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ORIGINAL ARTICLE

Effects of an oral bisphosphonate and three intravenous bisphosphonates on several cell types in vitro

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Abstract

Objective To analyze the influence of an oral bisphosphonate and compare the potency to intravenous bisphosphonates on various cell types as regards the rarity of bisphosphonate-associated osteonecrosis of the jaw (BP-ONJ) caused by oral bisphosphonate.

Materials and methods A viability assay (MTT), a migration assay (Boyden chamber), and an apoptosis assay (Caspase-Glo® 3/7) were performed to analyze the effect of bisphosphonates on human fibroblasts, umbilical vein endothelial cells (HUVEC), and osteoblasts.

Results Alendronate and intravenous bisphosphonates suppressed cell viability and migration, and induced apoptosis in all tested cell types. Alendronate had a greater impact than ibandronate on the characteristics in fibroblasts and osteoblasts but not as strong as zoledronate.

Conclusions The incidence of BP-ONJ in oral bisphosphonate treatment is reported to be much lower than that in intravenous bisphosphonates. However, the influences of alendronate on human cells were at least as strong as ibandronate, although it was lower than zoledronate.

Clinical relevance Alendronate showed strong enough effects to suppress human somatic cells and was comparable to certain intravenous bisphosphonates in potency. This study suggests that the lower incidence of BP-ONJ in alendronate treatment is not originated by its potency, but might be due to the low bioavailability of alendronate, lower dosing on a daily basis, and having no additional therapies.

Keywords Bisphosphonate · Alendronate · Bisphosphonate-associated osteonecrosis of the jaw · BP-ONJ · HUVEC · Fibroblasts · Osteoblasts

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Introduction

Bisphosphonates decrease bone resorption by their anti-osteoclastic activity. Their avid affinity to bone tissue and anti-resorption activity have made bisphosphonates a popular choice for treatment of bone diseases, such as osteoporosis, Paget's disease, multiple myeloma, and several tumors metastasizing into the bone tissue [1–5]. The anti-tumor property of bisphosphonates depends on anti-resorptive and anti-angiogenic effects and induction of tumor cell apoptosis [6–8]. Because of these effects, bisphosphonates are prescribed for bone malignancies as an effective treatment to reduce skeletal complications and the need for additional radiotherapy and chemotherapy and stabilizing operations [4]. Moreover, since alendronate was approved for postmenopausal osteoporosis prevention and treatment in 1995,

bisphosphonates became more widely used. The number of prescriptions for oral bisphosphonates was almost 15 million in the USA in 2012 [9].

The most common adverse effects of bisphosphonates are gastrointestinal effects, renal impairment, acute-phase reactions, and bisphosphonate-associated osteonecrosis of the jaw (BP-ONJ) [10]. There have been many attempts to identify and explore the nature of BP-ONJ since Marx reported the intraoral necrotic bony lesions in patients exposed to bisphosphonates in 2003 [11].

Bisphosphonates affect several types of somatic cells including keratinocytes, fibroblasts, human umbilical endothelial cells (HUVEC), endothelial progenitor cells, and osteoblasts, as well as osteoclasts [12–14] so that soft tissues and vessels surrounding bone are also compromised. Given that nitrogen-containing bisphosphonates suppress the mevalonate pathway by inhibiting enzymes involved in the synthesis of farnesyl diphosphate (FPP) [15] and that non-nitrogen-containing bisphosphonates are metabolically converted into cytotoxic analogues of adenosine triphosphate (ATP) [16, 17], it is reasonable to assume that bisphosphonates influence various cell types by the same process as in osteoclasts. Compromised functions in those cells may suppress bone formation and wound healing, and restrict the supply of nutrients. Therefore, bone and surrounding tissue viability may decline, contributing to the occurrence of BP-ONJ.

