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## **Bisphosphonates affect migration ability & cell viability of HUVEC, fibroblasts and osteoblasts in-vitro**

Christian Walter, Andreas Pabst, Thomas Ziebart, Marcus Oliver Klein, B Al-Nawas

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# ORAL DISEASES

## Bisphosphonates affect migration ability & cell viability of HUVEC, fibroblasts and osteoblasts in-vitro

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# Bisphosphonates affect migration ability & cell viability of HUVEC, fibroblasts and osteoblasts in-vitro

Walter C, Pabst A, Ziebart T, Klein MO, Al-Nawas B

Walter C. and Pabst A. contributed equally

Department of Oral- and Maxillofacial Surgery, plastic surgery; Johannes-Gutenberg-University Mainz, Germany (Head: Prof. Dr. Dr. W. Wagner)

Correspondence:

Dr. Dr. Christian Walter

Department of Oral and Maxillofacial Surgery

Johannes Gutenberg-University Mainz

Augustusplatz 2

55131 Mainz

Germany

Phone: 0049-(0)-6131-17 3050

Fax: 0049-(0)-6131-176602

Email: [walter@mkg.klinik.uni-mainz.de](mailto:walter@mkg.klinik.uni-mainz.de)

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1  
2 Abstract

3  
4 Objective: Bisphosphonate-associated-osteonecrosis of the jaw (BP-ONJ) is a side effect in  
5  
6 patients being treated with bisphosphonates. The bisphosphonates most often associated with  
7  
8 BP-ONJ are the highly potent nitrogen-containing bisphosphonates e.g. pamidronate or  
9  
10 zoledronate. In terms of BP-ONJ aetiology, several theories are being discussed: inhibition of  
11  
12 bone remodelling, effect on soft tissues, and antiangiogenic effect of bisphosphonates. The  
13  
14 aim of this in-vitro study was to investigate the effect of different potent bisphosphonates on  
15  
16 osteoblasts, fibroblasts and human umbilicord vein endothelial cells (HUVEC).

17  
18 Materials and Methods: Three nitrogen-containing bisphosphonates (ibandronate,  
19  
20 pamidronate and zoledronate) and one non-nitrogen-containing bisphosphonate (clodronate)  
21  
22 were compared concerning their potency on apoptosis induction (tunel), cell viability (calcein  
23  
24 assay) and migration potency (boyden chamber) on osteoblasts, fibroblasts and HUVEC.

25  
26 Results: The nitrogen-containing bisphosphonates, particularly pamidronate and zoledronate,  
27  
28 affect cell viability, cell migration and the induction of apoptosis of osteoblasts, fibroblasts  
29  
30 and HUVEC.

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32 Conclusions: These results support the theory that BP-ONJ is a multifactorially caused  
33  
34 disease because several cell lines of the oral cavity which are responsible for integrity and  
35  
36 wound healing are negatively affected by nitrogen-containing bisphosphonates. Perioperative  
37  
38 interruption of bisphosphonate application during dental surgical procedures - if possible -  
39  
40 might be feasible to promote better wound healing.

## Introduction

For over 30 years, bisphosphonates have been used in medicine (Bassett et al., 1969). They are separated into highly potent nitrogen-containing and low potent non-nitrogen-containing bisphosphonates. Through inhibition of osteoclasts, bisphosphonates prevent skeletal related events such as pathological fractures or hypercalcaemic episodes. Their positive effects are without controversy and increase the quality of life in these patients (Ruggiero et al., 2009).

The negative side effects can be separated into four major groups: acute-phase reactions, gastrointestinal effects, renal side effects and bisphosphonate associated osteonecrosis of the jaw (BP-ONJ) (Diel et al., 2007). The American Association of Oral and Maxillofacial Surgeons defines BP-ONJ as exposed bone in the maxillofacial region that has persisted for more than eight weeks in combination with a current or previous bisphosphonate therapy, and a lack of head and neck radiation in patient's history (Ruggiero et al., 2009). The frequency of BP-PNJ is higher in patients with malignancies and intravenously delivered, highly potent bisphosphonates such as pamidronate and zoledronate, and lower in patients receiving oral bisphosphonates due to osteoporosis (Ruggiero et al., 2009). Only a few cases of BP-ONJ have been reported in connection with the administration of non-nitrogen containing bisphosphonates such as clodronate (Montazeri et al., 2007).

