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## EFFECTS OF PAMIDRONATE ON HUMAN ALVEOLAR OSTEOBLASTS IN VITRO

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### Abstract

**Purpose**—Administration of bisphosphonates has recently been associated with the development of osteonecrotic lesions of the jaw (ONJ). To elucidate the potential contributions of osteogenic cells to the development and regeneration of ONJ, we have isolated primary cells from human alveolar and long/iliac bones, and examined the effects of pamidronate on cell viability, proliferation, osteogenesis and wound healing.

**Materials and Methods**—Primary human osteoblasts and bone marrow stromal cells were isolated from alveolar and iliac/long bone and marrow tissue. Cellular proliferation, alkaline phosphatase activity, apoptosis (TUNEL, Caspase-3, and DAPI assays) and wound healing in an *in vitro* scratch assay were assessed after exposure to pamidronate at a range of clinically relevant doses.

**Results**—Primary alveolar osteoblasts proliferated at significantly higher rates than long/iliac bone osteoblasts *in vitro*. Upon exposure of alveolar osteoblasts and long/iliac bone marrow stromal cells to pamidronate for more than 72h, we have observed significantly decreased cell viability, proliferation, osteogenesis and *in vitro* wound healing at  $6 \times 10^{-5}$  M pamidronate, with the induction of apoptosis in ~20% of cell population.

**Conclusions**—The remodeling activity of alveolar bone, indicated by higher proliferation of alveolar osteoblasts, could be negatively affected by exposure to high concentrations of

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pamidronate over extended periods of time. The absence of anabolic effects of pamidronate on alveolar osteoblasts, and induction of apoptosis in osteogenic cells could negatively affect bone balance at this site, and contribute to osteonecrosis of the jaw.

### Keywords

osteonecrosis of the jaw; pamidronate; alveolar osteoblasts; BMSCs; apoptosis

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## INTRODUCTION

Bisphosphonates (BPs), potent inhibitors of osteoclastic bone resorption, are widely used in the treatment of osteoporosis, Paget's disease, hypercalcemia of malignancy, multiple myeloma, and bone metastases associated with breast, prostate, lung, and other soft tissue tumors. Recently, the occurrence of exposed necrotic bone lesions in the jaw (osteonecrosis of the jaw, ONJ) has been associated with the use of nitrogen-containing BPs<sup>1, 2</sup>. Osteonecrosis of the jaw is defined by several professional societies as an area of exposed bone in the maxillofacial region that does not heal within 6–8 weeks after identification, in a patient who is receiving or has been exposed to a BP and has not had radiation therapy to the craniofacial region<sup>2–8</sup>.

At the present time the natural history and pathophysiology of ONJ development is not clear. In the majority of cases, ONJ has been diagnosed in the oncologic population, where patients are receiving high doses of BPs with concomitant chemotherapeutic agents and are often immunosuppressed. Oversuppression of bone turnover due to increased sequestration of BPs in the jaws, and compromised blood supply have been considered the initiating events in the development of the lesions<sup>1</sup>. In addition, inhibition of wound healing, surgically-induced trauma, specific anatomy of the oral cavity, the presence of a diverse microbial flora and on-going inflammatory processes have been proposed as potential contributing factors<sup>2, 9</sup>.

Differences in bone physiology between different sites in the skeleton could be implicated in the development of ONJ. Craniofacial bones differ from long bones in developmental lineage, arising from neural crest, and mechanism of formation by intramembranous ossification. Differences in matrix composition, bone morphogenic protein expression, osteoclastic bone degradation and bone marrow stromal cell (BMSC) compartment have been reported, and suggest functional differences in turn-over and mechanical properties of bone at different anatomic locations<sup>10–13</sup>. In a recent study, pamidronate treatment of primary human mandibular and iliac crest BMSCs isolated from the same donors indicated differences in survival, osteogenesis and osteoclast recruitment between the two sites, suggesting the possibility of dysregulation of mandibular bone homeostasis after exposure to BPs<sup>14</sup>. In previous studies, treatment of osteogenic cells isolated from the long/ilic bone with lower BPs concentrations (  $10^{-5}$  M) resulted in anabolic effects<sup>15–18</sup>. However exposure to higher concentrations (  $10^{-4}$  M) resulted in decreased cell proliferation, viability, protein synthesis and mineralization<sup>17, 19–21</sup>. Decreased cell viability and migration have also been reported for oral mucosal and endothelial cells after BP treatment, suggesting the possibility that ONJ is a multifactorial disease<sup>21–26</sup>.

Given the specific differences in cell responses to BPs, and physiological differences between bone at different locations, our aim was to assess the effects of BPs on primary human alveolar osteoblasts, which to our knowledge have not been reported previously. We have hypothesized that the osteogenic ability of residing osteoblasts, as well as transplanted cells, might be directly affected by BPs. We have chosen to study the effects of pamidronate, a common intravenously-administered bisphosphonate, linked to ONJ, that is

frequently used as a model compound in studies of BPs actions<sup>3, 23, 27-31</sup>. We have tested survival, proliferation, osteogenesis and wound healing of human osteoblasts isolated from the alveolar bone. In addition, we have extended our experiments to primary human osteogenic populations from the long/iliac bone and marrow, in order to compare their response to populations derived from intramembranous bone. We have treated the cells with a range of pamidronate concentrations ( $10^{-10}$  –  $10^{-4}$  M), and report for the first time concentration-dependent inhibitory effects of pamidronate on primary human alveolar bone osteoblasts including induction of apoptosis.

## MATERIALS AND METHODS

### Materials

Pamidronate, ascorbic acid 2-phosphate, dexamethasone,  $\beta$ -glycerophosphate, paraformaldehyde and formalin-free fixative (Accustain) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), basic fibroblast growth factor (bFGF), 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI), gentamicin, Fungizone®, Dulbecco's PBS (D-PBS), non-essential amino acids and trypsin/EDTA were obtained from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's media: nutrient mixture F-12 (DMEM/F-12) was from Lonza (Basel, Switzerland). All other substances were of analytical or pharmaceutical grade and obtained from Sigma-Aldrich.

### Isolation and Culture of Primary Human Cells

Primary human osteoblast-like cells (“osteoblasts”) were isolated from discarded alveolar (n=7) and long/iliac bone surgical procedures (n=5). The study was approved by the Columbia University IRB. The bone tissue was minced, thoroughly washed to remove any remaining soft tissue, and placed in 6-well plates to initiate explant cultures. The culture medium consisted of DMEM/F12 supplemented with 20% FBS, 50  $\mu$ g/mL gentamicin and 0.08% Fungizone®, and was changed twice per week. Long/iliac BMSCs were isolated from the bone marrow aspirate samples obtained from Lonza (n=2) or by washing and collecting the marrow fraction from the long bone sample (n=1). Adherent cells were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 1 ng/mL bFGF, 50  $\mu$ g/ml gentamicin and 0.08% Fungizone® according to the previously established protocols<sup>32</sup>. All cultures were incubated at 37°C and 5% CO<sub>2</sub>. Upon confluence, cells were dissociated from the plates by trypsinization, counted and subcultured at a density of 5 000 cells/cm<sup>2</sup> for three passages. Osteoblast proliferation was compared between different tissue sources at passage one. Third passage cells were used in all other experiments, and exposed to a range of pamidronate concentrations, as noted in specific assays.

### Proliferation Assay

Two thousand cells were seeded per well of 96-well plates (6,000 cells/cm<sup>2</sup>) in 100  $\mu$ L of DMEM/F12 supplemented with 10% FBS, 50  $\mu$ g/mL gentamicin and 0.08% Fungizone®, which was changed every two days. 100 mM pamidronate stock solution was prepared in deionized water and further diluted to final concentrations in culture media. The effect of pamidronate ( $10^{-10}$  –  $10^{-4}$  M) on cell proliferation was evaluated at 24h, 48h, 72h and 168h after the addition to the cultures. Proliferation was measured by CellTiter96® Assay according to the manufacturer's instructions (Promega, Madison, WI). Briefly, MTS/PMS reagent was added directly to the culture media, incubated at 37°C for 1.5h and the absorbance was measured at 490 nm.