However, the occurrence of BP-ONJ in oral bisphosphonate treatment is reported to be much lower than that in intravenous bisphosphonates [18–28]. Therefore, it is important to explore the influence of oral bisphosphonate and compare the potency to intravenous bisphosphonates on various cell types to clarify the rarity of BP-ONJ caused by alendronate. This study investigated the differences between the potency of oral and intravenous bisphosphonates on human somatic cells and the effects of those bisphosphonates. Four bisphosphonates (alendronate, zoledronate, ibandronate, and clodronate) were chosen to compare their effects on cell viability, migration, and apoptosis. Human fibroblasts, human osteoblasts, and HUVEC were selected because of their role in bone remodeling and wound healing.

Materials and methods

Cell culture

Human periodontal ligament fibroblasts (Lonza, Basel, Switzerland: No. CC-7049), HUVEC (Lonza, Basel, Switzerland: No. CC-2517A), and human osteoblasts (Promocell, Heidelberg, Germany: No. C-12720) were purchased from the respective companies. Cells were cultured according to the manufacturer's instructions. Growth medium was changed every 2–3 days and subcultured with Accutase solution (Sigma-Aldrich, St. Louis, USA) when they reached 70–80% confluence. Cells of passages 4 to 7 were used in experiments. Human periodontal ligament fibroblasts were cultivated with growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, CA, USA), 1% penicillin-streptomycin-neomycin antibiotic mixture (PSN), 1% L-glutamine, 10% fetal calf serum (FCS), and 1 ng/ml basic fibroblast growth factor (bFGF). HUVEC were grown with endothelial basal medium (EBM2; Lonza, Basel, Switzerland), supplemented with EGM-2 SingleQuot™ Kit (Lonza, Basel, Switzerland). Human osteoblasts were cultured with Osteoblast Growth Medium (Promocell, Heidelberg, Germany) with SupplementMix (Promocell, Heidelberg, Germany). Cells were maintained in a humidified incubator with 5% CO_2 and 95% air at 37 °C.

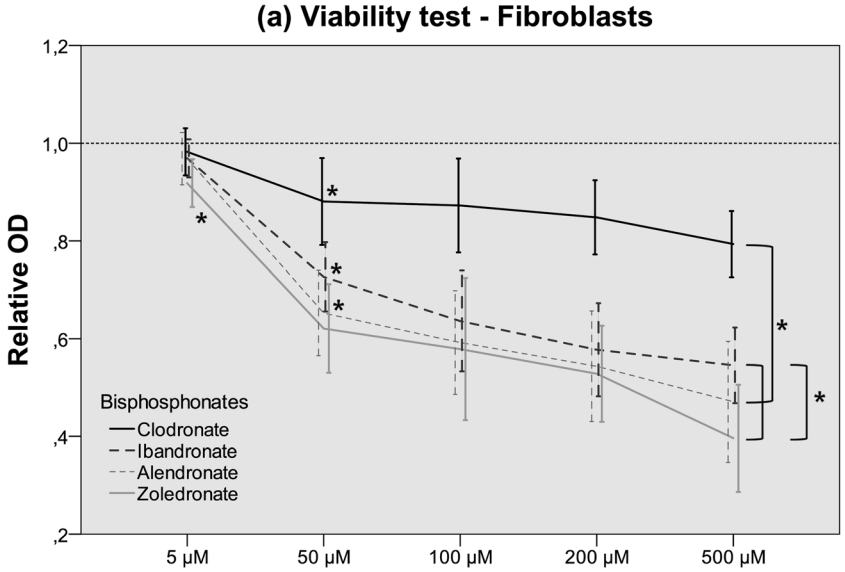
Fig. 1 Cell viability test in fibroblasts (**a**), HUVEC (**b**), and osteoblasts (**c**). Relative optical densities (OD) were expressed compared to the control group set to 1.0. Vertical bars represent standard deviations and asterisks indicate statistical significance between groups or compared to the control group ($P < 0.05$). The concentrations higher than the asterisks displayed significant differences compared to the control group