Several theories on BP-ONJ development are being discussed in the literature (Allen and Burr, 2009). The most common theory concentrates on the impact of bisphosphonates on bone, especially on osteoclasts. Osteoclasts are the main cellular target of bisphosphonates, inhibiting enzymes of the mevalonate pathways (nitrogen containing bisphosphonates) or synthesizing ATP derivatives (non nitrogen containing bisphosphonates) (Frith et al., 1997, Rogers et al., 2000). In addition to osteoclasts, osteocytes (Allen and Burr, 2008) and osteoblasts (Walter et al., 2009) are influenced by bisphosphonates. The consequence is suppressed bone remodelling with microdamage to the bone (Mashiba et al., 2005). The fact that, with only one exception (Polizzotto et al., 2006), BP-ONJ appears almost exclusively in

1  
2 the jaws still remains unclear. A possible reason is the higher remodelling rate of the jaw,  
3 particularly at the alveolar ridge, resulting in higher accumulation of bisphosphonate in  
4 comparison to other sites of the skeleton (Allen and Burr, 2009). In addition to bone, some  
5 authors focus on the soft tissues in the aetiology of BP-ONJ, since adequate wound healing is  
6 lacking in these patients (Reid et al., 2007). Failed wound healing might result in exposed and  
7 secondary necrotic bone. A similar mechanism could be discussed in patients with periodontal  
8 disease, where the soft tissues are disturbed by inflammation, and no appropriate soft tissue  
9 layer covering the bone is present (Walter et al., 2008). It is difficult to explain cases of BP-  
10 ONJ at the mylohyoid ridge which lack a trigger factor without taking the impact on the soft  
11 tissues into account. Bisphosphonates might be released from the underlying bone and may  
12 compromise the very thin mucosal layer in this area. The pressure of the tongue against this  
13 ridge, and the influence of the mylohyoid muscle might affect the compromised soft tissue  
14 layers so that a defect in the covering soft tissue evolves. Next to the oral keratinocytes  
15 (Landesberg et al., 2008, Rubegni and Fimiani, 2006), fibroblasts and blood vessel cells are  
16 involved in this process (Walter et al., 2009). The reduced potency of the latter is desired in  
17 terms of suppressed angiogenesis tumor therapy (Green and Clezardin, 2002). For some  
18 bisphosphonates, a proapoptotic effect on cancer cells is reported (Santini et al., 2003).  
19 Therefore bisphosphonates might inhibit several cell types necessary for wound healing, such  
20 as osteoblasts, fibroblasts and vessel cells. In summary, a broad range of cells and their  
21 functions is affected by bisphosphonates, and not just osteoclasts. Since BP-ONJ frequencies  
22 differ among the several bisphosphonates, the impact on cell viability, cell migration and  
23 apoptosis of osteoblasts, fibroblasts and endothelial cells was analyzed.  
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## Materials and Methods

Cell culture: Cell cultures were prepared and maintained according to standard cell culture procedures. Commercially available human umbilicord vein endothelial cells (HUVEC; Lonza, Basel, Switzerland) were cultured in an endothelial basal medium (EBM) supplemented with 1µg/ml hydrocortisone, 12 µg/ml bovine brain extract, 50 µg/ml gentamicin, 50 ng/ml amphotericin-B, 10 ng/ml epidermal growth factor and 10% fetal calf serum (FCS).

Human osteogenic cells (HOB; Promocell, Heidelberg, Germany), were cultivated in a solution composed of Dulbecco's Modified Eagle's Medium (DMEM) with 1% PSN, 1% L-glutamin and 10% FCS. Before the experiments, the osteogenic cells were positively characterized by immunohistochemical expression of alkaline phosphatase and osteocalcin (labelled streptavidin-botin/horseradish peroxidase).

Fibroblasts (Lonza, Basel, Switzerland) were grown in Stroma Cell Growth Medium (SCGM) with 1% Penicillin-Streptomycin-Neomycin antibiotic mixture (PSN), 10% FCS, and 500ng basic fibroblast growth factor per 500ml medium.

All cell cultures were incubated and cultured in an incubator with 5% CO<sub>2</sub> and 95% air at 37° C till the third passage of cell generation. Cells were passaged at regular intervals depending on their growth characteristics using 25% trypsin (Seromed Biochrom, Berlin, Germany).

