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ORIGINAL ARTICLE

Influence of bisphosphonates on the adherence and metabolism of epithelial cells and gingival fibroblasts to titanium surfaces

Fernanda Gonçalves Basso[1,2,3] · Taisa N. Pansani[1,3] · Diana G. Soares[1,3] · Lais M. Cardoso[1,3] · Josimeri Hebling[1,3] · Carlos Alberto de Souza Costa[1,3]

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Abstract
Objectives To evaluate the effects of sodium alendronate (SA) and zoledronic acid (ZA), on the adhesion and metabolism of epithelial cells and gingival fibroblasts to titanium surfaces considering cell functions related to an effective mucosal barrier around the implant.
Materials and methods Cells were seeded onto titanium discs and incubated for 24 h. Then, serum-free DMEM containing selected bisphosphonates (0, 0.5, 1, or 5 μM) was added for 24 and 48 h. Factors related to the achievement of an effective mechanical and immunological barrier—cell adhesion, viability, collagen epidermal growth factor, and immunoglobulin synthesis—were evaluated. Data were analyzed by Kruskal-Wallis and Mann-Whitney tests as well as by ANOVA and Tukey's tests, ($\alpha = 0.05$).
Results The presence of bisphosphonates culminated in lower cell adhesion to the titanium discs, particularly for SA at 5 μM (40%) and ZA at all concentrations (from 30 to 50%, according to increased concentrations). Reduced cell viability occurred after exposing these cells to ZA (40%); however, only 5 μM SA-treated cells had decreased viability (30%). Reduced synthesis of growth factors and collagen was observed when cells were treated with ZA (20 and 40%, respectively), while about 70% of IgG synthesis was enhanced.
Conclusion Bisphosphonates negatively affected the adhesion and metabolism of oral mucosal cells, and this effect was related to the type of bisphosphonate as well as to concentration and period of treatment.
Clinical relevance The negative effects of bisphosphonates on oral mucosal cells can hamper the formation of an effective biological seal in osseointegrated implants.

Keywords Fibroblast · Implantology · Bisphosphonate · Connective tissue biology

Introduction

After intake, bisphosphonates show highly selective affinity for mineralized tissues, especially for hydroxyapatite crystals (1), and the release of these drugs can be triggered by physiological bone remodeling or by inflammatory events, leading to pathological resorption (2). Once released from these tissues, the cellular effects of drugs show poor specificity (3–6). As a main effect, bisphosphonates inhibit the maturation of pre-osteoclasts into osteoclasts and also induce the apoptosis of these cells, preventing bone loss (7–9). Secondary cellular effects, however, have been extensively described, such as high toxicity to osteoblasts, fibroblasts, and epithelial cells (3–6, 10–14), and these events have been associated with the development of bisphosphonate-related osteonecrosis of the jaws (BRONJ) (15, 16).

Some studies have assessed the local and systemic factors related to the development of this condition (7, 15, 17, 18). Administration route and potency (type of bisphosphonate) have been described as the main factors associated with these adverse effects (2, 15). Previous studies have also demonstrated that these

✉ Fernanda Gonçalves Basso
fergbasso@gmail.com

[1] Araraquara School of Dentistry, Universidade Estadual Paulista – UNESP, Araraquara, SP, Brazil

[2] Department of Physiology and Pathology, School of Dentistry, Araraquara, São Paulo State University (Unesp), Araraquara, SP, Brazil

[3] Araraquara School of Dentistry, Campus Araraquara, São Paulo State University (Unesp), Araraquara, SP, Brazil

factors are related to the toxicity of these drugs to several cell types (3–6, 10–14).

Due to these events, the insertion of oral osseointegrated implants into patients undergoing bisphosphonate treatment is controversial (19–21). This controversy is based on the development of BRONJ lesions in patients receiving ongoing bisphosphonate therapy, as well as to the failure of oral implant treatment in these patients (19–22).

The success of oral implants is related primarily to bone formation and biological sealing (23); therefore, local or systemic factors can interfere with the integration of these components into the bone tissue (osteointegration) or with the adhesion of oral mucosa, thus contributing to implant failure.