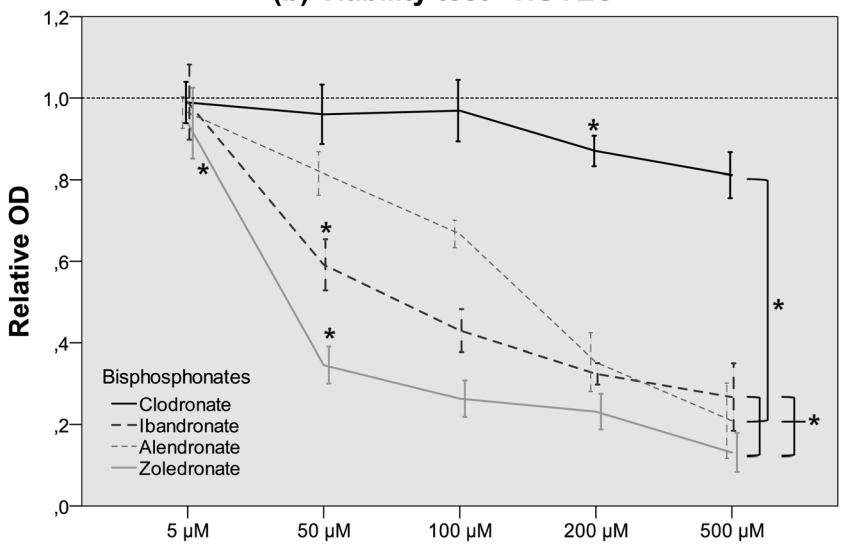
Bisphosphonates

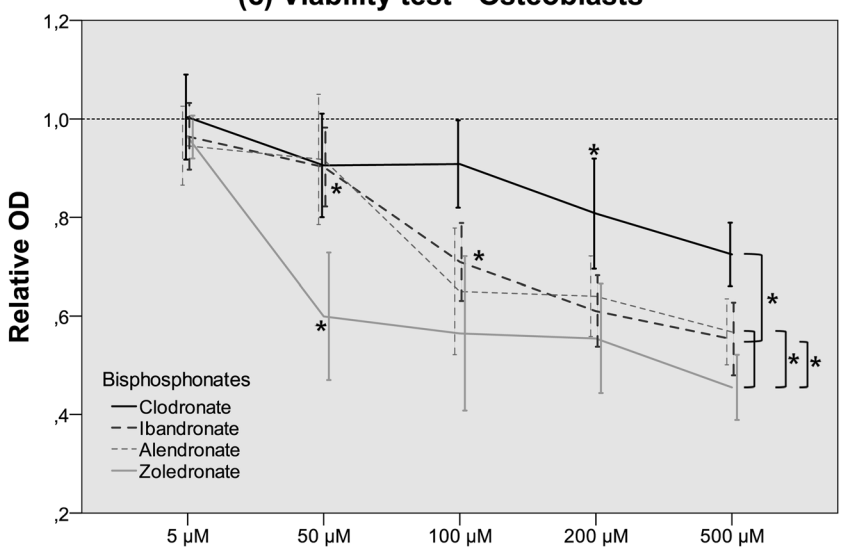
Zometa (zoledronate, Novartis Pharma, Basel, Switzerland), Bonviva (ibandronate, Roche, Basel, Switzerland), and Alendron (alendronate, Hexal AG; Novartis group, Holzkirchen, Germany) were used as nitrogen-containing bisphosphonates and Bonefos (clodronate, Bayer, Leverkusen, Germany) was used as a non-nitrogen-containing bisphosphonate. The alendronate tablet was pulverized and dissolved in phosphate-buffered saline (PBS) and then the pH of the solution was adjusted and sterilized by filtration. Control was incubated with the growth medium mentioned above only.