Calcein viability assay: To analyze the effect of bisphosphonates on cell viability, we evaluated a Calcein Viability Assay. HUVEC, fibroblasts and human osteoblasts were cultivated in standard cell culture as described. All cells were incubated in standard 24-well culture plates with different bisphosphonates (clodronat, ibandronate, pamidronate, zoledronate) in gradient concentrations (0 µmol, 5 µmol, 50 µmol, 100 µmol, 200 µmol, 500 µmol) for 72 hours. This range of concentrations was selected because in-vivo concentrations in plasma shortly after zoledronate infusion are about 5 µmol, and concentrations in bone shortly after infusion can be 100 fold higher (Chen et al., 2002). After removing the used

1  
2 culture medium from the wells, they were filled with 500µl fresh culture medium and 7µl  
3 Calcein-AM (Invitrogen™, Eugene, Oregon, USA) per well. The plates were incubated in a  
4 dark cell incubator for 1 hour. Subsequently, the Calcein-AM medium was also removed and  
5  
6 cells were detached with 500 µl Trypsin-EDTA solution per well. 200 µl of the cell- Trypsin  
7  
8 solution was carried in standard black 24-well cell culture plates and the extent of cell  
9  
10 viability was measured by Microplate Fluorescence Reader (BioTek FLx800, Bad  
11  
12 Friedrichshall, Germany) with an extinction wave length of 480nm and an emission wave  
13  
14 length of 520 nm.  
15

16  
17 TUNEL assay: For the apoptosis TUNEL-Assay, the three cell lines (HUVEC, HOB, NHDF,  
18  
19  $5 \times 10^4$ ) in EBM, DMEM and SCGM medium were seeded to 6 well plates. After 24 hours, all  
20  
21 cell lines were incubated with 50µmol bisphosphonates (clodronate, ibandronate, pamidronate  
22  
23 and zoledronate) including a negative control group for 72 hours. Cells were solved by  
24  
25 Trypsin-EDTA and centrifuged onto cover slips by 2000 rpm for 5 minutes. Then cells were  
26  
27 fixed with 3% paraformaldehyd for 10 minutes at room temperature (RT), permeabilized with  
28  
29 0.1% Triton X-100, 0.1% sodium citrate for 2 minutes on ice, washed twice with PBS, pH  
30  
31 7.4, and incubated for one hour at 37° C in the dark with a TUNEL reaction mixture  
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33 (Boehringer, Ingelheim, Germany), for in situ detection of cell death. After washing twice  
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35 with PBS, pH 7.4, cells were incubated at RT with the Hoechst solution for 5 minutes. All  
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37 Hoechst-positive nuclei as well as TUNEL-positive nuclei were visualized using a Zeiss  
38  
39 Axioplan fluorescence microscope. Apoptosis was expressed as a percent of fragmented  
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41 Hoechst-positive nuclei versus total Hoechst-positive nuclei, and as a percent of TUNEL-  
42  
43 positive nuclei versus total Hoechst-positive nuclei and are shown as fold increase versus  
44  
45 control.  
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47 Migration Assay (Boyden-Chamber): To examine the effects of bisphosphonates on the  
48  
49 migration ability of HUVEC, osteoblasts and fibroblasts, a 24-well Boyden Chamber Assay  
50  
51 system (ThinCert™, pore size 8µm translucent, Greiner BioOne, Essen, Germany) with a  
52

1  
2 pore size of 8µm was used according to the manual (Greiner BioOne, Essen, Germany). All  
3  
4 cell lines were incubated for 72 hours with different bisphosphonates and 50 µmol. Cells were  
5  
6 harvested, washed twice in PBS and re-suspended in cell culture medium at a final  
7  
8 concentration of  $10^6 \text{ ml}^{-1}$ . After 24 hours, the cells were stained with Calcein-AM  
9  
10 fluorescent dye (invitrogen™, Eugene, Oregon, USA). Thereafter, the culture medium was  
11  
12 removed from the inserts, and the inserts were transferred to the wells of a new 24-well plate  
13  
14 containing 500µl Trypsin-EDTA per well. This plate was incubated for 10 minutes in a cell  
15  
16 culture incubator at 37°C and 5% CO<sub>2</sub>. The inserts were removed, and 200 µl of the Trypsin-  
17  
18 EDTA solution, now containing the detached migratory cells, was transferred from each well  
19  
20 of the 24 well-plate to a well of a flat-bottom black 24-well plate. For quantification of cell  
21  
22 migration, we used a Microplate Fluorescence Reader (BioTek FLx800, Bad Friedrichshall,  
23  
24 Germany) with an extinction of 480 nm and emission of 520 nm. For each cell line and each  
25  
26 bisphosphonate the experiments were conducted 12 times.

