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ORIGINAL ARTICLE

The influence of bisphosphonates on viability, migration, and apoptosis of human oral keratinocytes—in vitro study

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Abstract Bisphosphonate-associated osteonecrosis of the jaw (BP-ONJ) is one of the most often seen side effects in patients treated with bisphosphonates, presenting clinically as a non-healing wound. One theory of BP-ONJ etiology describes a negative effect on soft tissues, especially on keratinocytes, which play an important role in oral wound healing and oral soft tissue regeneration. A high cell viability of keratinocytes, which can migrate to the affected location, is essential for wound healing. The aim of this in vitro study was to investigate the effect of differently potent bisphosphonates on human oral keratinocytes (HOK).

Three nitrogen-containing bisphosphonates (ibandronate, pamidronate, and zoledronate) and one non-nitrogen-containing bisphosphonate (clodronate) were compared concerning their potency on cell viability (calcein assay and MTT assay), migration ability (Boyden chamber migration assay and scratch wound proliferation assay), and apoptosis (TUNEL assay) of HOK.

The nitrogen-containing bisphosphonates, particulary highly potent pamidronate and zoledronate preparations, had a strong negative influence on cell viability, migration ability, and apoptosis of HOK. The non-nitrogen-containing clodronate even increased cell viability in higher concentrations.

This study demonstrates that bisphosphonates have a strong influence on HOK on different cellular levels like cell viability, migration ability, and apoptosis rate. The results support the theory that BP-ONJ is a multifactorially caused disease.

Furthermore, this in vitro study confirms the theory that perioperative interruption of bisphosphonate application during dental surgical procedures might be feasible to promote better tissue regeneration and wound healing.

Keywords Bisphosphonate · Osteonecrosis of the jaws · Etiology · Keratinocytes

Introduction

The negative side effects of bisphosphonates can be classified into four main groups: acute phase reactions, gastrointestinal effects, renal side effects, and bisphosphonate-associated osteonecrosis of the jaw (BP-ONJ) [1], the latter of which was first described by Marx in 2003 [2] and has since increased in frequency [3]. The American Association of Oral and Maxillofacial Surgeons defines BP-ONJ as exposed bone in the maxillofacial region that has persisted for more than 8 weeks in combination with current or previous bisphosphonate therapy and a lack of head and neck radiation in the patient's history [4].

To date, several theories on the development of BP-ONJ are being discussed in the literature [5]. The most common theory attributes the condition to reduced bone remodeling due to bisphosphonate-induced osteoclast inhibition. In addition to osteoclasts and osteocytes, osteoblasts are

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influenced by bisphosphonates as well [6, 7]. This theory is supported by the development of osteonecrosis under therapy with anti-RANKL antibodies, which also influence bone remodeling and are used in oncologic patients as well [8–11].

Bisphosphonates have verified antiangiogenic and apoptotic effects [6, 7, 12] which are therapeutically used in oncologic patients. In addition, a negative influence on epithelial progenitor cells which might also result in a reduced angiogenesis and an avascular necrosis has been described [13].

Some authors focus on the soft tissues in the etiology of BP-ONJ since adequate wound healing is lacking in these patients [14]. Fibroblasts and mature endothelial cells are involved in this process and are negatively influenced by bisphosphonates [6, 7, 13]. For sufficient wound healing, the viability of these cell lines is essential. Reduced wound healing may result in necrotic and exposed bone. A similar mechanism could be discussed in BP-ONJ patients with periodontal disease, where the soft tissues are disturbed by inflammation [15]. If bisphosphonates are constantly released from the underlining bone, the very thin mucosal layer may be compromised, including the superficial layer of keratinocytes. The aim of the present study was to analyze the effects of different nitrogen- and non-nitrogen-containing bisphosphonates on human oral keratinocytes (HOK) which are also involved in oral mucosa and gastrointestinal mucosa wound healing.

Materials and methods

Cell culture

Cell cultures were prepared and maintained according to standard cell culture procedures. Commercially available HOK (ScienCell, Carlsbad, USA) were cultured in a keratocyte growth medium (Provitro, Berlin, Germany) supplemented with 500 ng 500 ml^{-1} medium basic fibroblast growth factor, 10 ng ml^{-1} epidermal growth factor (recombinant human), 5 μg ml^{-1} insulin (recombinant human), 0.004 ml ml^{-1} bovine pituitary extract, and 1 μg ml^{-1} hydrocortisone.