### Alkaline Phosphatase Activity Assay

Alkaline phosphatase (AP) activity of the cells was assessed as an early marker of osteogenesis<sup>33, 34</sup> in control medium (DMEM/F12 supplemented with 10% FBS, 50  $\mu\text{g}/\text{mL}$  gentamicin and 0.08% Fungizone®) and osteogenic medium (control medium supplemented with 10 mM  $\beta$ -glycerophosphate, 100 nM dexamethasone and 0.05 mM L-ascorbic acid 2-phosphate) 24h and 168h after the addition of pamidronate ( $10^{-10}$  –  $10^{-4}$  M) to the media. For quantitative assays, cells were seeded and cultured in 96-well plates, as described above for the proliferation assay. The AP activity was determined by following the conversion of p-nitrophenyl phosphate to p-nitrophenol (Sigma-Aldrich)<sup>32</sup>. Cells were lysed by adding 100  $\mu\text{L}$  of a buffer containing 2-amino-2-methylpropanol and p-nitrophenylphosphate (Sigma-Aldrich) to each well, and incubated at 37°C until the development of color. Reaction time was noted for AP activity calculation. Reactions were stopped by adding 50  $\mu\text{L}$  0.2 M NaOH. Absorbance was measured at 405 nm, and the AP activity was calculated from the standard curve and normalized to the reaction time and number of cells per well in each group. Cultures were fixed with Accustain, stained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride), and the number of cells in randomly sampled fields was counted using fluorescent microscope (IX81, Olympus, Center Valley, PA) and Metamorph imaging software (Molecular Devices, Sunnyvale, CA). For qualitative assessments, cells were seeded into 48-well plates (6,000 cells/cm<sup>2</sup>) and cultured in osteogenic and control media with pamidronate, as described above. AP activity was detected by histological staining according to the manufacturer's instructions (Fast Blue RR Salt staining, Sigma-Aldrich).

### TUNEL Assay

TUNEL assay, the most commonly used *in situ* test for apoptosis, was selected to probe for apoptosis induction as in our previous study<sup>24</sup>. Cells were seeded into Permax chamber slides (Nunc, Rochester, NY) as described above for the proliferation assay, and incubated with pamidronate (0 M,  $10^{-5}$  M,  $6 \times 10^{-5}$  M and  $10^{-4}$  M) for 24h and 72h. Positive controls were obtained by incubating cells with 1  $\mu\text{M}$  staurosporine (Sigma-Aldrich) for 6h. TUNEL assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN). Slides were fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature, washed and permeabilized with 0.1% sodium citrate, 0.1% Triton X-100 solution for 2 minutes on ice. After washing, slides were incubated with enzyme and labeling solution for 1 hour at 37°C in a humidified chamber. All slides were counterstained with DAPI to detect pyknotic nuclei in the cells that have undergone apoptosis, and mounted with Vectashield mounting solution (Vector Labs, Burlingame, CA). TUNEL positive cells, total number of cells and nuclear condensation were assessed under the fluorescent microscope (IX81, Olympus, Center Valley, PA).

### Caspase-3 Assay

Induction of apoptosis at high concentrations of pamidronate was confirmed by measuring activity of Caspase-3, a key enzyme in the execution of the apoptotic cascade that mediates DNA condensation, DNA fragmentation, and cell blebbing<sup>35</sup>. Alveolar osteoblasts and BMSCs were grown to 70% confluence in 6-well plates and treated with pamidronate ( $10^{-4}$  M and  $3 \times 10^{-4}$  M) for 24h, 48h, 72h and 96h. Positive controls were generated by incubation of cells with 1  $\mu\text{M}$  staurosporine for 6h. Cells were collected in the culture medium and centrifuged at  $5,000 \times g$  for 5 minutes. Caspase-3 activity was assayed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, cell pellets were lysed in the buffer provided in the kit and stored at  $-80^\circ\text{C}$ . Upon thawing, samples were centrifuged at  $13,400 \times g$  for 10 minutes at  $4^\circ\text{C}$ , and assayed in 96-well plates by adding the Caspase-3 substrate (Acetyl-Asp-Glu-Val-Asp p-nitroanilide) and incubating overnight at  $37^\circ\text{C}$ . Absorbance was read at 405 nm, and the caspase activity was calculated from the

standard curve and normalized to the total protein content determined using a Bradford reagent based protein assay (Sigma-Aldrich). A standard curve was generated using known concentrations of bovine serum albumin (Bio-Rad, Hercules, CA).

### Wounding Assay

The *in vitro* model of wound healing was used to examine the effects of pamidronate treatment on the ability of alveolar osteoblasts and BMSCs to migrate and proliferate in a monolayer culture<sup>24</sup>. Cells were seeded into 6-well plates, grown to confluence and preincubated with pamidronate (0M,  $10^{-5}$  M,  $3 \times 10^{-5}$  M,  $6 \times 10^{-5}$  M and  $10^{-4}$  M) for 24h. Wounds were created with a standardized protocol using a trimmed comb containing 7 tines spaced 2.5 mm apart (Goody Products Inc., Peachtree City, GA) that had been washed with 70% ethanol and dried prior to use. Wounded monolayers were briefly washed, and the culture media were returned to the cells. Migration of the cells into wounded areas of the monolayer (percentage of overgrown area) was assessed by microscopy over four days.

### Statistical Analyses

Statistical analyses were done by one-way and two-way ANOVA, followed by Tukey-Kramer HSD post-hoc test using Statistica software (StatSoft, Tulsa, OK). Significance level was set at 0.05.

## RESULTS

### Effects of Pamidronate on Cell Proliferation

Primary human osteoblasts were isolated from samples of alveolar and long/iliac bone tissue by preparation of explant cultures. Proliferation of primary cells at passage 1 was markedly higher in alveolar osteoblasts (5-fold increase in cell number, n=4) than the long/iliac bone osteoblasts (<2-fold increase in cell number, n=3) (Fig. 1). Alveolar osteoblasts continued to proliferate over three passages, whereas osteoblasts from the long/iliac bones demonstrated only limited growth, which arrested before the second passage in all tested samples (n=5). Bone marrow stromal cells (n=3) were isolated from the marrow aspirates obtained from the long/iliac bones, and cultured in medium supplemented with 1 ng/ml bFGF. Under these conditions, BMSCs exhibited slightly lower growth rate than alveolar osteoblasts (Fig. 1), and were used in subsequent studies.

Addition of  $10^{-4}$  M pamidronate to culture medium significantly decreased alveolar osteoblast viability after 72h and 168h (7 days) of treatment compared to all other concentrations (Fig. 2A,  $p < 0.05$ ), and induced cell detachment in 3 of 4 tested samples. Cell proliferation was significantly inhibited in this group after 72h and 168h compared to all other concentrations (Fig. 2C,  $p < 0.001$ ). Similarly, treatment of BMSCs with  $10^{-4}$  M pamidronate for 48h, 72h and 168h, as well as treatment with  $6 \times 10^{-5}$  M pamidronate for 168h significantly decreased cell viability (Fig. 2B,  $p < 0.001$ ) compared to the lower concentrations. BMSCs proliferation was significantly inhibited with  $10^{-4}$  M pamidronate after 72h and 168h, and with  $6 \times 10^{-5}$  M pamidronate after 168h (Fig. 2D,  $p < 0.001$ ) compared to the lower concentrations. We have not observed any significant anabolic effects at any of the pamidronate concentrations tested. Slightly increased BMSCs viability, noted in the lower pamidronate concentration groups ( $10^{-7}$  –  $3 \times 10^{-5}$  M) at 48h and 72h, was transient and declined by 168h of treatment.

### Effects of Pamidronate on Alkaline Phosphatase Activity

The effect of pamidronate supplementation on alkaline phosphatase (AP) activity, an early marker of osteogenic cell phenotype was assessed quantitatively at 24 and 168h. Alkaline phosphatase activity values of alveolar osteoblasts in control medium supplemented with  $3 \times$

$10^{-5}$  M,  $6 \times 10^{-5}$  M and  $10^{-4}$  M pamidronate were 45%, 39% and 27% of the unsupplemented group, and differed significantly from all lower concentration groups after 168h (Fig. 3A,  $p < 0.001$ ). Alveolar osteoblasts cultured in osteogenic medium exhibited significantly lower AP activities with  $6 \times 10^{-5}$  M and  $10^{-4}$  M pamidronate (39% and 16% of the unsupplemented group) as compared to all lower concentration groups after 168h (Fig. 3A,  $p < 0.001$ ). We have not detected anabolic effects of pamidronate supplementation on alveolar osteoblast AP activity in any of the concentration groups. AP activity per number of cells in the wells (Fig. 3B) and histological stains of AP activity (Fig. 3C) indicated that the total decrease per well resulted from the lower cell numbers, as well as from lower amounts of AP activity expressed per cell.

### Pamidronate and Cell Apoptosis

In order to examine pamidronate toxicity further, we have evaluated apoptosis by TUNEL assay, DAPI staining (Fig. 4A–E, G–K), and quantitation of caspase-3 activity (Fig. 4F, L). A significant increase in TUNEL positive apoptotic alveolar osteoblasts (16%) was seen in cultures supplemented with  $10^{-4}$  M pamidronate after 72h when compared to other concentrations and incubation times (Fig. 4A, B, E,  $p < 0.001$ ). Similarly, BMSCs cultures exhibited a significant increase in apoptotic cells (12%) with  $10^{-4}$  M pamidronate at 72h, compared to other concentrations and incubation times (Fig. 4G, H, K,  $p < 0.001$ ). In both cell types, counterstaining with DAPI showed nuclear condensation, the cells exhibited stressed morphology and started detaching from the culture surface at 72h (Fig. 4C, D, I, J).