27  
28 Statistical analysis: The hypothesis was that incubation of bisphosphonates with varying  
29  
30 potencies has a distinct effect on cell viability, cell migration ability and apoptosis of  
31  
32 osteoblasts, fibroblasts and endothelial cells. As a control, non-incubated cells were used.  
33  
34 Continuous variables are expressed as mean +/- standard deviation. Comparisons between  
35  
36 groups were analysed by ANOVA (post hoc test: Tukey) for experiments with more than two  
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38 subgroups (SPSS version 17.0). P values <0.05 were considered statistically significant.  
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2 Results

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4 Calcein viability-assay (figure 1 a-c):

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6 Osteoblasts: At lower concentrations (5 – 50  $\mu\text{mol}$ ), the impact of zoledronate on osteoblasts  
7  
8 was the strongest compared to the other bisphosphonates, and was significant compared to all  
9  
10 bisphosphonates except for clodronate, which depended on concentration. At 5 $\mu\text{mol}$ , only  
11  
12 zoledronate had a significant reduction on cell viability compared to the control ( $p=0.006$ ).

13  
14 Osteoblasts were affected by clodronate, which turned out to be significant compared to the  
15  
16 control at 100 $\mu\text{mol}$  ( $p=0.036$ ). There were no further significant differences between  
17  
18 pamidronate and zoledronate, and there were no differences at any concentration between  
19  
20 ibandronate and clodronate.

21  
22 Fibroblasts: There was no significant difference at 5 $\mu\text{mol}$  between the groups. At 50 $\mu\text{mol}$ , the  
23  
24 differences between the control and all the other bisphosphonates ( $p=0.002$  up to 0.037) were  
25  
26 significant. From this concentration level and higher, the difference between clodronate and  
27  
28 all the other bisphosphonates were significant. The differences between the nitrogen  
29  
30 containing bisphosphonates were significant as of 200 $\mu\text{mol}$ , with a significant difference  
31  
32 between pamidronate and ibandronate ( $p<0.001$ ), and pamidronate and zoledronate ( $p=0.03$ ).  
33  
34 At 500 $\mu\text{mol}$  there is no longer a difference between pamidronate and zoledronate, but a  
35  
36 significant difference between ibandronate and clodronate ( $p<0.001$ ), and ibandronate and  
37  
38 pamidronate ( $p=0.008$ ) was found.

39  
40 HUVEC: There were no significant differences at 5 $\mu\text{mol}$ . At 200 $\mu\text{mol}$ , clodronate has a  
41  
42 significant impact on the cells ( $p=0.047$ ). All other bisphosphonates turned out to have a  
43  
44 significant impact at 50 $\mu\text{mol}$  ( $p<0.001$ ). The differences between zoledronate and all the other  
45  
46 bisphosphonates was significant as well ( $p<0.001$ ). At a concentration of 100 $\mu\text{mol}$ , the  
47  
48 difference of the impact of zoledronate and pamidronate was no longer significant.

49 TUNEL-Assay (figure 2):

1  
2 Osteoblasts: Zoledronate and pamidronate had the greatest impact but there was no significant  
3  
4 difference between these two bisphosphonates. The effect of ibandronate was significantly  
5  
6 weaker ( $p < 0.001$  compared to zoledronate, and  $p = 0.006$  compared to pamidronate). There  
7  
8 was no significant difference of clodronate compared to the control, and no significant  
9  
10 difference compared to ibandronate.

11 Fibroblasts: All differences between the respective bisphosphonates were statistically  
12  
13 significant ( $p$  between  $p < 0.001$  and  $0.007$ ). Zoledronate had the strongest impact, followed by  
14  
15 pamidronate, ibandronate and clodronate. The level of apoptosis was nearly 77% for  
16  
17 zoledronate and 61% for pamidronate.

18  
19 HUVEC: Pamidronate and zoledronate had the strongest effect ( $p < 0.001$ ) but there was no  
20  
21 significant difference between these two bisphosphonates ( $p = 0.522$ ). Ibandronate and  
22  
23 clodronate had a considerably smaller but nevertheless significant effect on the apoptosis rate  
24  
25 ( $p < 0.001$  for ibandronate and  $p = 0.005$  for clodronate). The impact difference between  
26  
27 ibandronate and clodronate was not significant ( $p = 0.351$ ).

28  
29 Boyden Migration Assay (figure 3):

30  
31 For each cell type, the ANOVAs conducted showed significant differences among the  
32  
33 experimental groups and control group. Each ANOVA yielded a  $p$ -value  $< 0.001$ .

34  
35 Osteoblasts: Only the nitrogen containing bisphosphonates inhibited the migration ability of  
36  
37 osteoblasts. There was no significant difference between zoledronate, pamidronate and  
38  
39 ibandronate.

40  
41 Fibroblasts: All bisphosphonates inhibited the migration ability, save clodronate. The  
42  
43 strongest effect was measured for pamidronate ( $p < 0.001$  compared to all other  
44  
45 bisphosphonates). There was no significant difference between zoledronate and ibandronate.