The cell culture was incubated and cultured in an incubator with 5% CO_2 and 95% air at 37°C until the third passage of cell generation. Cells were passaged at regular intervals depending on their growth characteristics using 25% trypsin (Seromed Biochrom, Berlin, Germany).

Cell viability

To analyze the effects of bisphosphonates on cell viability of HOK, we used a previously described calcein viability assay and a 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [7].

Calcein viability assay

HOK were cultured in standard cell culture as described. All cells were incubated in standard 24-well culture plates with different bisphosphonates (clodronate, ibandronate, pamidronate, and zoledronate) in gradient concentrations (0, 5, 50, 100, 200, and 500 μmol/l) for 72 h. This range of concentrations was selected because in vivo concentrations in plasma shortly after zoledronate infusion are about 5 μmol/l, and concentrations in bone shortly after infusion can be 100-fold higher [16]. After removing the used culture medium from the wells, they were filled with 500 μl fresh culture medium and 7 μl Calcein AM (Invitrogen™, Eugene, USA) per well. The plates were incubated in a dark cell incubator for 1 h. Subsequently, the Calcein AM medium was also removed, and cells were detached with 500 μl Trypsin–EDTA solution per well; 200 μl of the cell–Trypsin solution was carried in standard black 24-well cell culture plates, and the extent of cell viability was measured by a Microplate Fluorescence Reader (BioTek FL×800, Bad Friedrichshall, Germany) with an extinction wavelength of 480 nm and an emission wavelength of 520 nm.

MTT assay

Cells were transferred into six-well plates (100,000 cells/well). After 24 h, the cells were incubated with bisphosphonates (clodronate, ibandronate, pamidronate, and zoledronate) in increasing concentrations (0, 5, 50, 100, 200, and 500 μmol/l) for 24 h. The cell viability of HOK was evaluated with the MTT colorimetric assay (Sigma, München, Germany). Viable cells ferment tetrazolium bromide to formazan that can be measured after cell lysis photometrically at 550 nm. The experiments were performed in triplicate.

Migration assay (Boyden chamber)

To examine the effects of bisphosphonates on the migration ability of human oral keratinocytes, a 24-well Boyden Chamber Assay system (ThinCert™, pore size 8 μm translucent, Greiner BioOne, Essen, Germany) was used according to the manual. The cells (HOK) were incubated for 72 h with different bisphosphonates at a concentration of 50 μmol/l. Cells were harvested, washed twice in phosphate-buffered saline (PBS), and re-suspended in cell culture medium at a final concentration of 10^6 ml^{-1}. After 24 h, the cells were stained with Calcein AM fluorescent dye (Invitrogen™). Thereafter, the culture medium was removed from the inserts, and the inserts were transferred to

the wells of a new 24-well plate containing 500 μl Trypsin–EDTA per well. This plate was incubated for 10 min in a cell culture incubator at 37°C and 5% CO_2. The inserts were removed, and 200 μl of the Trypsin–EDTA solution, now containing the detached migratory cells, was transferred from each well of the 24-well plate to a well of a flat-bottom black 24-well plate. For quantification of cell migration, we used a Microplate Fluorescence Reader (BioTek FL×800, Bad Friedrichshall, Germany) with an extinction of 480 nm and emission of 520 nm. For each bisphosphonate, the experiments were conducted 12 times.

Scratch wound assay

Cells were seeded on tissue culture plastic in Petri dishes (Nunc, Langenselbold, Germany); at 80% cell confluence, a scratch wound using a sterile pipette tip was performed, and the size of the gap was measured and set up to 100%. The cells were incubated with 50 μmol/l of the previously mentioned bisphosphonates, and the closing of the scratch wound was measured for 72 h or until the scratch wounds were closed. The experiment was performed six times for each bisphosphonate.

TUNEL assay

For the apoptosis terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, HOK (5×10^4) were seeded to six-well plates. After 24 h, the cells were incubated with 50 μmol/l bisphosphonates (clodronate, ibandronate, pamidronate, and zoledronate) including a negative control group for 72 h. Cells were solved by Trypsin–EDTA and centrifuged onto cover slips with 778 g for 5 min. Then, cells were fixed with 3% paraformaldehyd for 10 min at room temperature (RT), permeabilized with 0.1% Triton X-100, 0.1% sodium citrate for 2 min on ice, washed twice with PBS, pH 7.4, and incubated for 1 h at 37°C in the dark with a TUNEL reaction mixture (Boehringer, Ingelheim, Germany) for in situ detection of cell death. After washing twice with PBS, pH 7.4, cells were incubated at RT with Hoechst solution for 5 min. All Hoechst-positive nuclei as well as TUNEL-positive nuclei were visualized using a Zeiss Axioplan fluorescence microscope. Apoptosis was expressed as a percent of fragmented Hoechst-positive nuclei versus total Hoechst-positive nuclei and as a percent of TUNEL-positive nuclei versus total Hoechst-positive nuclei and are shown as fold increase versus control.