Induction of apoptosis was further indicated in both cell types by significant increases in caspase-3 activity. Alveolar osteoblasts cultured with  $10^{-4}$  M pamidronate exhibited significantly higher caspase-3 activity at 72h and 96h compared to 24h and 48h (Fig. 4F,  $p < 0.001$ ). Similarly,  $3 \times 10^{-4}$  M pamidronate induced a gradual increase in caspase-3 activity in alveolar osteoblasts, which was significantly higher at 72h and 96h compared to 24h (Fig. 4F,  $p < 0.001$ ). Caspase-3 activity levels measured in BMSCs were more variable, but exhibited a similar gradually-increasing trend over 72h as seen in the alveolar osteoblasts. Significantly increased caspase-3 activity was detected in BMSCs cultures treated with  $3 \times 10^{-4}$  M pamidronate for 72h compared to 24h (Fig. 4L,  $p < 0.01$ ).

### Pamidronate and Wound Healing

An *in vitro* model of wound healing was used to examine the ability of alveolar osteoblasts and BMSCs to migrate and proliferate during pamidronate treatment (Fig. 5). Confluent cell monolayers were preincubated with pamidronate ( $3 \times 10^{-5}$ ,  $6 \times 10^{-5}$  and  $10^{-4}$  M) for 24h, wounded by scratching, and the healing was observed daily. For both cell types, cell migration was noted 24h after wounding and continued until 72h, when the wound was closed in the control (unsupplemented) groups (Fig. 5) and the groups with  $3 \times 10^{-5}$  M pamidronate. Alveolar osteoblasts exposed to  $6 \times 10^{-5}$  M pamidronate behaved similarly to control cultures (Fig. 5A), whereas BMSCs exhibited only ~20% wound closure at this concentration (maintained upon longer incubation) (Fig. 5B). Both cell types failed to heal the wound at  $10^{-4}$  M pamidronate, and the monolayers completely detached 48h after wounding (72h days of pamidronate treatment, similar to our observations in proliferation studies) (Fig. 5).

## DISCUSSION

Our work was motivated by the increasing frequency of ONJ, a clinical condition associated with the use of nitrogen-containing BPs, previous traumatic injury, and several other risk factors. To date, the majority of knowledge regarding ONJ is based on case report studies<sup>1, 2, 36</sup>, and the true incidence, etiology, pathogenesis, and natural history of this

condition have yet to be elucidated. In addition, restoration of oral tissues is challenging<sup>37</sup>, and in order to develop effective treatments, a fundamental understanding of cellular responses in the environment of diseased tissue is needed. Our group and others have previously reported the effects of BPs on oral mucosa cells, fibroblasts, vascular cells and bone cells isolated from non-oral sites<sup>21–26</sup>. Given the developmental and physiological differences between bone at different locations, the present study was aimed at assessing the specific effects of pamidronate on primary human alveolar and long/iliac bone osteoblasts. Our results show for the first time that pamidronate treatment inhibited cell viability, proliferation, osteogenesis and *in vitro* wound healing, and induced apoptosis in primary human alveolar osteoblasts. The exposure to  $10^{-4}$  M threshold concentration for more than three days was shown to be necessary for the inhibitory actions to take effect *in vitro*. Similar responses were found with primary human BMSCs from long/iliac bone marrow.

We have studied a wide range of pamidronate concentrations, which was based on reported maximum concentrations in the serum immediately after a 4h 90 mg pamidronate infusion ( $\sim 10^{-5}$  M)<sup>38</sup> and previous reports<sup>14, 17, 20, 21, 29, 39</sup>. As noted in previous work and supported by animal experiments<sup>24, 27, 40, 41</sup>, a range of 50 to 500 ng/mg (or 214 to 2 140 nmol/g) seems a reasonable estimate of the mean pamidronate concentration in bone tissue in humans that were treated for a period of 4 years with monthly infusions totaling 90 mg of the drug. Assuming a volume of 1 mL per g of bone, this amount corresponds to a concentration of 0.2 – 2 mM. Local concentrations of bisphosphonates in bone vary widely<sup>42, 43</sup>, and it has been speculated that due to the high rate of remodeling, alveolar bone could accumulate increased amounts of BPs. Recent studies indicate that cells in the bone microenvironment other than osteoclasts could be affected by bisphosphonates, when they are released from the mineralized surface<sup>39, 43, 44</sup>.

We have noted significant differences in the proliferative potential of alveolar and long/iliac bone osteoblasts (Fig.1), which corresponded with previous reports of rat calvarial osteoblasts<sup>45</sup> and human maxillofacial BMSCs<sup>10</sup> exhibiting higher proliferation than the same cell types isolated from the iliac/long bones. Even though our observations were limited by the relatively small number of samples originating from different donors, the observed differences were consistent. Due to the limited cell growth, we were unable to evaluate the effects of pamidronate on long bone osteoblasts. We have extended our studies to BMSCs from the long/iliac bone, a cell population commonly studied as a model for bone repair, in order to evaluate their healing potential in the presence of pamidronate.

Upon exposure of alveolar osteoblasts and BMSCs to pamidronate, we have noted considerable cell toxicity at  $6 \times 10^{-5}$  M pamidronate concentrations, which was accompanied by complete cell detachment in most cultures by 168h of treatment (Fig.2). The inhibitory effects on cell viability were highly consistent between different donors, and were in agreement with previous reports of human long bone<sup>17, 19, 21</sup> and mouse calvarial osteoblasts<sup>29</sup>. Specific differences in cell sensitivity between the studies could possibly result from different subpopulations that are present in the primary cell preparations, including osteocytic cells. We have noted a somewhat higher sensitivity of BMSCs to pamidronate in comparison to alveolar osteoblasts, and a slight transitory increase in BMSC viability at 48h and 72h of treatment with lower pamidronate concentrations (Fig.2).

We have not been able to observe significant anabolic effects of pamidronate on either cell type during 168h treatment, in contrast to previous work reporting anabolic effects of BPs on long/iliac osteoblasts and BMSCs<sup>15, 16, 18</sup>. Our findings with long/iliac BMSCs are in agreement with a recent study, where a decrease in mandibular BMSCs viability was found with increasing pamidronate concentrations, whereas iliac bone BMSCs exhibited an increased cell viability at low concentrations, and a dose-dependent decrease at higher

concentrations<sup>14</sup>. Interestingly, osteogenic populations tested in the current study were more sensitive than oral mucosa cells in our previous work, which did not proliferate but remained viable in the presence of  $10^{-4}$  M pamidronate<sup>24</sup>.

In addition to decreased cell proliferation and viability, pamidronate treatment also decreased alkaline phosphatase activity of alveolar osteoblasts (Fig. 3). Alkaline phosphatase activity is an early marker of the osteogenic phenotype, which increases at the onset of bone matrix maturation phase during *in vitro* and *in vivo* osteogenesis. We have detected a slightly higher threshold of pamidronate inhibition ( $6 \times 10^{-5}$  M) with the cells cultured in osteogenic medium as compared to the cells in control medium ( $3 - 10^{-5}$  M). This difference could possibly be attributed to L-ascorbic acid supplementation, which has been shown to promote bone matrix deposition and continuous proliferation of human osteoblasts during osteogenesis *in vitro*<sup>34</sup>, and might partially rescue the effects of pamidronate. The absence of anabolic effects on alkaline phosphatase activity with lower pamidronate concentrations corresponds to previous studies with mice calvarial osteoblasts<sup>29</sup> and human mandibular BMSCs<sup>14</sup>. In order to understand the broader effects of pamidronate on osteogenesis of alveolar osteoblasts, further studies should be aimed at testing an array of early and late markers of osteoblastic phenotype, such as collagen type I synthesis, expression of bone matrix proteins (i.e. bone sialoprotein, osteocalcin) and potential for matrix mineralization.

One of the main mechanisms of BPs actions is the induction of osteoclast apoptosis<sup>46</sup>. Recent reports suggested apoptosis of mice calvarial osteoblasts and human long bone osteoblasts after treatment with pamidronate<sup>25, 29</sup>, and led us to explore if considerably decreased cell survival observed in our cultures could result from the induction of apoptosis. We used three complementary methods to evaluate the cells undergoing apoptosis: TUNEL assay to evaluate nuclear fragmentation, DAPI staining to visualize chromatin condensation, and caspase-3 assay to detect activation of apoptotic cascade. We observed significant increases in the number of apoptotic cells after 72h pamidronate treatment both in alveolar osteoblasts and in BMSCs (Fig.4). Notably, levels of apoptosis detected by TUNEL and caspase-3 assays were corresponding (15–20%) and suggested that only a part of cell population undergoes programmed cell death. In previous studies, 80% of mice calvarial osteoblasts and 40% of human long bone osteoblasts underwent apoptosis when exposed to pamidronate for 48h and 72h<sup>25, 29</sup>.

