.33 ± 2.61 | 109.04 ± 20.74 | 50.67 ± 46.38 | 87.71 ± 23.90 | 67.98 ± 47.58 | 18.87 ± 32.68 | 97.64 ± 18.05 | 73.35 ± 35.46 |

**Table 3** Percentage of osteoid, mineralized bone, total bone, soft connective tissue, blood clot, and bone/dentin fragments within the extraction socket in four groups. Means ± SD

| Healing period | Group | Osteoid | Mineralized bone | Total bone | Soft connective tissue | Blood clot | Bone/ dentin fragments |
|---|---|---|---|---|---|---|---|
| 1 week | Control | 1.56 ± 1.13 | 0.13 ± 0.23 | 1.69 ± 1.33 | 55.43 ± 14.67 | 42.70 ± 16.04 | 0.16 ± 0.28 |
| | ALDN_ 0.5 | 2.00 ± 1.45 | 0.31 ± 0.15 | 2.31 ± 1.50 | 61.21 ± 9.78 | 36.45 ± 10.35 | 0.02 ± 0.04 |
| | ALDN_1 | 0.63 ± 0.65 | 0.35 ± 0.14 | 0.98 ± 0.78 | 51.43 ± 11.03 | 47.38 ± 11.59 | 0.20 ± 0.24 |
| | ALDN_2 | 1.28 ± 0.15 | 0.21 ± 0.18 | 1.49 ± 0.21 | 53.73 ± 13.39 | 44.45 ± 13.09 | 0.31 ± 0.41 |
| 2 weeks | Control | 6.90 ± 1.28 | 10.96 ± 2.37 | 17.87 ± 1.92 | 82.10 ± 1.89 | 0.00 ± 0.00 | 0.02 ± 0.04 |
| | ALDN_ 0.5 | 6.99 ± 1.16 | 10.63 ± 4.47 | 17.62 ± 3.99 | 82.26 ± 3.82 | 0.00 ± 0.00 | 0.11 ± 0.19 |
| | ALDN_1 | 7.09 ± 1.47 | 9.05 ± 2.13 | 16.14 ± 3.60 | 83.85 ± 3.60 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | ALDN_2 | 6.74 ± 2.72 | 8.28 ± 2.53 | 15.03 ± 0.19 | 84.92 ± 0.28 | 0.00 ± 0.00 | 0.04 ± 0.08 |
| 8 weeks | Control | 0.55 ± 0.48 | 35.68 ± 32.61 | 36.24 ± 33.06 | 30.42 ± 28.32 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | ALDN_ 0.5 | 1.75 ± 2.13 | 24.9 ± 22.63 | 26.71 ± 23.71 | 39.9 ± 34.99 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | ALDN_1 | 2.27 ± 2.59 | 50.08 ± 15.49 | 52.35 ± 13.01 | 47.61 ± 13.05 | 0.00 ± 0.00 | 0.03 ± 0.05 |
| | ALDN_2 | 2.25 ± 1.37 | 48.63 ± 16.34 | 50.88 ± 15.05 | 49.12 ± 15.05 | 0.00 ± 0.00 | 0.00 ± 0.00 |

with that at 2 weeks ($P = 0.006$). Similar values for ALN and control groups were observed at 1 week and 8 weeks. Mean BMD at 2 weeks was higher for ALN than for the control group, without statistical significance.

## Discussion

The bone resorption process following tooth extraction has significant consequences in implant dentistry. Whereas tissue modeling appears to be a comparatively fast process, remodeling of the newly formed hard tissue is seemingly slow and unpredictable [32–35]. In this context, we hypothesized that treatment with BP would attenuate bone resorption and benefit the normal osseous healing process of the extraction socket in dogs. ALN treatment, however, increased vertical distance between the lingual and the buccal bony crest as compared with control. Furthermore, the morphometric analysis indicated a lower bone area in the buccal than in the lingual bone wall of the extraction sockets.

Under normal healthy conditions, osteoclasts are responsible for the vertical loss of both buccal and lingual bone crest of extraction socket [12, 13]. Complete loss of bundle bone is related to the catabolic changes caused by the disruption of the vascular blood supply from periodontal ligament, leading to the significant osteoclastic activity [36]. Our results frequently showed crestal bone surface covered with $TRAP^+$ cells in ALN sites. As the BPs are known to suppress the osteoclast activity, this was not expected. Nevertheless, an anabolic response of the skeleton may be related to an increase in TRAP

**Table 4** Bone volume, relative bone volume, and bone mineral density of buccal bone in four groups, as measured on the micro-CT. All values are expressed as means ± SD

| | Healing period | Control | Alendronate (mg/mL) | | | *F* | *P* |
|---|---|---|---|---|---|---|---|
| | | | ALN_0.5 | ALN_1 | ALN_2 | | |
| Bone volume ($mm^3$) | 1 week | 14.12 ± 4.14 | 13.75 ± 4.50 | 15.49 ± 4.02 | 14.29 ± 3.23 | 0.178 | 0.910 |
| | 2 weeks | 8.39 ± 1.03 | 10.98 ± 2.77 | 10.91 ± 5.49 | 10.72 ± 3.78 | 0.582 | 0.635 |
| | 8 weeks | 17.25 ± 2.80 | 18.64 ± 7.58 | 20.65 ± 6.23 | 20.99 ± 5.15 | 0.343 | 0.795 |
| Relative bone volume (%) | 1 week | 62.25 ± 7.92 | 61.60 ± 9.05 | 58.85 ± 10.19 | 62.47 ± 16.16 | 0.109 | 0.954 |
| | 2 weeks | 55.55 ± 2.05 | 57.29 ± 4.99 | 56.30 ± 6.64 | 56.62 ± 7.42 | 0.094 | 0.962 |
| | 8 weeks | 79.35 ± 13.18 | 82.65 ± 9.35 | 98.30 ± 25.00 | 81.75 ± 8.97 | 1.543 | 0.242 |
| Bone mineral density (mg HA/$mm^3$) | 1 week | 5604.23 ± 411.24 | 5517.02 ± 436.81 | 5680.88 ± 341.46 | 5549.44 ± 283.97 | 0.742 | 0.542 |
| | 2 weeks | 5699.48 ± 116.23 | 5920.84 ± 183.80 | 5867.50 ± 364.38 | 5830.93 ± 310.22 | 0.644 | 0.598 |
| | 8 weeks | 6382.23 ± 221.48 | 6358.91 ± 173.81 | 6388.26 ± 245.93 | 6170.31 ± 284.85 | 1.179 | 0.349 |