Therefore, the achievement of high-quality biological sealing by the adhesion of oral mucosal cells to implant surfaces is crucial for the maintenance of these prosthetic components, since it provides a physical and immunological barrier, preventing bacterial invasion and also providing esthetics for these components (23).

Since reports in the literature have already demonstrated the toxicity of bisphosphonates to oral mucosal cells, the failure of implant therapy in patients under bisphosphonate treatment may be due to a direct toxic effect of these drugs (5, 6, 12, 13). However, the fact that not all patients receiving bisphosphonate therapy show negative outcomes for oral implants (19–22) suggests that these failures can also be related to the types and regimens of bisphosphonates.

Therefore, this study evaluated the effects of two bisphosphonates, sodium alendronate (SA) and zoledronic acid (ZA), on the adhesion and metabolism of epithelial cells and gingival fibroblasts to titanium surfaces.

Material and methods

Disc preparation

Commercially pure titanium discs (grade IV, 13 × 1.5 mm) were obtained and ground wet with 400-, 600-, and 1200-grit silicon carbide paper (3 M do Brasil, Sumaré, SP, Brazil) to obtain standardized surfaces. Surface roughness was evaluated by means of a confocal microscope (ULS 4000, Olympus Corporation, Center Valley, PA, USA). Then, discs were ultrasonically cleaned in 95% ethanol and deionized water and were sterilized in an autoclave.

Cell culture

Human epithelial cells (HaCaT - CLS 300493) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA, USA), containing antibiotic Pen/Strep solution (1%, Gibco) and 10% fetal bovine serum (FBS, Gibco) in a humidified incubator (37 °C, 5% CO_2). Gingival fibroblasts were isolated from a healthy young individual during a tooth extraction procedure, according to a protocol approved by the university's Ethics Committee (CAAE: 1432113.7.0000.5416). For cell isolation, gingival tissue was subjected to enzymatic digestion for 24 h, with collagenase type I (3 mg/mL; Worthington Biochemical Corp., Lakewood, NJ, USA) in serum-free culture medium containing antibiotic/antimycotic solution (1%, Gibco) in a humidified incubator (37 °C, 5% CO_2) (Thermo Fisher Scientific, Waltham, MA, USA).

For the experimental procedures, titanium discs were placed at the bottoms of wells of 24-well plates. Then, 1 mL of culture medium was added to each well, and epithelial cells or gingival fibroblasts were seeded onto the discs (1×10^5 cells/disc) and incubated for 24 h.

Bisphosphonates

Twenty-four hours after being seeded, the culture medium was replaced by serum-free DMEM containing different concentrations of sodium alendronate (SA) or zoledronic acid (ZA) (0.5, 1, or 5 μM) (Sigma-Aldrich, St. Louis, MO, USA). The drugs were maintained in contact with cells for 24 or 48 h.

Cell adhesion

The adhesion of epithelial cells and fibroblasts after treatment with SA or ZA was evaluated by scanning electron microscopy (SEM). Samples were processed according to standard protocol previously reported and were maintained in a desiccator for 7 days, covered with gold, and analyzed by scanning electron microscopy (Inspect Scanning Electron Microscope - S50; FEI, Hillsboro, OR, USA) (5).

Expression of cell adhesion molecules H-CAM and actin and determination of adherent cell number

The adhesion of epithelial cells was also qualitatively evaluated by the expression of cell adhesion molecules (H-CAM/CD44), while the adhesion and cytoskeletal conformation of fibroblasts were analyzed by the expression of actin by immunofluorescence. Cells were fixed in 4% formalin for 24 h at 4 °C. Then, these samples were washed three times in cold PBS (5 min each) and were incubated with 3% bovine serum albumin (BSA) solution for 1 h, then washed with PBS. Primary antibodies for CD44 (mouse anti-CD44, 100 μg/mL in 3% BSA; Santa Cruz Biotechnology Inc., Dallas, TX, USA) or anti-actin conjugated with Alexa Fluor 630 nm (1:100, Molecular Probes, Carlsbad, CA, USA) was added to each sample and incubated for 1 h. Epithelial cells were also stained with secondary antibody (goat anti-mouse IgG [Molecular Probes], conjugated with AlexaFluor 488 nm - 1:100 in 1% BSA) for 2 h. A DNA intercalator (Hoechst, 1:5000 – Molecular Probes) was used to identify cells. Positive fluorescence was detected by an inverted fluorescence

microscope (EVOS FL Image System, Thermo Fisher Scientific).