The large decrease in cell viability after 72h pamidronate treatment (Fig.2), suggests that apoptosis may not be the only mechanism of pamidronate toxicity in human alveolar osteoblasts and BMSCs. It has previously been suggested that pamidronate could affect cells by mechanisms other than mevalonate pathway inhibition, possibly by forming toxic complexes on the cell surface<sup>30, 31, 47</sup>. For example, treatment of cells with three times higher pamidronate concentration did not significantly increase or accelerate apoptosis induction in either alveolar osteoblasts or BMSCs (Fig.4 F,L). (deleted text)

Finally, we aimed to assess whether pamidronate treatment would affect wound healing responses of alveolar osteoblasts and iliac/long bone BMSCs in an *in vitro* model, since traumatic injury has been associated with the development of ONJ. We observed an absence of wound healing by alveolar osteoblasts and BMSCs at  $10^{-4}$  M pamidronate, accompanied by complete cell detachment from the culture plates (Fig.5). Additionally, BMSCs exhibited incomplete wound closure at  $6 \times 10^{-5}$  M pamidronate, presumably due to limited cell proliferation at this concentration (Fig.2).

Notably, results from the current study differ significantly from our previous observations with mouse oral mucosa cells<sup>24</sup>, where apoptosis was not noted in oral mucosa cells after



treatment with the same concentrations of pamidronate. Additionally, both intramembranous and endochondral derived osteogenic cells exhibited higher sensitivity to pamidronate than oral mucosa cells, which exhibited inhibited healing responses, but reached complete wound closure after 5 days of incubation<sup>24</sup>. In recent studies with human cells, treatment with  $5 \times 10^{-5}$  M pamidronate resulted in apoptosis of human oral fibroblasts; however the same concentration did not cause apoptosis but resulted in senescence of human oral keratinocytes<sup>22, 23</sup>.

At the present time it is unclear whether the ONJ lesion initiates in the bone or the soft tissue. Our data indicating a high proliferation activity and sensitivity of alveolar osteoblasts to pamidronate is in line with the previous work suggesting dysregulation of mandible bone homeostasis<sup>14</sup>, and development of ONJ from within the bone (“inside-out” theory). However, taken together with the growing amount of work indicating potential adverse effects of BPs on the soft tissues<sup>21–25</sup>, it seems likely that ONJ is a multifactorial disease, initiated and/or negatively affected by surgically-induced trauma.

## CONCLUSIONS

Pamidronate inhibited cell viability, proliferation and wound healing, and induced apoptosis in primary human alveolar osteoblasts and iliac/long bone BMSC. In light of the high remodeling rates of alveolar bone, our data suggest that pamidronate treatment could exert negative effects if high concentrations ( $10^{-4}$  M or above) were reached *in vivo* for prolonged periods of time. Significant differences in proliferation of primary osteoblasts were observed during early passages *in vitro*, possibly reflecting higher remodeling activity of alveolar bone compared to iliac/long bone. The absence of anabolic effects of pamidronate, and induction of apoptosis in osteogenic cell populations could negatively affect bone balance, with implications to ONJ development and treatment. Future work is needed to further assess the molecular mechanisms of pamidronate toxicity in human alveolar osteoblasts, and to develop strategies for counteracting the inhibitory effects of pamidronate.

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## ABBREVIATIONS

<b>BPs</b>	Bisphosphonates
<b>ONJ</b>	Osteonecrosis of the jaw
<b>BMSCs</b>	Bone marrow stromal cells