Cell viability test

MTT colorimetric assay (Sigma-Aldrich, Saint Louis, USA) was performed to evaluate the effects of bisphosphonates on cell viability. Cells were seeded into 12- or 24-well plates with densities of 10,000 cells/cm^2 for fibroblasts, 15,000 cells/cm^2 for HUVEC, and 13,000 cells/cm^2 for osteoblasts. The assay was conducted in triplicate with four replicates for each experiment. Twenty-four hours later, phenol-free growth medium was added to the wells with increasing concentrations (0, 5, 50, 100, 200, and 500 μM) of each bisphosphonate (zoledronate, ibandronate, alendronate, clodronate). Seventy-two hours after the addition of bisphosphonates, MTT solution was supplemented to each well and incubated for another 4 h. Afterwards, the wells were rinsed and cell lysis buffer (isopropanol and 2 N HCL, 49:1) was added to lyse the cells


(a) Viability test - Fibroblasts
Relative OD
1,2
1,0
,8
,6
,4
,2
Bisphosphonates
Clodronate
Ibandronate
Alendronate
Zoledronate
5 µM
50 µM
100 µM
200 µM
500 µM


(b) Viability test - HUVEC
Relative OD
1,2
1,0
,8
,6
,4
,2
,0
Bisphosphonates
Clodronate
Ibandronate
Alendronate
Zoledronate
5 µM
50 µM
100 µM
200 µM
500 µM


(c) Viability test - Osteoblasts
Relative OD
1,2
1,0
,8
,6
,4
,2
Bisphosphonates
Clodronate
Ibandronate
Alendronate
Zoledronate
5 µM
50 µM
100 µM
200 µM
500 µM

in each well. After 30 min of incubation, the contents of the wells were photometrically measured by a microplate reader (Synergy HT; BioTek, Winooski, USA) at 570 nm.

Cell migration test

The Boyden chamber assay system (24-well ThinCertTM, Greiner Bio-One, Kremsmünster, Austria) was performed to analyze the migration ability. Twenty-five-square centimeter flasks were used with seeding densities of 10,000 cells/cm^2 for fibroblasts, 15,000 cells/cm^2 for HUVEC, and 13,000 cells/cm^2 for osteoblasts. The assay was conducted in triplicate with three replicates for each experiment. Twenty-four hours after the seeding, fresh growth medium containing 0 and 50 μM bisphosphonate (zoledronate, ibandronate, alendronate, clodronate) was added. After 72 h of incubation, the cells were harvested and the final cell number for each group was adjusted to 5×10^5 cells/ml in serum-free medium. Each well was filled with 600 μl of cell culture medium with chemoattractant (fetal calf serum) and 24-well ThinCert™ cell culture inserts were placed in the wells. Then 200 μl of cell suspension was added to the 24-well ThinCert™ cell culture insert and maintained in cell incubator for 24 h. After the staining with Calcein-AM fluorescent dye (Invitrogen, MA, USA), the cells in the upper and lower compartments were carefully washed with PBS and the inserts were transferred into a new black 24-well plate filled with 500 μl of Accutase (Sigma-Aldrich, St. Louis, USA). Ten minutes later, the cells on the bottom of the lower chamber were detached and the quantification of fluorescence was determined by a microplate fluorescence reader (Synergy HT; BioTek, Winooski, USA) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Apoptosis test

Caspase 3/7 activity was measured with the Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA) to evaluate the effects of bisphosphonates on cell apoptosis. Cells were seeded into transparent 96-well plates with densities of 15,000 cells/cm^2 for fibroblasts, 20,000 cells/cm^2 for HUVEC, and 15,000 cells/cm^2 for osteoblasts. The assay was conducted in triplicate with six replicates for each experiment. After 24 h of incubation, each well was rinsed with PBS and further incubated for 72 h with fresh culture medium containing 0 and 50 μM bisphosphonate (zoledronate, ibandronate, alendronate, clodronate). Afterwards, the Caspase-Glo® substrate and buffer mixture was added to each well of the plates and incubated for 1 h at room temperature. The content in each well of the transparent 96-well plate was transferred into a new white-walled 96-well plate and the luminescence was measured by a microplate reader (Synergy HT; BioTek, Winooski, USA) according to the manufacturer's protocol.