Statistical analysis

Comparisons between groups were analyzed by ANOVA (post hoc test: Tukey) for experiments with more than two subgroups (SPSS version 17.0). P values<0.05 were considered statistically significant. Continuous variables were expressed as mean ± standard deviation in the line graph.

Results

Cell viability

The viability assays demonstrated a strong negative influence of nitrogen-containing bisphosphonates. Clodronate and ibandronate showed a lower influence than pamidronate and zoledronate. Clodronate, which does not contain nitrogen, increased cell viability in higher concentrations.

Calcein viability assay

Clodronate increased cell viability of HOK, becoming statistically significant at a concentration of 100 μmol/l compared to the control group ($p=0.014$). The nitrogen-containing bisphosphonates induced a reduction in cell viability. The strongest negative influence was evaluated for the highly potent zoledronate, which turned out to be significant compared to control group at a concentration of only 5 μmol/l ($p=0.008$). Compared to the control group, pamidronate showed a significant difference at a concentration of 100 μmol/l ($p=0.013$) and ibandronate at a concentration of 500 μmol/l ($p=0.006$). There were no further significant differences between the nitrogen-containing bisphosphonates (Fig. 1).

MTT assay

Similar results were assessed with the MTT test. All used nitrogen-containing bisphosphonates had a very strong negative influence on cell viability of HOK. They reduced cell viability at very low concentrations. Among the nitrogen-containing bisphosphonates, ibandronate had the weakest influence on cell viability. The strongest negative influence on cell viability was induced by the highly potent, nitrogen-containing bisphosphonate pamidronate, followed by zoledronate. The difference in cell viability turned out to be significant for pamidronate and zoledronate at a concentration of 5 μmol/l compared to the control group (each $p<0.04$), and the effect of ibandronate turned out to be significant compared to the control group at a concentration of 100 μmol/l ($p=0.04$). The non-nitrogen-containing clodronate increased cell viability at concentrations of 100 μmol/l and 500 μmol/l significantly compared to the control group (each $p<0.001$) whereas there was no significance at a concentration of 200 μmol/l (Fig. 2).

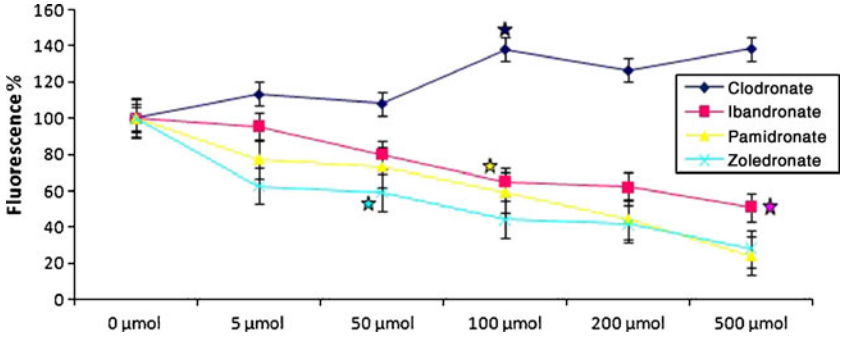

Fig. 1 Calcein viability assay. Cell viability test for human oral keratinocytes influenced by clodronate, ibandronate, pamidronate, and zoledronate at different concentrations compared to a control set up to 100%. *Stars* indicate concentrations with a statistically significant change of cell viability compared to the control. (*X*-axis=bisphosphonate concentrations, *Y*-axis=cell viability compared to control group expressed as a percent)

Migration assay (Boyden chamber)

The Boyden chamber migration assay again revealed a stronger negative influence on migration ability for the highly potent, nitrogen-containing bisphosphonates. All nitrogen-containing bisphosphonates induced a statistically significant inhibition of migration ability ($p<0.04$). Pamidronate and zoledronate had the strongest influence, which was statistically significant compared to the non-nitrogen-containing clodronate ($p<0.05$). There was no significant difference for ibandronate compared to clodronate. Pamidronate also demonstrated a significantly stronger influence on migration ability than the other bisphosphonates (Fig. 3).