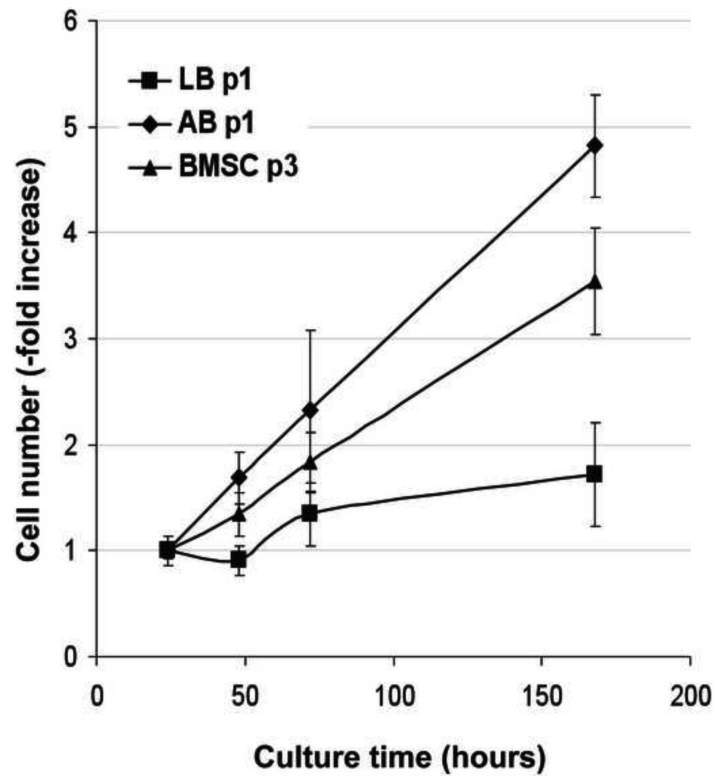
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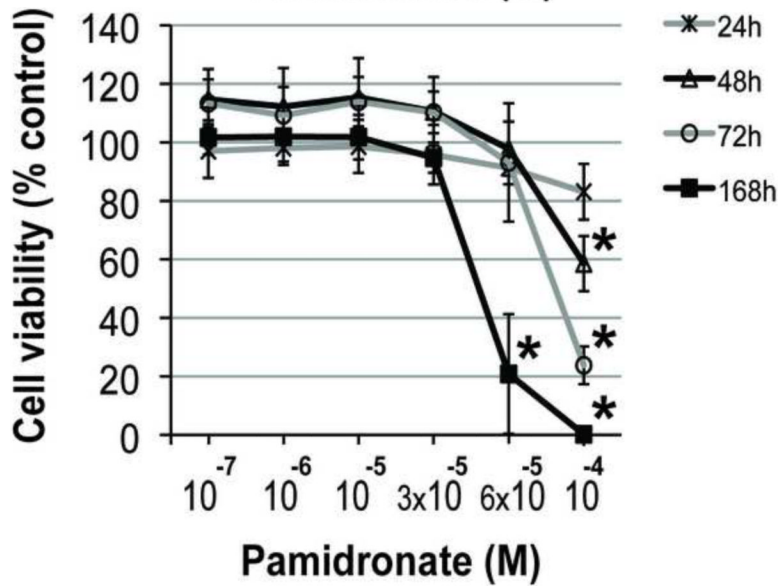
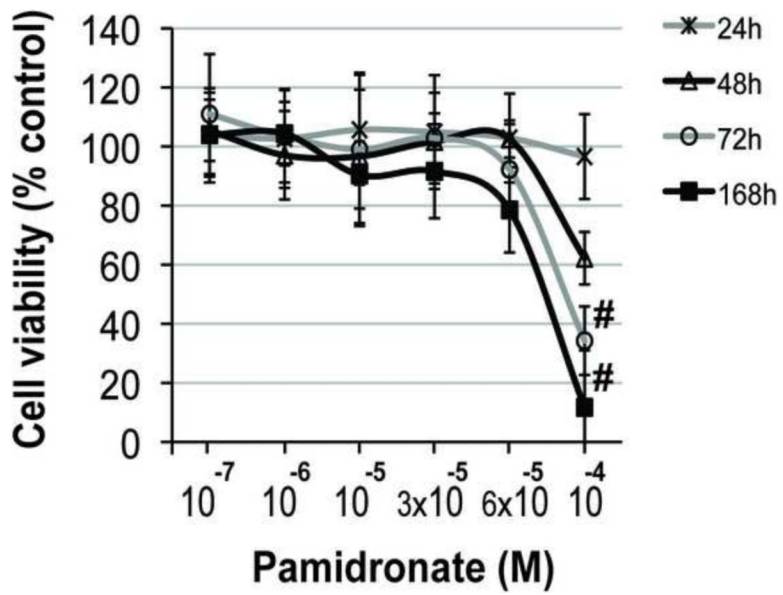
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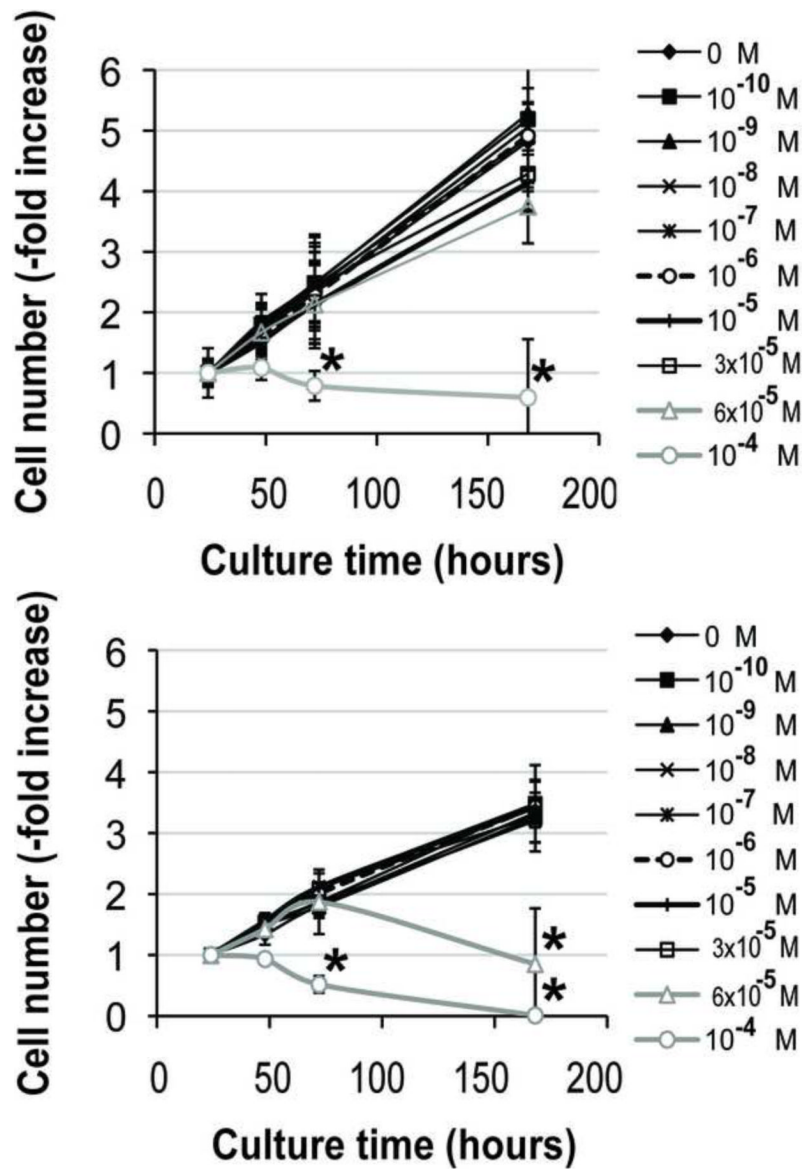
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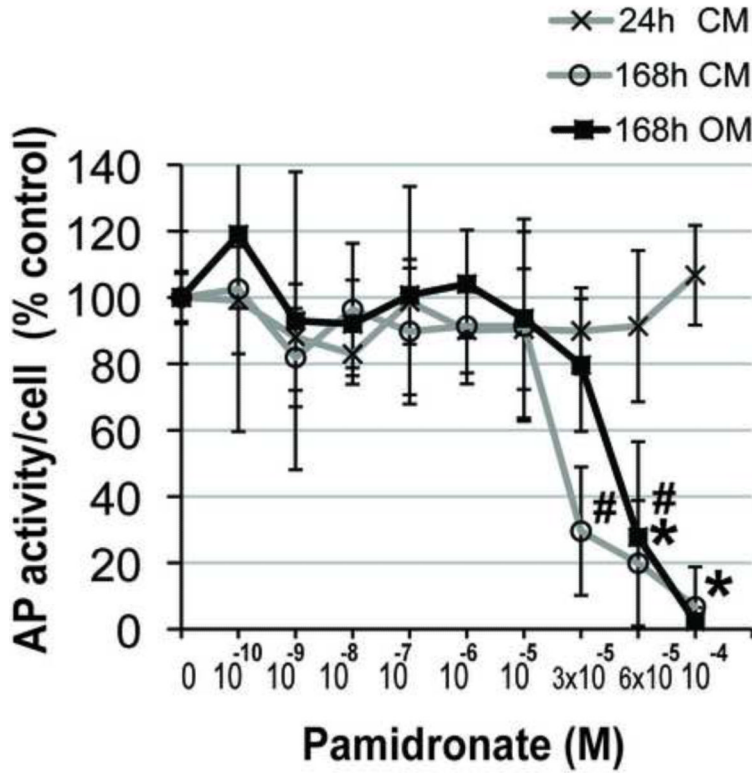
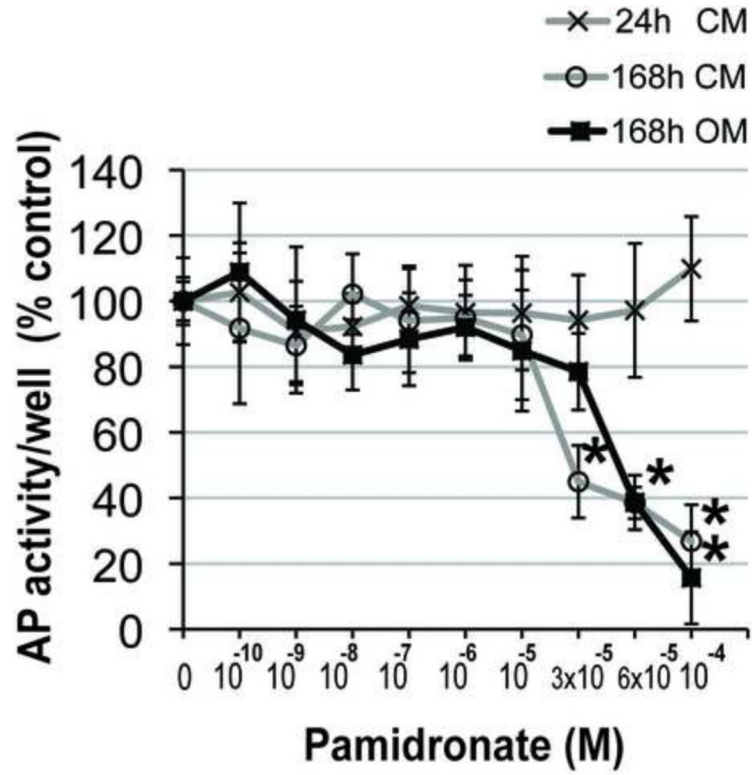


**Figure 1. Primary alveolar bone osteoblasts, long bone osteoblasts and BMSCs proliferation**  
Data represent averages  $\pm$  SD of measurements (n=4) obtained from four alveolar bone (AB), three iliac/long bone (LB) and three BMSCs samples.

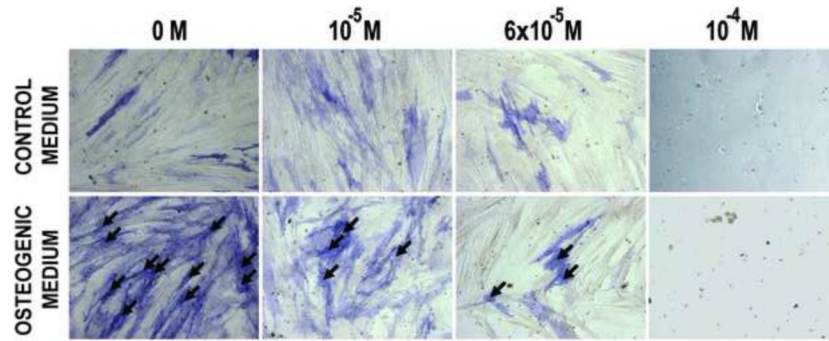




**Figure 2.** Effects of pamidronate on osteogenic cell viability (A, B) and proliferation (C, D) High concentrations ( $6 \times 10^{-5}$  M) of pamidronate significantly affected survival and decreased cell proliferation of alveolar osteoblasts (A, C) and BMSCs (B, D) in comparison to lower concentration groups (#,  $p < 0.05$ ) (\*,  $p < 0.001$ ). Data represent averages  $\pm$  SD of measurements ( $n=4$ ) obtained from four alveolar bone and three BMSCs samples.