Statistical analysis

The results were analyzed by analysis of variance (ANOVA) with the post hoc test Tukey or Kruskal-Wallis test followed by Mann-Whitney test as post hoc test, with SPSS statistic software 15.0. The α-level was set to 0.05 in ANOVA and it was set to 0.005 by the Bonferroni correction in case of Kruskal-Wallis with Mann-Whitney test. Each box plot shows values between the 25th and 75th percentile and the median value was expressed as the black line within the box. Whiskers indicate 1.5 times the value of interquartile range (IQR) and circles represent outliers within 3 IQR, while x represents outliers over 3 IQR.

Table 1 Cell viability in fibroblasts, HUVEC, and osteoblasts

	Bisphosphonates	5 μM	50 μM	100 μM	200 μM	500 μM	*P value
Fibroblasts	Clodronate[a]	0.983 ± 0.048	0.881 ± 0.091*	0.873 ± 0.096	0.848 ± 0.076	0.793 ± 0.068	0.005
	Ibandronate[b]	0.969 ± 0.039	0.727 ± 0.071*	0.636 ± 0.103	0.577 ± 0.095	0.545 ± 0.078	0.001
	Alendronate[c]	0.969 ± 0.054	0.652 ± 0.087*	0.592 ± 0.106	0.544 ± 0.113	0.471 ± 0.124	0.000
	Zoledronate[c]	0.918 ± 0.049*	0.621 ± 0.091	0.579 ± 0.146	0.528 ± 0.099	0.396 ± 0.110	0.050
HUVEC	Clodronate[a]	0.989 ± 0.051	0.960 ± 0.073	0.969 ± 0.076	0.870 ± 0.038*	0.811 ± 0.056	0.004
	Ibandronate[b]	0.990 ± 0.039	0.591 ± 0.063*	0.430 ± 0.053	0.324 ± 0.026	0.267 ± 0.083	0.000
	Alendronate[c]	0.965 ± 0.087	0.815 ± 0.053*	0.667 ± 0.034	0.353 ± 0.072	0.209 ± 0.092	0.001
	Zoledronate[d]	0.939 ± 0.071*	0.346 ± 0.045	0.264 ± 0.045	0.232 ± 0.044	0.131 ± 0.048	0.034
Osteoblasts	Clodronate[a]	1.004 ± 0.086	0.905 ± 0.129	0.908 ± 0.157	0.554 ± 0.111*	0.455 ± 0.066	0.000
	Ibandronate[b]	0.964 ± 0.068	0.902 ± 0.132*	0.710 ± 0.128	0.640 ± 0.082	0.568 ± 0.067	0.014
	Alendronate[b]	0.946 ± 0.080	0.917 ± 0.080	0.650 ± 0.079*	0.610 ± 0.073	0.533 ± 0.074	0.001
	Zoledronate[c]	0.963 ± 0.044	0.599 ± 0.105*	0.564 ± 0.089	0.808 ± 0.112	0.725 ± 0.064	0.000

The values were normalized relative to control. The same superscript letter indicates statistical insignificance, and different letters indicate statistical significance

*Indicates statistical significance ($\alpha = 0.05$) of the concentration and higher, compared to control