46  
47 HUVEC: All bisphosphonates inhibited the migration ability of HUVEC. Zoledronate and  
48  
49 pamidronate had the strongest effect, but there was no significant difference between these  
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2 two bisphosphonates. There were similar results for the significantly less potent clodronate  
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4 and ibandronate with no significant difference between them.  
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2 Discussion

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4 In addition to reduced bone remodelling several theories regarding the aetiology of  
5 bisphosphonate osteonecrosis of the jaw are being discussed in literature (Allen and Burr,  
6 2009). The range of aetiological possibilities has been broadened with the realization that not  
7 only osteoclasts, but rather several cell types are affected by bisphosphonates, as shown in  
8 this study.  
9

10 Clodronate, representing the non-nitrogen containing bisphosphonates, has nearly no impact  
11 on the apoptosis rate of the investigated cell lines. The effect of clodronate on cell viability of  
12 HUVEC fibroblasts and osteoblasts was not as large compared to the other bisphosphonates,  
13 yet still yielded significant results in higher concentrations. As well, the effect on cell  
14 migration was marginal and statistically significant only for HUVEC, so that the over all  
15 effect of clodronate is minimal, which could explain the rarity of BP-ONJ in patients  
16 receiving clodronate. Among the nitrogen containing bisphosphonates represented by  
17 ibandronate, pamidronate and zoledronate, pamidronate and zoledronate had the greatest  
18 effect on all cell types and functions. One exception is the effect of zoledronate and  
19 Pamidronat on the migration rate of fibroblasts with a way higher effect of pamidronat. The  
20 apoptosis rate in cells incubated with ibandronate is either as high as the cells incubated with  
21 clodronate (osteoblasts, HUVEC) or is little higher (fibroblasts). A similar results was  
22 obtained for the cell viability, with an effect just as high as clodronate for osteoblasts. The  
23 effect on fibroblasts and HUVEC was stronger, but not as strong as the effect of zoledronate  
24 and pamidronate. These last two bisphosphonates had the greatest impact on the apoptosis  
25 rate, the cell viability and the migration ability of all cell lines with only a few exceptions.  
26

27 Similar results regarding migration ability and cell viability have previously been described  
28 (Walter et al., 2009). A migration assay was performed with a scratch wound assay, and  
29 results showed that clodronate had had the weakest effect on all cell types, which is similar to  
30 the present study. The cell migration was most strongly inhibited by zoledronate and  
31

1  
2 pamidronate, with the exception of fibroblast cell migration of where ibandronat had a greater  
3  
4 effect than zoledronate. A similar effect is seen in the present study with the greatest effect of  
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6 pamidronate on fibroblast cell migration. The cell viability test in the previously published  
7  
8 study (Walter et al., 2009) conducted with the MTT-test clodronate evaluated the weakest  
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10 effect on all tested cell types as well. Among the nitrogen containing bisphosphonates,  
11  
12 pamidronate and zoledronate had a greater effect on the cell viability compared to  
13  
14 ibandronate. The cell viability measured with the calcein assay seems to be higher compared  
15  
16 to the ones evaluated with the MTT-test. This is well in accordance with the underlying test  
17  
18 methods, as MTT is a metabolism test and therefore might be more sensitive than calcein  
19  
20 staining alone. It should however be noted, that in these in vitro experiments for all cell types  
21  
22 and functions tested, the influence of the different bisphosphonates is reproducible. This is  
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24 even of more significance as the effect ranking of the bisphosphonates is also nearly the same  
25  
26 in these experiements.

**Deleted:** which may indicate some variability is due to the test procedure

**Deleted:** However,

27 This in vitro study verified an impact on osteoblasts, fibroblasts and HUVEC thus supporting  
28  
29 the idea of a multifactor pathology of BP-ONJ. It supports the clinical theory of damage to  
30  
31 osteogenic and blood vessel cells and soft tissue repair.  
32  
33 These results should be verified in an animal model and might be verified in specimens from  
34  
35 patients with BP-ONJ.

**Deleted:** This study verified an impact on osteoblasts, fibroblasts and HUVEC thus supporting the idea of a multifactor pathology of BP-ONJ.