Scratch wound assay

Again, the nitrogen-containing bisphospohonates had the strongest negative influence on cell migration and proliferation ability of HOK. As of day 2, all nitrogen-containing bisphosphonates showed a significant difference compared to the control group (each $p<0.005$). Pamidronate and zoledronate showed a significant difference compared to clodronate as of day 1 (pamidronate, $p=0.01$; zoledronate, $p=0.001$). Between pamidronate and zoledronate, there was no significance before day 3. The non-nitrogen-containing clodronate also decreased the migration ability of HOK, becoming statistically significant as of day 3 ($p<0.001$; Fig. 4).

TUNEL assay

The nitrogen-containing pamidronate had the strongest negative influence, followed by zoledronate and ibandronate. Non-nitrogen-containing clodronate had the lowest influence on apoptosis of keratinocytes. There was no further statistical significance between the nitrogen-containing bisphosphonates ($p>0.05$). Pamidronate and zoledronate showed a significance compared to the non-nitrogen-containing clodronate (each $p<0.05$; Fig. 5).

Discussion

One theory on the etiology of BP-ONJ is the adverse impact of bisphosphonates on the integrity of the mucosal layer [5,

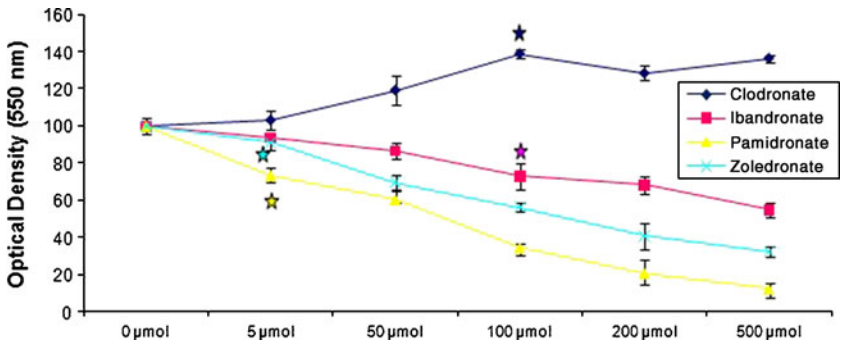

Fig. 2 MTT assay. Cell viability test for human oral keratinocytes influenced by clodronate, ibandronate, pamidronate, and zoledronate at different concentrations compared to a control set up to 100%. *Stars* indicate concentrations with a statistically significant change of cell viability compared to the control. (*X*-axis=bisphosphonate concentrations, *Y*-axis=cell viability compared to control group in percent)