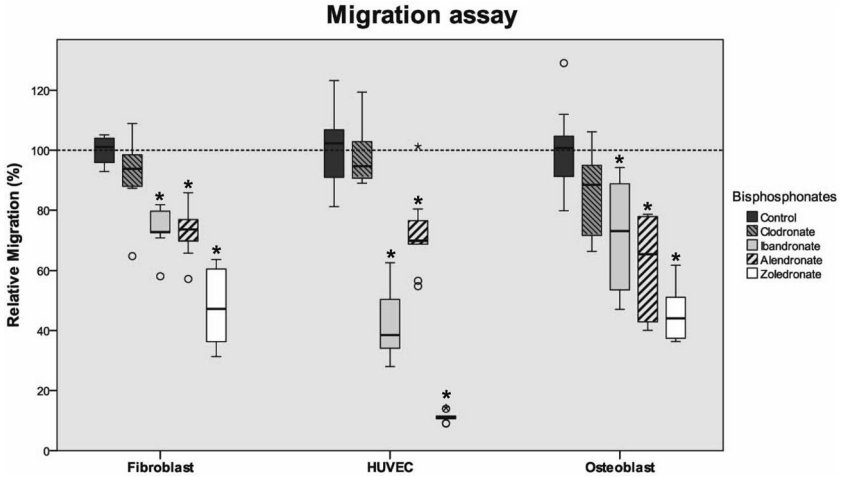


Fig. 2 Cell migration test in fibroblasts, HUVEC, and osteoblasts—Boyden chamber assay. Relative migration rates were expressed compared to the control group set to 100%. Asterisks indicate statistical significance in comparison to the control group ($P < 0.005$)

Results

Cell viability (Fig. 1 and Table 1)

In fibroblasts, the zoledronate group was the most affected one, followed by alendronate and ibandronate. However, no significance was found between zoledronate and alendronate ($P = 0.146$), and alendronate was significantly more potent than ibandronate ($P = 0.047$). The alendronate and ibandronate groups showed significant differences to control at 50 μM and higher ($P \leq 0.001$), while the zoledronate group showed a difference at 5 μM ($P = 0.016$) and higher. At 500 μM, the cell viability in the zoledronate, alendronate, ibandronate, and clodronate group decreased by 61, 53, 46, and 21%, respectively.

In HUVEC, ibandronate was more potent than alendronate except at 500 μM ($P < 0.001$). Alendronate was the third most potent bisphosphonate to decrease the viability. However, no significance was observed between them at 500 μM ($P = 0.336$). Alendronate and ibandronate significantly affected HUVEC viability at 50 μM ($P \leq 0.001$) and higher, whereas 5 μM zoledronate significantly suppressed cell viability ($P = 0.034$). The influence of zoledronate was the most potent, with 87% decreased cell viability at 500 μM compared to the control group.

In osteoblasts, zoledronate also showed the strongest influence and reduced the cell viability by 55% at 500 μM compared to control. Alendronate and ibandronate were statistically similar in their effects ($P = 0.998$). Cell viability decreased by 43% by alendronate, 45% ibandronate, and 27% clodronate, at 500 μM.

Cell migration (Fig. 2 and Table 2)

In fibroblasts, zoledronate was the most effective; the migration rate was reduced by 51% compared to the control group ($P < 0.001$). Alendronate was the second strongest, reducing cell migration rate by 27%, following zoledronate. However, alendronate was statistically indifferent from ibandronate ($P = 0.796$), which decreased the migration rate by approximately 26%. Clodronate failed to decrease the migration of fibroblasts.

The migration of HUVEC in the zoledronate group was significantly higher than that in the alendronate and ibandronate groups ($P < 0.001$). The migration rate decreased by 88%, followed by ibandronate (58%), alendronate (27%), and clodronate (2%). Ibandronate was significantly more potent than alendronate ($P < 0.001$).

In osteoblasts, although no statistical significance was found between alendronate and ibandronate ($P = 0.043$),

Table 2 Cell migration in fibroblasts, HUVEC, and osteoblasts (%)

	Clodronate		Ibandronate		Alendronate		Zoledronate	
	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value
Fibroblasts	92.9 ± 12.7^{a}	0.136	$*74.3 \pm 7.4^{b}$	0.001	$*73.3 \pm 8.8^{b,c}$	0.001	$*48.9 \pm 12.7^{d}$	0.000
HUVEC	98.1 ± 9.6^{a}	0.605	$*41.8 \pm 11.1^{b}$	0.000	$*72.6 \pm 13.7^{c}$	0.000	$*11.4 \pm 1.8^{d}$	0.000
Osteoblasts	85.4 ± 14.3^{a}	0.043	$*71.7 \pm 17.9^{b}$	0.001	$*61.0 \pm 15.9^{b}$	0.000	$*46.0 \pm 8.7^{c}$	0.000