36  
37 The distribution of bisphosphonates in patients with BP-ONJ is reflected by this study's  
38  
39 results. Within the natural limitations of in vitro studies, these data support the theory that  
40  
41 bisphosphonates influence soft tissue cells such as vessel cells and fibroblasts, in addition to  
42  
43 osteoblasts. Although the half-life of bisphosphonates can be more than ten years (Wang et  
44  
45 al., 2007), the finding that soft tissue cells are affected by bisphosphonates could indicate that  
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47 interruption of bisphosphonate treatment to promote better wound healing in the oral cavity  
48  
49 may be reasonable.  
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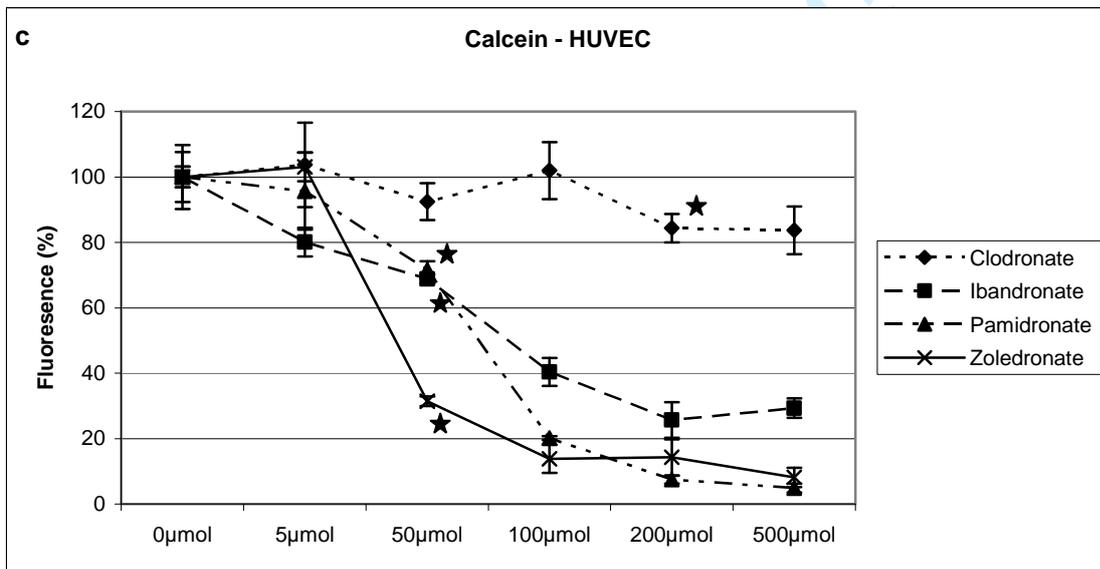
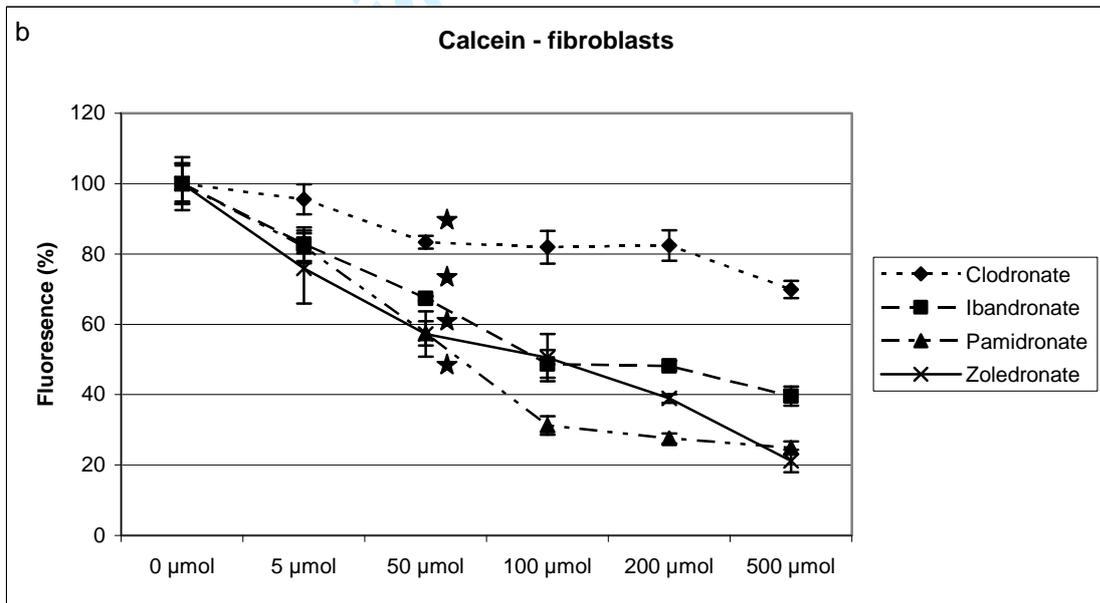
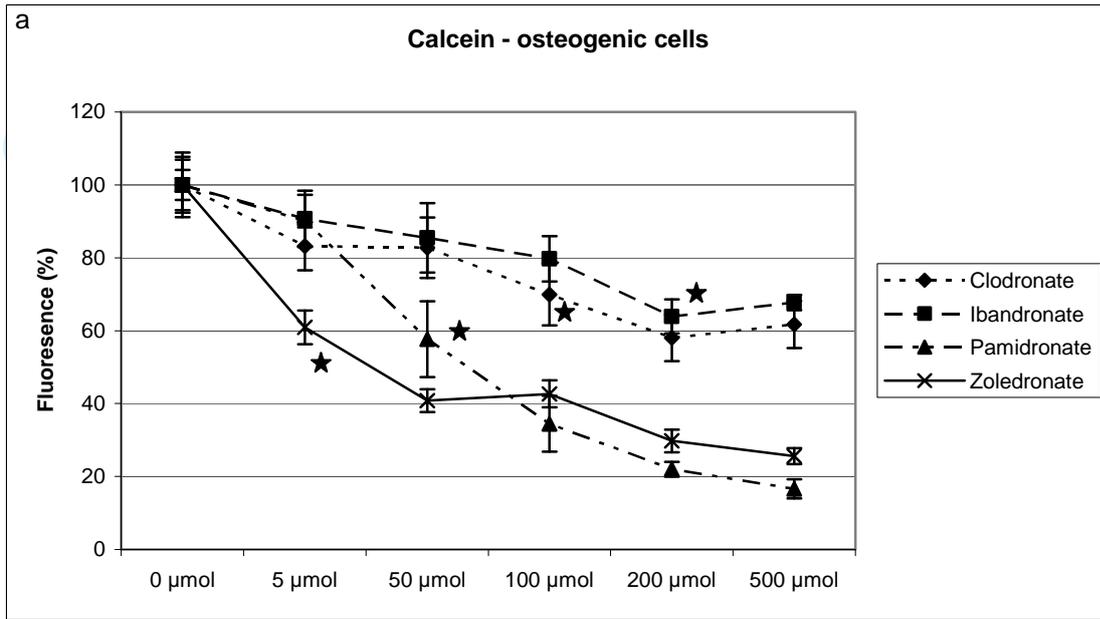
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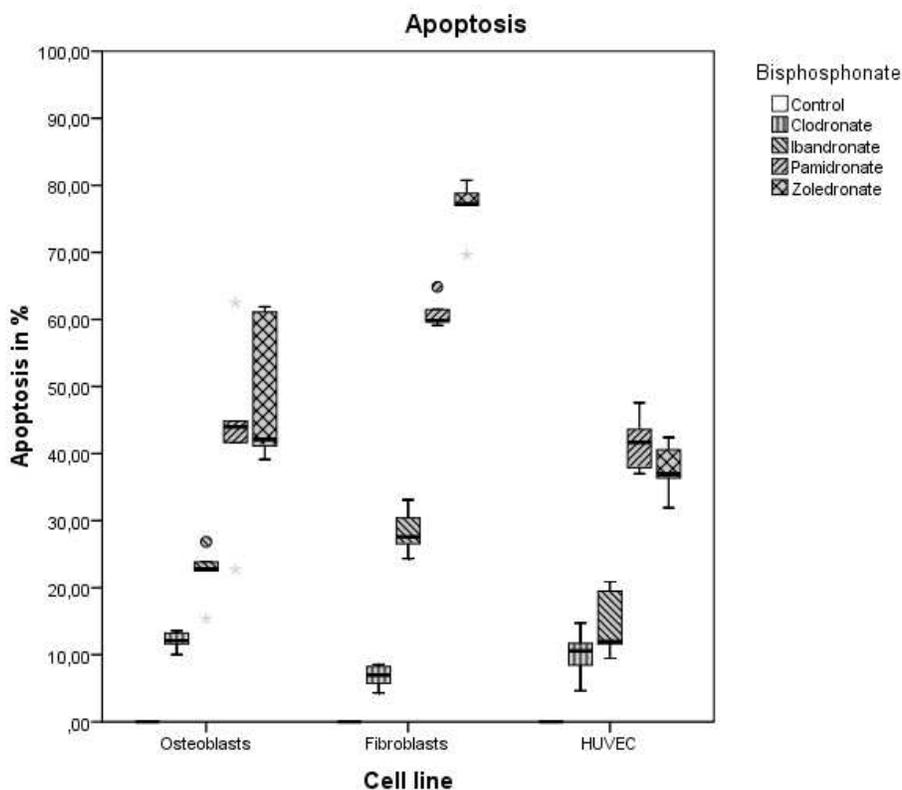
Figure 1: Cell viability test for osteogenic cells (a), fibroblasts (b) and human umbilicord vein endothelial cells (c) influenced by clodronate, ibandronate, pamidronate and zoledronate at different concentrations compared to a control set to 100%. Asterix indicate concentrations that are significantly different from control.