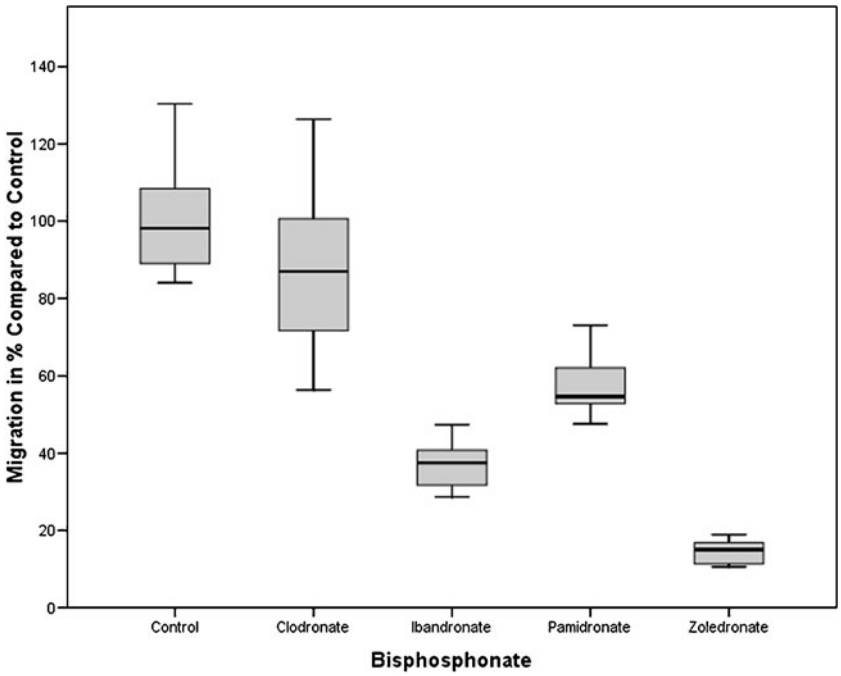

Fig. 3 Migration assay (Boyden chamber). Boyden chamber migration assay for human oral keratinocytes influenced by 50 μmol/l clodronate, ibandronate, pamidronate, and zoledronate compared to a control without bisphosphonates. The *black bar* in the middle of each box represents the median. The *box* includes all values between the 25th and the 75th percentiles. *Whiskers* indicate values still within 1.5 interquartile range (IQR)

14, 17], resulting in defective wound healing and thereby enabling bacterial contamination of the underlying bone. Affected cell lines particularly include gingival fibroblasts and oral keratinocytes. In addition to the negative influence on gingival fibroblasts, as described previously [6, 7], we have demonstrated the negative influence of bisphosphonates on HOK in the form of reduced viability, reduced migration ability, and an increased apoptosis rate.

Keratinocytes are influenced by nitrogen-containing bisphosphonates at the used concentrations between 5 μmol/l and 100 μmol/l which could be demonstrated by the MTT assay. Scheper et al. also demonstrated the negative influence on keratinocyte cell viability by zoledronate in similar concentrations [18, 19]. Interestingly, we also were able to demonstrate a positive effect on cell viability of HOK for clodronate with the calcein assay and MTT assay. This effect of clodronate has already been described for other cell lines, such as osteoblasts for example [7, 20–23]. This phenomenon of increased cell viability could only be demonstrated for the non-nitrogen-containing clodronate.

Zoledronate had the strongest negative influence on migration ability of HOK, followed by ibandronate and pamidronate. A positive effect of clodronate on migration or proliferation ability of HOK, as demonstrated for the viability assay, could not be detected.

Pamidronate (50 μmol/l) induced the highest rate of apoptosis in HOK, 70%, followed by zoledronate and ibandronate. The rate of apoptosis after clodronate incubation

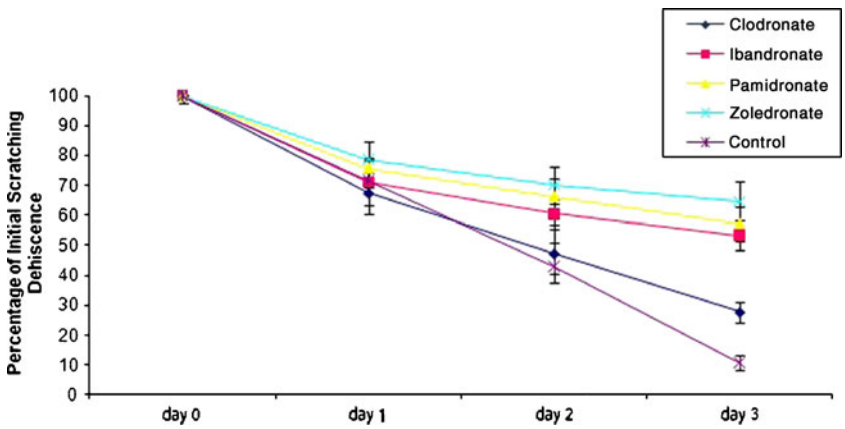

Fig. 4 Scratch wound assay. Migration and proliferation tested by scratch wound assay. Wound closure is demonstrated in percentage in relation to the initial dehiscence caused by the scratching. (*X*-axis=day of measurement, *Y*-axis=wound closure compared to control group expressed in percent)