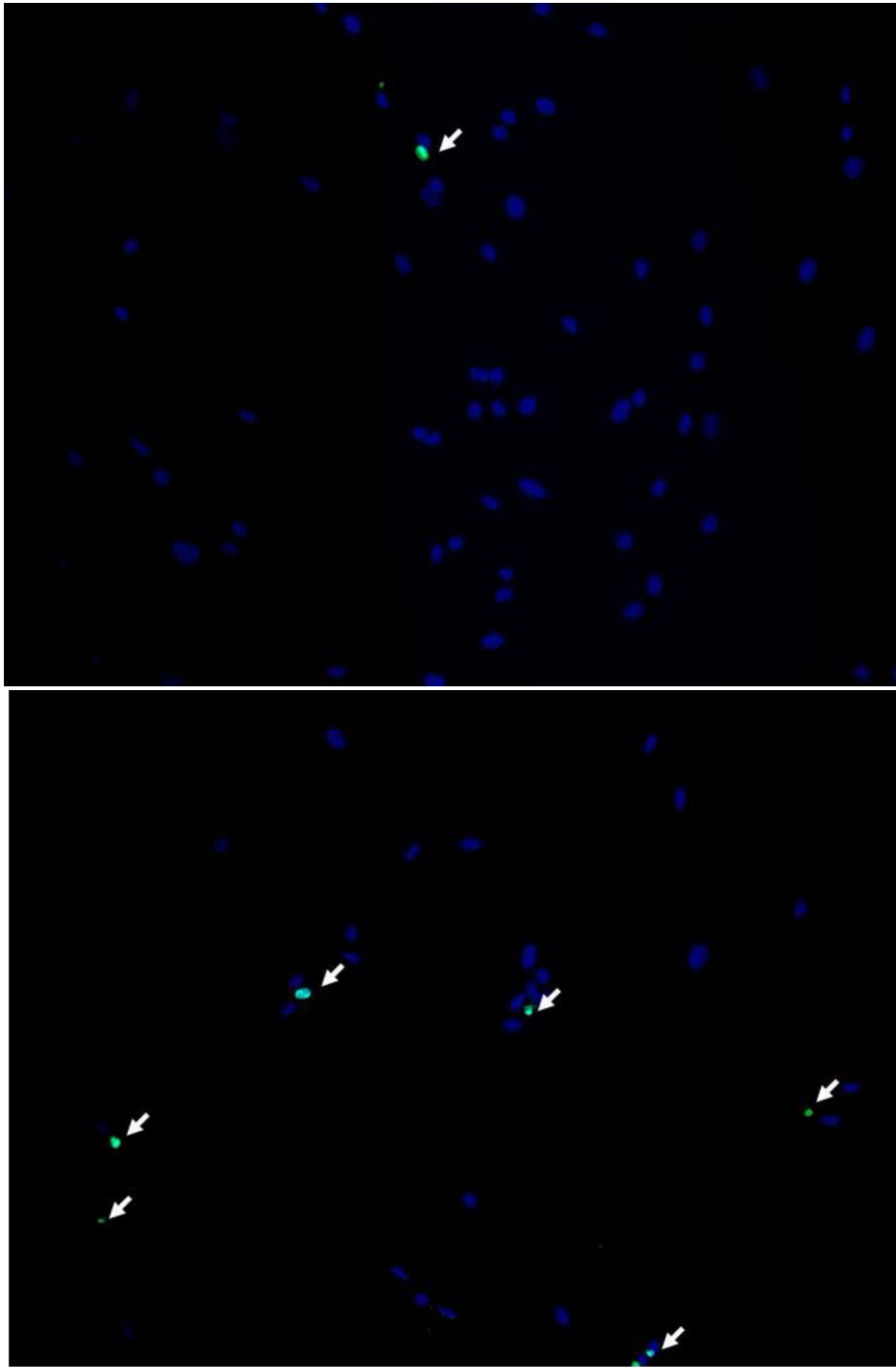


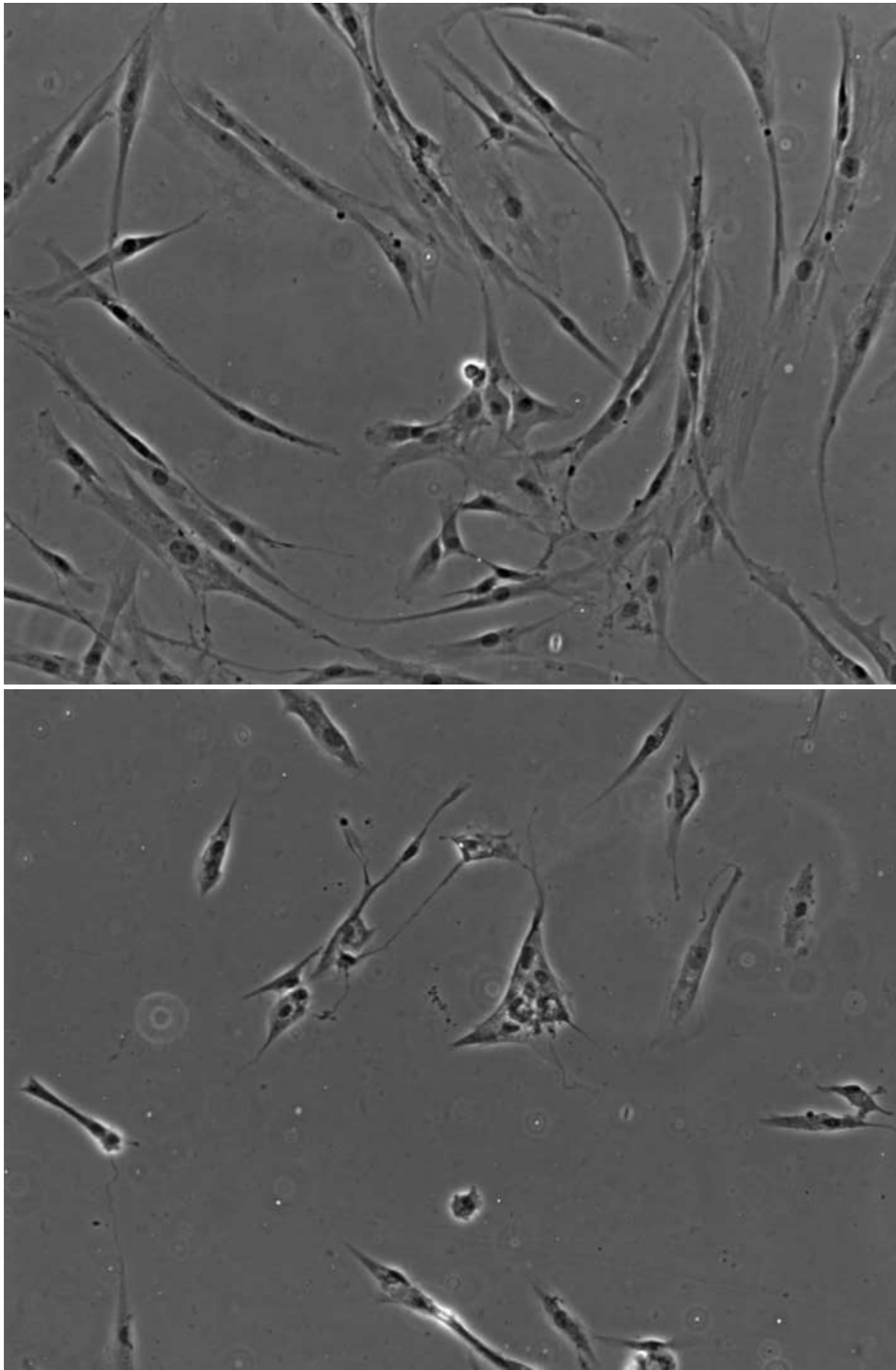


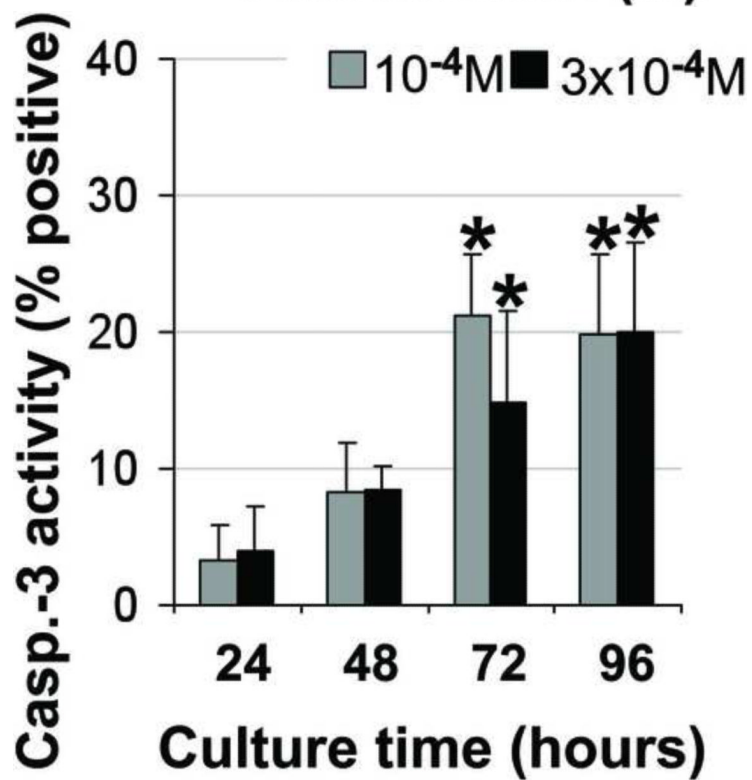
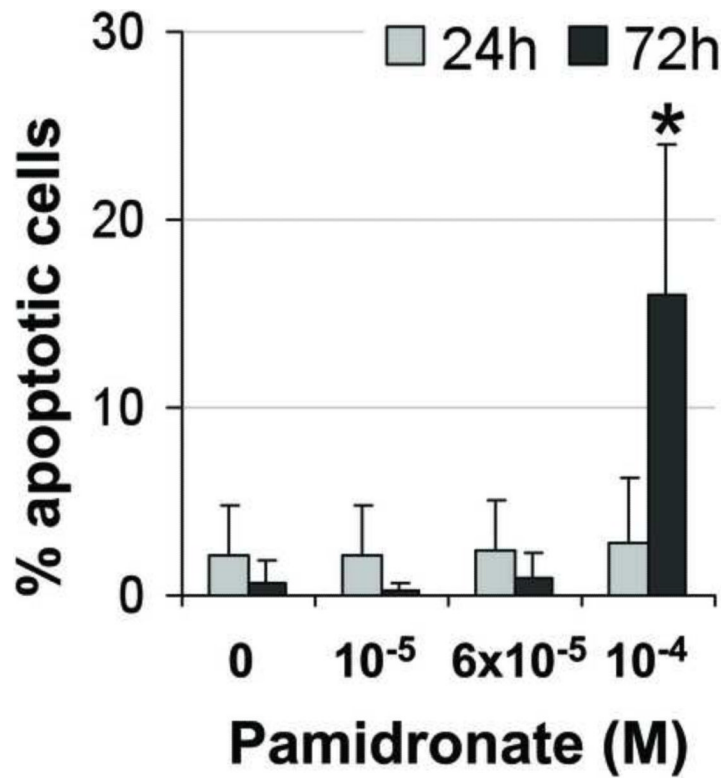