expression [37]. One possible explanation for an increased vertical distance is that the measured buccal bone height is a relative value where lingual crest served as a reference. As the lingual bone was well maintained and the resorption of the buccal crest proceeded, the vertical distance increased.

A similar difference between the buccal and the lingual bones was also observed using morphometric analysis. The resorption of the bony walls of the extraction socket occurred in two overlapping phases: (i) resorption of bundle bone and replacement with woven bone and (ii) resorption from the outer surfaces of both bony walls in conjunction with severed blood vessels, orchestrated by osteoclasts in the periosteum [2]. Possibly, the suppression of osteoclasts by ALN hindered neovascularization, induced by angiogenic factors in the MMP-1 pathway [38, 39]. The structure of the extraction socket might explain a different pattern of area alterations between the lingual and buccal bones. Thick lingual bone is composed of both bundle and lamellar bones, while most of the thin buccal bone wall is a tooth-depending structure. Furthermore, the presence of BMUs on the lingual sites at 2-week healing period indicated its bone modeling/remodeling activity. Yamamoto-Silva et al. [17] observed Sharpey's fibres among the mineralized matrix at the wall of the alveoli without signs of resorption 3 weeks after tooth extraction. ALN treatment demonstrated some beneficial effect and to some extent attenuated loss of the lining bone into which the periodontal ligament fibers were inserted even at 8-week healing period.

The pattern of bone healing assessed by μCT was comparable with the lingual sites, but not to the buccal sites in most of the specimens. In contrast to the two-dimensional morphometric measurements, the volumetric assessment by μCT was obtained using neighboring teeth as a reference. The VOI obtained by μCT also included newly formed bone within the extraction socket, which is difficult to distinguish from the pristine bone. Thus, the assessment of the buccal bone wall alterations was limited. A similar pattern of bone formation was observed for all groups. Nahles et al. [40] described an alternating activity of osteoblasts in the extraction socket of humans. Some osteoblasts that become inactive, namely bone-lining cells, can differentiate again into active osteoblasts in response to the osteoinductive signals [41]. The values at 2-week and 8-week healing periods were higher for ALN-treated groups, but none of the concentrations performed significantly better when compared with the control; statistically significant difference was obtained only for the ALN groups pooled at 2-week healing period. In this study, we aimed at assessing a dose-related effect during an early healing period. The use of three ALN concentrations per animal though contributed to the lower power of the study. It is thus possible that the significance would be reached with an increased "*n*" per a treatment modality and healing period.

Three concentrations of ALN were applied in the present study since optimal concentration for local use varies for different BPs [42]. Furthermore, high dosages of BP might have cytotoxic effects on the osteoblasts [43]. Topical application of relatively low ALN concentrations was preferable, as the local necrosis should be resolved during the healing process. Still, an averaging of three ALN concentrations within the same animal cannot be fully neglected. It is, however, possible that the effects of the ALN in the present study were even diminished by the post-extraction bleeding and recruitment of monocytes/macrophages [44]. The effects of ALN could be enhanced using a delivery system. Nevertheless, the combined application of BP may affect the resorption of the bone filler and indirectly enhance the healing process of the extraction socket [45].

Formation of new bone from the bony walls in all groups started from the 2-week healing period onwards, in line with the previous reports [1, 2]. Nitrogen-containing BP inhibited bone resorption without decreasing but increasing the number of osteoclasts [14, 46, 47]. Continuous BP therapy in tooth extraction model decreased $TRAP^{+}$ osteoclasts on the bone surface and increased mononuclear and non-attached osteoclasts [14, 48]. The non-attached osteoclasts are not able to resorb bone but may be involved in bone remodeling and activation of osteoblasts [49]. The amount of newly formed bone within the socket between the groups was similar and increased at 8 weeks. In all groups, new bone matured and sealed the entrance of the extraction socket. Enhanced bone formation observed in the ALN_1 and ALN_2 groups may facilitate correct placement and osseointegration of dental implants. Local application of ALN did not affect the alterations of the ridge dimensions is a dose-dependent manner. In fact, the differences in μCT were significant only between control and pooled ALN-treated groups. As this is an underpowered study, the overall conclusions have to be interpreted with care. The provided basis could serve to design a study with sufficient statistical power, allowing more robust scientific conclusions.

## Conclusions

Within the limitations of the present study, which is underpowered, ALN treatment alone did not prevent resorption of the buccal bone of the extraction socket. The preservation of the lingual bone area and formation of new bone within the socket seem to benefit from the ALN treatment, while control sockets showed better preservation of the buccal bone areas. Different ALN concentrations, however, failed to elicit a dose-response relationship. Future studies should evaluate the effect of timing of the ALN application and the implant placement on the healing of the extraction socket.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** The study protocol was approved from the Ethics Committee of the Rof Codina Foundation, Lugo, Spain (AELU001/17/INVMED(02)OUTROS(04)/AGC/01).

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

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