Figure 2: TUNEL assay for osteogenic cells, fibroblasts and human umbilicord vein endothelial cells influenced by 50 $\mu$ mol clodronate, ibandronate, pamidronate or zoledronate compared to a control without bisphosphonates. The black bar in the middle of each box represents the median. The box includes all values between the 25<sup>th</sup> and 75<sup>th</sup> percentile. Whiskers indicate values still within 1.5 interquartile range (IQR). Outliers within 3 IQR are represented as circles, outliers over 3 IQR are represented as asterix.

Figure 3: Boyden chamber migration assay for osteogenic cells, fibroblasts and human umbilicord vein endothelial cells influenced by 50 $\mu$ mol clodronate, ibandronate, pamidronate or zoledronate compared to a control without bisphosphonates. The black bar in the middle of each box represents the median. The box includes all values between the 25<sup>th</sup> and 75<sup>th</sup> percentile. Whiskers indicate values still within 1.5 interquartile range (IQR). Outliers within 3 IQR are represented as circles.

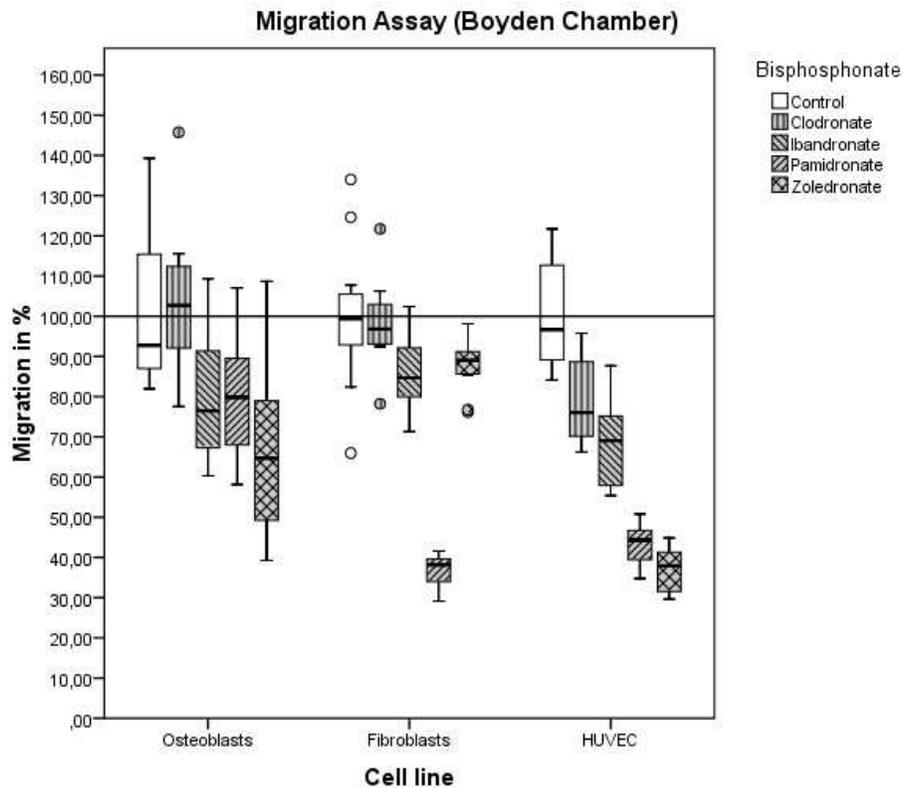


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Tunel assay for osteogenic cells, fibroblasts and human umbilicord vein endothelial cells influenced by 50µmol clodronate, ibandronate, pamidronate or zoledronate compared to a control without bisphosphonates. The black bar in the middle of each box represents the median. The box includes all values between the 25th and 75th percentile. Whiskers indicate values still within 1.5 interquartile range (IQR). Outliers within 3 IQR are represented as circles, outliers over 3 IQR are represented as asterix.

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Boyden chamber migration assay for osteogenic cells, fibroblasts and human umbilicord vein endothelial cells influenced by 50 $\mu$ mol clodronate, ibandronate, pamidronate or zoledronate compared to a control without bisphosphonates. The black bar in the middle of each box represents the median. The box includes all values between the 25th and 75th percentile. Whiskers indicate values still within 1.5 interquartile range (IQR). Outliers within 3 IQR are represented as circles.