The values were normalized relative to control. The same superscript letter indicates statistical insignificance, and different letters indicate statistical significance. $\alpha = 0.005$ by the Bonferroni correction

*Indicates statistical significance compared to control

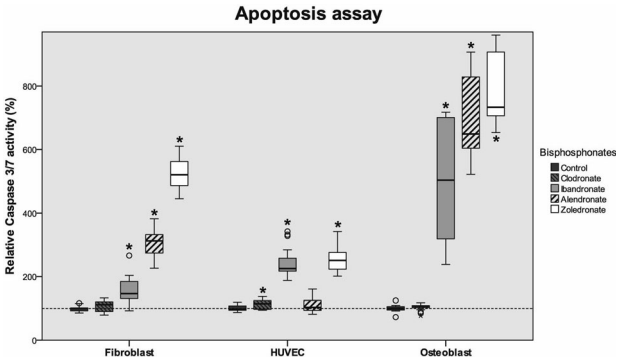


Fig. 3 Cell apoptosis test for fibroblasts, HUVEC, and osteoblasts—Caspase-Glo® 3/7 assay. Relative caspase 3/7 activity rates were expressed compared to the control group set to 100%. Asterisks indicate statistical significance in comparison to the control group ($P < 0.005$)

alendronate was more potent than ibandronate. Alendronate decreased the migration rate by 39% compared to the control group, 54% for zoledronate, and 29% for ibandronate.

Cell apoptosis (Fig. 3 and Table 3)

In fibroblasts, zoledronate was the strongest bisphosphonate at increasing the apoptosis. It increased fivefold compared to the control group and was significantly different compared to all the other bisphosphonates (all $P < 0.001$). Alendronate produced a threefold increase ($P < 0.001$), followed by ibandronate and clodronate.

In HUVEC, increased apoptotic enzyme activity was also found. Zoledronate produced a 2.5-fold increase, followed by ibandronate, clodronate, and alendronate ($P < 0.001$). However, there was no significant difference between zoledronate and ibandronate ($P = 0.226$). Alendronate failed to show a significant difference compared to control ($P = 0.192$) and clodronate ($P = 0.265$).

In osteoblasts, alendronate increased apoptotic enzyme activity 6.9-fold, following 7.9-fold in zoledronate, compared to control. No statistical significance was found between zoledronate and alendronate. Ibandronate was less effective than alendronate. The influence of clodronate was the weakest and it was not statistically significant compared to control ($P = 0.239$).

Discussion

Bisphosphonates primarily target osteoclast activities and aim to control abnormal bone metabolism caused by various bone malignancies or osteoporosis and therefore change the bone turnover. As one of the side effects of bisphosphonates, BP-ONJ was reported, and the etiology has been investigated. One of the causes next to the impaired bone turnover is the influence of bisphosphonates on several cell types around bone tissue [29–31]. Bisphosphonates that have currently been taken or dissociated from the underlying bone during resorption [17] compromise the function of fibroblasts and vessel cells, subsequently impairing re-epithelialization of oral mucosa and nutrition supply. Along with the fragile and vulnerable oral environment, due to thin mucosal coverage, microflora, eating, and frequent dental procedures, compromised cell functions and viability are considered to contribute to the occurrence of BP-ONJ. Therefore, fibroblasts, osteoblasts, and HUVEC were chosen to verify the effects of alendronate and compare with intravenous bisphosphonates.

The bisphosphonate-associated osteonecrosis usually occurs after the intake of nitrogen-containing bisphosphonates. But the frequency in patients taking alendronate, that is administered orally, is surprisingly low [25–28].