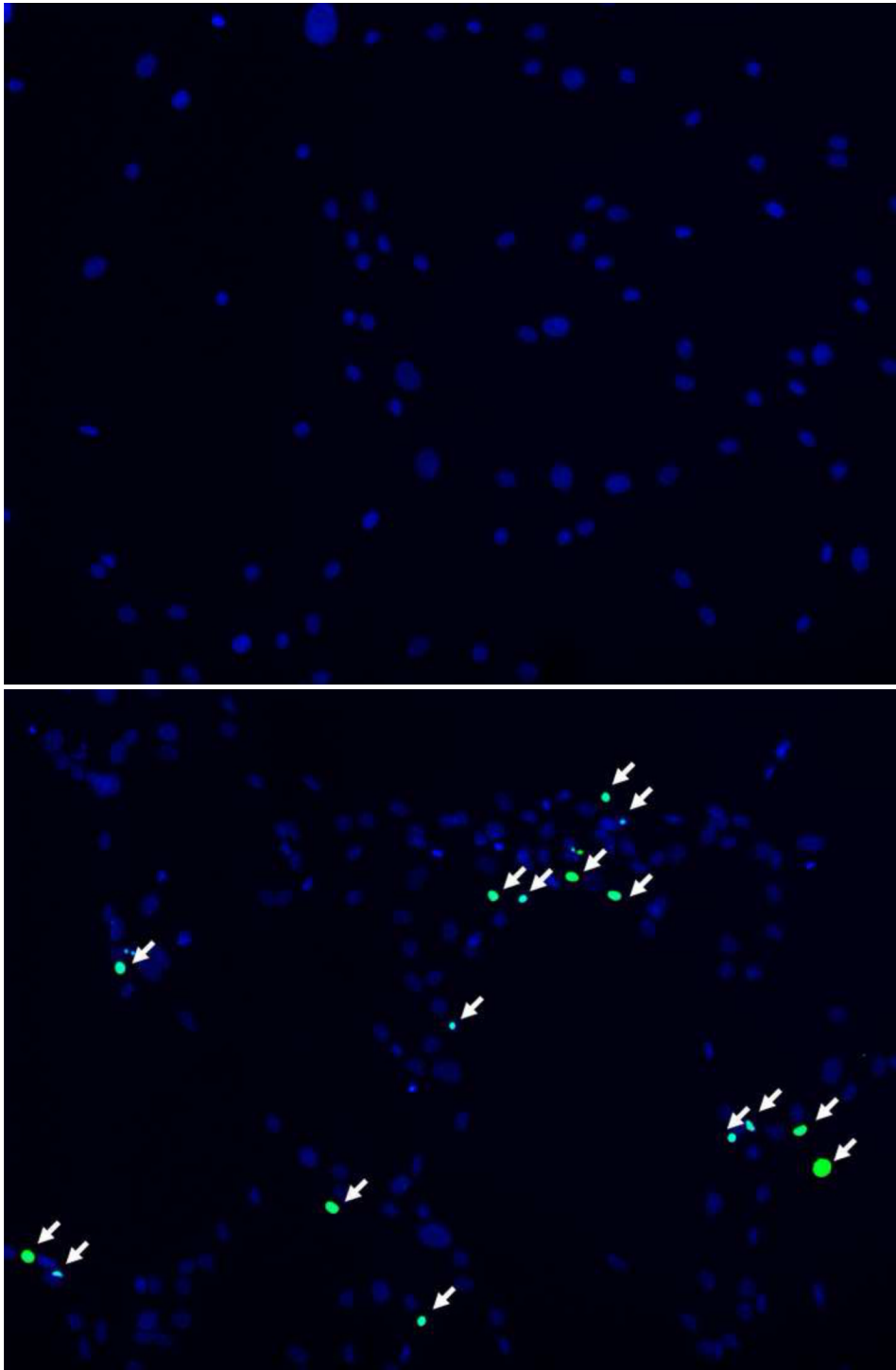
**Figure 3. Effects of pamidronate on alveolar osteoblast osteogenesis**

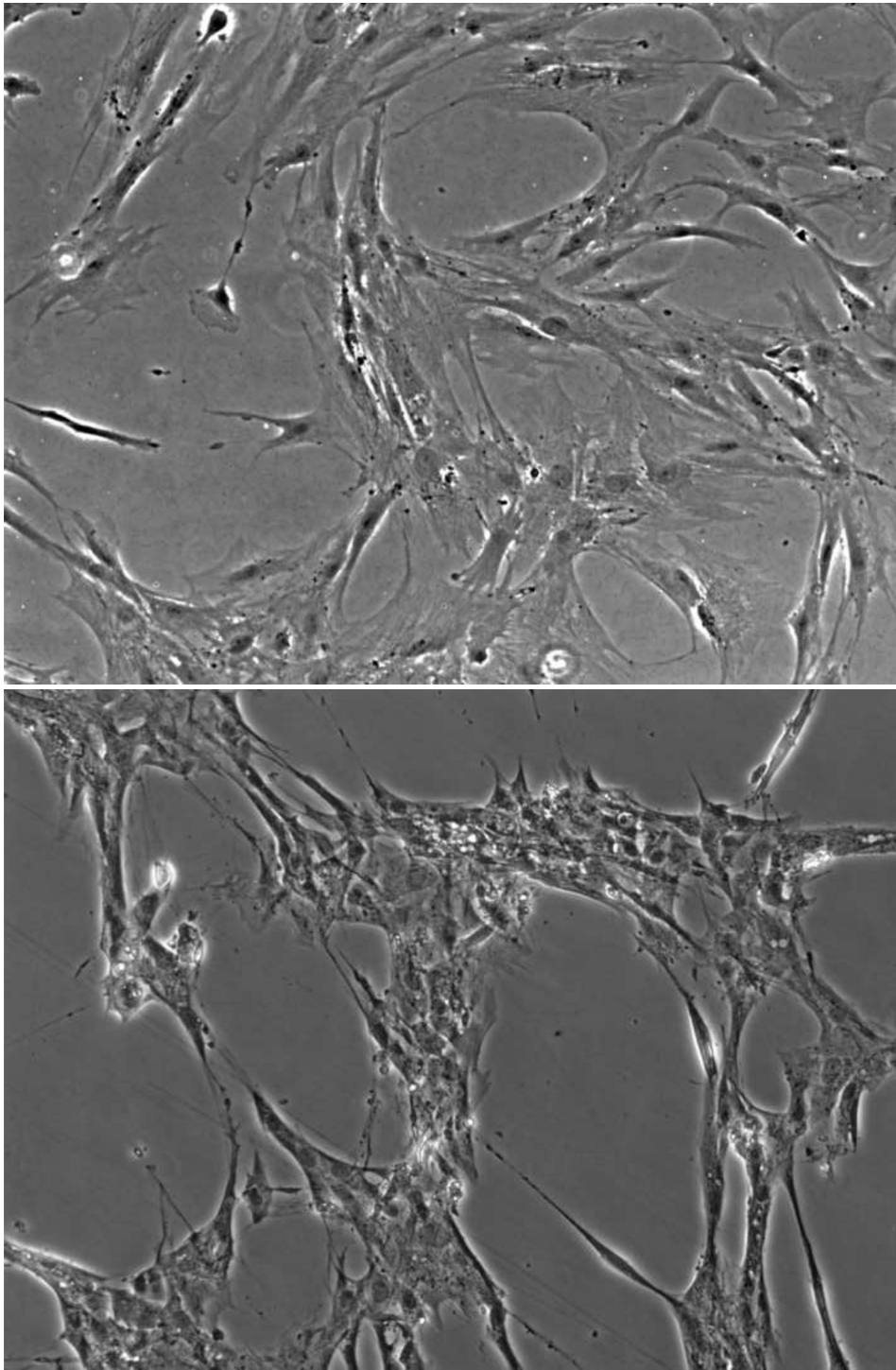
(A, B) Quantitative assessments of AP activity indicated significant decrease at higher pamidronate concentrations (#,  $p < 0.01$ ) (\*,  $p < 0.001$ ). The cells were cultured 168h in unsupplemented medium (CM) and in medium with osteogenic supplements (OM). Data represent averages  $\pm$  SD of measurements ( $n=4$ ) obtained from two alveolar bone samples. (C) Histological stains (Fast Blue RR Salt) indicated a decrease in the number of cells strongly expressing AP activity (black arrows) at higher pamidronate concentrations. Magnification 100 $\times$ .