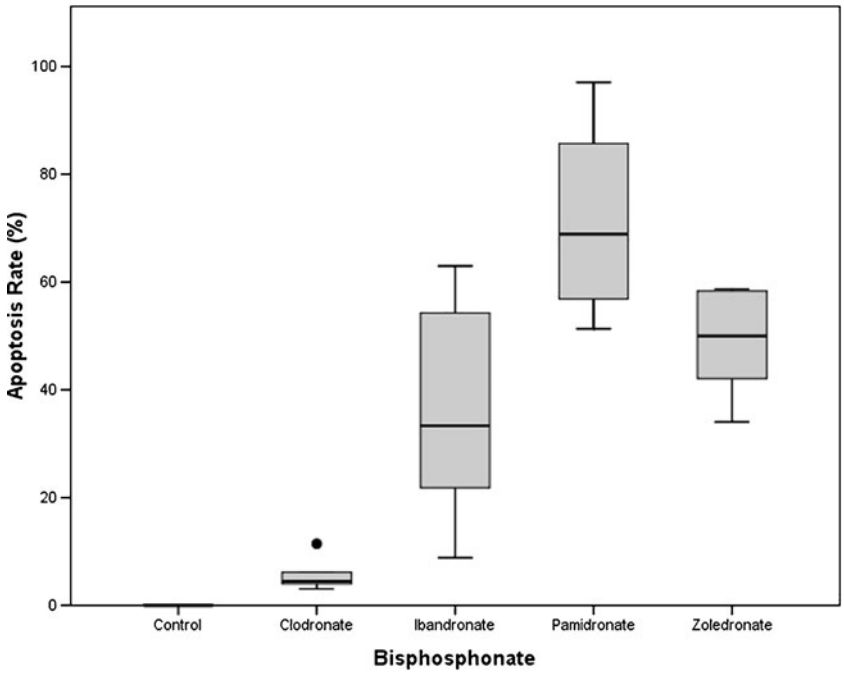

Fig. 5 TUNEL apoptosis assay. TUNEL assay for human oral keratinocytes influenced by 50 μmol/l clodronate, ibandronate, pamidronate, and zoledronate compared to a control without bisphosphonates. The *black bar* in the middle of each box represents the median. The *box* includes all values between the 25th and the 75th percentiles. *Whiskers* indicate values still within 1.5 interquartile range (IQR). Outliers within 3 IQR are represented as *circles*

was nearly 0%, demonstrating the marginal influence of clodronate on HOK again.

These results are supported by the literature. Scheper et al. also demonstrated the influence of zoledronate on cell proliferation and apoptosis of keratinocytes in low concentrations (0.25–3 μmol/l) by MTS and MTT assays [18].

In in vitro studies, other authors already described a proapoptotic effect of bisphosphonates on mouse keratinocytes [24] and cells of the gastrointestinal tract [25]. Clinically, the influence of bisphosphonates on the gastrointestinal tract [26] such as esophagitis in patients taking oral medication has also been described [27]. Further symptoms are mucosal inflammation and ulcerations of oral cavity [28], esophagus, stomach, and the small intestine [1, 29]. For this reason, direct damage of cells by oral application is discussed, through inhibition of the farnesyltransferase of the mevalonat pathway, resulting in a decrease of geranylgeraniol, which is necessary among others for membrane localization of intracellular GTP-binding proteins [30]. In addition, the synthesis of cell components might be affected [24, 31]. Similar to the continuous bisphosphonate contribution from the bone, especially at sites of very thin mucosa such as the mylohyoid line, might affect keratinocytes and gingival fibroblasts, resulting in defective wound healing and exposed bone.

The bisphosphonates associated with BP-ONJ are the nitrogen-containing bisphosphonates, most notably pamidronate and zoledronate [2, 3, 32], followed by ibandronate and alendronate [3, 33]. A few case reports of BP-ONJ have been published in patients taking non-nitrogen-containing bisphosphonates such as clodronate [34]. A reason for this distribution might be the different impact of these bisphosphonates on human oral keratinocytes as shown in these experiments. In all assays, the highly potent, nitrogen-containing bisphosphonates ibandronate, pamidronate, and zoledronate had a strong negative effect on human oral keratinocytes compared to non-nitrogen-containing clodronate. In the comparison of the nitrogen-containing bisphosphonates, zoledronate and pamidronate had the biggest effect on human oral keratinocytes. The results presented may explain the higher occurrence of BP-ONJ in patients receiving nitrogen-containing bisphosphonates as compared to patients receiving non-nitrogen-containing bisphosphonates. This study's data could support the idea of a multifactorial genesis of BP-ONJ and supports the theory that mucosal layer in oral cavity might have an important role in BP-ONJ etiology and pathophysiology. Therefore, an atraumatic surgical procedure to not further harm the soft tissues might be feasible.

Finally, these results suggest that it may be prudent to interrupt bisphosphonate treatment if oral surgery is necessary in order to protect the soft tissues and promote better wound healing.

Conflict of interests No funding was received for this study. Christian Walter and Bilal Al-Nawas have received speaker fees from Roche and Christian Walter received funding from Novartis for a different study.

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