Alendronate prescriptions comprised over 1.3×10^8 of the total of 2×10^8 bisphosphonate defined daily doses in Germany in 2011 [32, 33], and the total annual administered dose of alendronate is much higher than oncologic doses of zoledronate and ibandronate [34, 35]. Nevertheless, the incidence of BP-ONJ in patients treated using oral bisphosphonates (0.0007–0.34%) [25–28] is much lower than that in patients receiving intravenous bisphosphonates (1.2–18.6%) [18–24]. Considering these facts, the potency of alendronate can be presumed much lower than that of intravenous bisphosphonates; however, alendronate had strong inhibitory effects that are enough to

Table 3 Cell apoptosis in fibroblasts, HUVEC, and osteoblasts (%)

	Clodronate		Ibandronate		Alendronate		Zoledronate	
	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value
Fibroblasts	109.2 ± 16.7^{a}	0.055	$*157.6 \pm 40.9^{b}$	0.001	$*302.9 \pm 40.9^{c}$	0.000	$*526.1 \pm 47.9^{d}$	0.000
HUVEC	$*114.0 \pm 13.2^{a}$	0.002	$*242.8 \pm 48.5^{b}$	0.000	109.1 ± 20.4^{a}	0.192	$*257.7 \pm 42.1^{b}$	0.000
Osteoblasts	104.2 ± 14.1^{a}	0.239	$*511.0 \pm 165.4^{b}$	0.000	$*624.1 \pm 199.7^{b,c}$	0.000	$*753.6 \pm 139.3^{c}$	0.000

The values were normalized relative to control. The same superscript letter indicates statistical insignificance, and different letters indicate statistical significance. $\alpha = 0.005$ by the Bonferroni correction

*Indicates statistical significance compared to control

compromise cell viability and migration capacity, and induce cell apoptosis in this study. Alendronate was even more potent than ibandronate in fibroblasts and osteoblasts, although ibandronate showed stronger influences in HUVEC and zoledronate was measured as the most potent bisphosphonate throughout the experiment. McLeod et al. reported that alendronate suppressed cell proliferation at 100 μM in human fibroblasts and 10 μM in oral keratinocytes, although zoledronate showed stronger effects on both cell types [14]. Martins et al. also found that alendronate inhibited the proliferation of human fibroblasts and human osteogenic sarcoma cells at over 10 μM with the incubation of 24 h [36].

Based on these findings, it can be surmised that there could be several contributing factors involved in the rarity of BP-ONJ caused by alendronate. First, because of the low bioavailability of alendronate, which is assumed approximately 0.6% and varies according to the conditions upon drug use and the gastric pH [37], the amount of alendronate absorbed decreases considerably compared to the initial dose of alendronate. Second, considering the low daily dosing of alendronate, it takes an extended time to accumulation in bone tissue to a level that is high enough to induce BP-ONJ, while dissociation of the bisphosphonates from the bone occurs simultaneously during the administration period. On the other hand, zoledronate is usually administered intravenously once every 3–4 weeks with high dose for tumor patients so that the initial impact from the bisphosphonate could be more tremendous and might reach the level to induce BP-ONJ, although dissociation of the drug molecules occurs afterwards. Therefore, these factors may affect the low incidence of BP-ONJ caused by alendronate, regardless of its drug potency. Lastly, chemotherapy and radiotherapy in tumor patients may also help explain the higher incidence of BP-ONJ in intravenous bisphosphonate therapy, in case these additional therapies had been accompanied, because of suppressed immune system and bone viability.

The results from this study suggest that the potency of alendronate in compromising various cell activities as oral bisphosphonate is comparable to certain intravenous bisphosphonates. Thus, these findings address that the rarity of BP-ONJ in alendronate treatment may be caused by not only its potency, but also the low bioavailability of alendronate [37], lower dosing of alendronate on a daily basis, and having no additional therapies. Additionally, this study is also in agreement with the theory that bisphosphonates detrimentally affect not only osteoclasts, but also several other somatic cell types. These negative influences may act as a critical factor in the pathogenesis of BP-ONJ and the strongest negative effects of zoledronate help understand the higher incidence of BP-ONJ in tumor patients receiving intravenous bisphosphonates.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent is not required for this study.

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