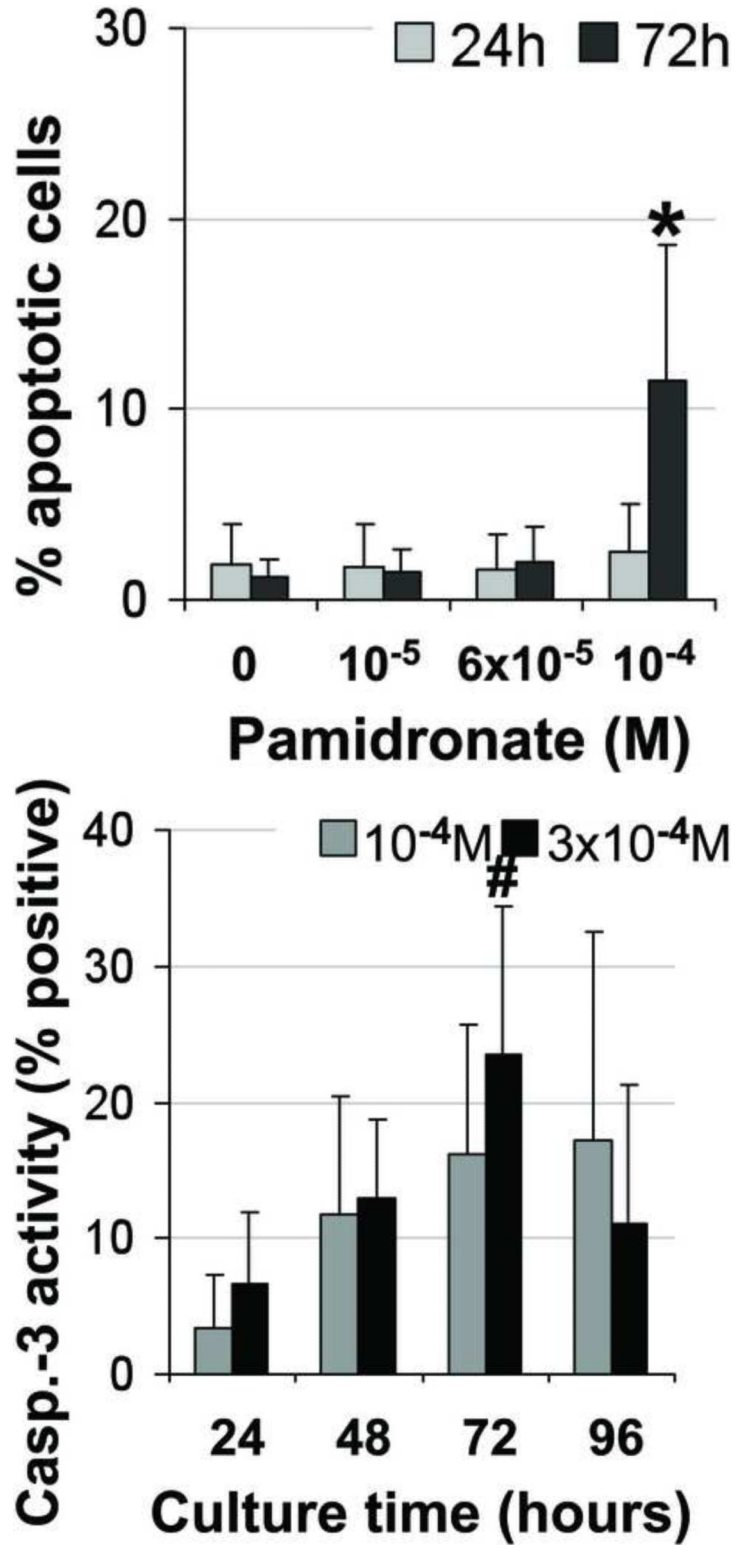
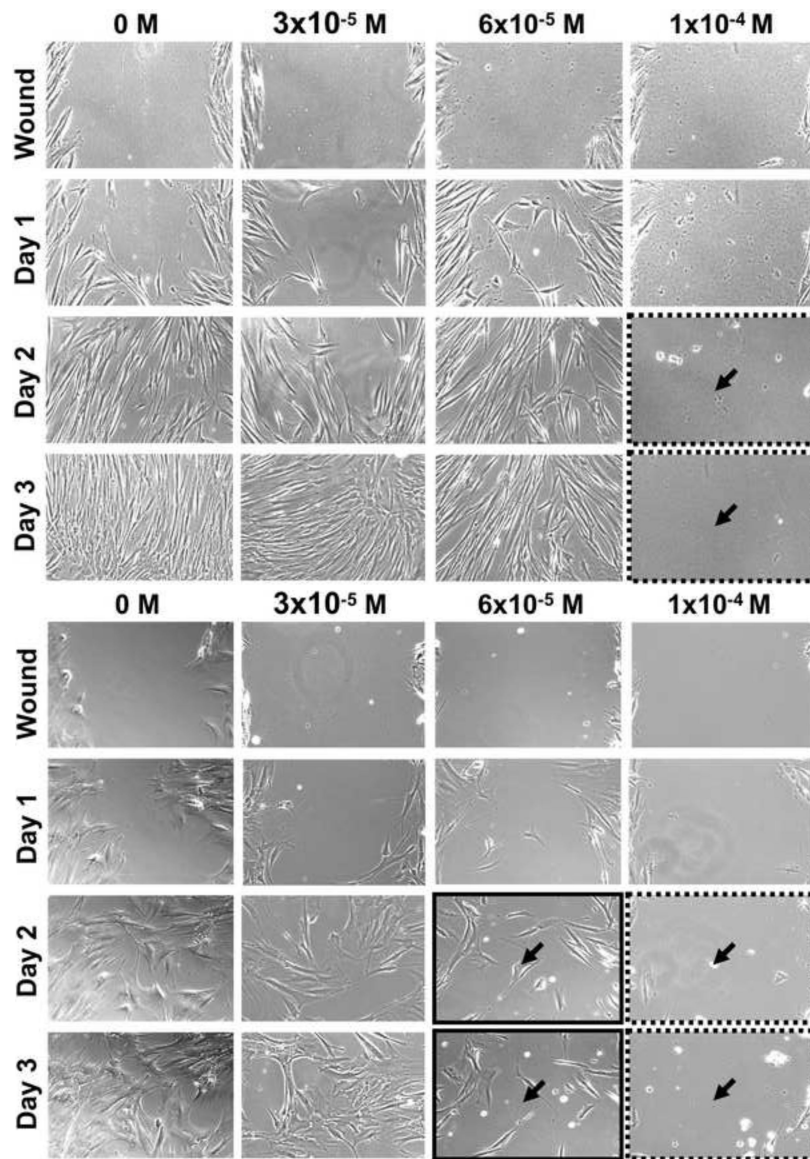


Figure 4. Induction of apoptosis in alveolar osteoblast (A, B, C, D, E, F) and BMSC (G, H, I, J, K, L) treated cultures  
Tunel assay (green stain) indicated the presence of apoptotic cells (white arrows) in the total cell population (blue stain - nuclei) treated with 10<sup>-4</sup> M pamidronate (B, H) compared to

untreated controls (**A, G**). Corresponding bright-field images are shown for pamidronate treated (**D, J**) and untreated cultures (**C, I**). Magnification 100×. Quantitative assessments of the Tunel slides (**E, K**) and measurements of caspase-3 activity (**F, L**) indicated a significant increase in the apoptotic cells after 72h of treatment with  $10^{-4}$  M pamidronate compared to lower concentrations (\*,  $p < 0.001$ ). A similarly increasing trend with significant increases of caspase-3 activity after 72h was found with three-times higher pamidronate concentration (#,  $p < 0.01$ ) (\*,  $p < 0.001$ ). Data represent averages  $\pm$  SD of measurements (n=3–4) obtained from three alveolar bone and three BMSCs samples.





**Figure 5. Wound healing assay**

Alveolar osteoblasts (A) and BMSCs (B) were pretreated with pamidronate for 24h, wounded and continuously monitored over several days. Pamidronate toxicity (black arrows) limited wound healing by BMSCs at  $6 \times 10^{-5}$  M pamidronate (solid brackets), and completely inhibited wound healing at  $10^{-4}$  M in both cell types (dotted brackets). Representative images are shown from testing of two alveolar bone and two BMSC samples. Magnification 100 $\times$ .