The number of cells that remained attached to the titanium surface after treatment with SA and ZA at different concentrations was determined by fluorescence microscopy by nuclei staining with the DNA intercalator (Hoechst, 1:5000 – Molecular Probes). Four photomicrographs of each sample were obtained, and these images were analyzed with ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Cell viability

The viability of cultured cells was evaluated by the MTT assay (14). To each sample, a solution of DMEM containing 10% of MTT salt (5 mg/mL in phosphate buffer) was added. Cells were incubated for 4 h in a humidified incubator (37 °C, 5% CO_2). During this period, the enzyme succinic dehydrogenase of viable cells promoted the cleavage of MTT salt, resulting in the formation of purple formazan crystals. These crystals were then dissolved in acidified isopropanol. An aliquot of 200 μL of each sample was transferred to a 96-well plate, and absorbance of each sample was evaluated by spectrophotometry (570 nm; Synergy H1, Bio Tek, Winooski, VT, USA). The viability of each sample was determined according to the mean absorbance of the control group (100% viability).

Collagen synthesis

Total collagen secreted by gingival fibroblasts was determined by the Sirius Red method. Briefly, an aliquot (400 μL) of the culture medium that remained in contact with cells was added to an equal volume of 0.1% direct red solution (Sigma-Aldrich) in saturated picric acid. Samples were incubated for 1 h under agitation (400 rpm, Thermomixer – Eppendorf, Hamburg, Germany) and centrifuged at 10,000 rpm for 10 min (Microcentrifugal 5415R, Eppendorf). Then, supernatant was discarded, and samples were washed with hypochlorous acid (0.1 M) followed by centrifugation (10,000 rpm/10 min). The total collagen pellet was resuspended in sodium hydroxide (0.5 M). An aliquot of 200 μL of each sample was transferred to a 96-well plate and then subjected to absorbance determination (562 nm, Synergy H1, Bio Tek).

EGF and VEGF synthesis

Synthesis of epidermal growth factor (EGF) by epithelial cells and vascular endothelial growth factor (VEGF) by gingival fibroblasts was assessed by the enzyme-linked immunosorbent assay (ELISA), with standardized kits (DuoSet, R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's instructions. Briefly, aliquots of culture medium in contact with cells during treatment were stored at -20 °C until analysis. Then, plates were prepared by overnight incubation with primary antibodies (1 μg/mL) at room temperature. Plates were washed three times with 1% washing solution and treated with Reagent Diluent solution (BSA) for 1 h. Samples and standard curve aliquots (100 μL) were added to the plates and incubated for 2 h, followed by washing and incubation with secondary antibodies (100 ng/mL) for an additional 2 h.

As a next step, plates were subjected to washing and incubation with a 100-μL quantity of 1:40 streptavidin solution for 20 min, followed by washing, incubation with reagent solution, and stop solution. Then, the absorbance of each sample was read in a spectrophotometer at 455 nm (Synergy H1, Bio Tek). The concentration of this growth factor for each sample was determined according to a standard curve containing known EGF or VEGF concentrations.

Synthesis of IgG

Synthesis of immunoglobulin G by epithelial cells was detected by ELISA assay (R&D Systems), according to protocol described above.

Statistical analysis

Data on cell adhesion, cell viability, synthesis of collagen, EGF, and VEGF were analyzed by Kruskal-Wallis and Mann-Whitney tests, while results of IgG synthesis were analyzed by ANOVA and Tukey's tests ($\alpha = 0.05$).

Results

Cell adhesion by SEM

Results demonstrated that treatment of epithelial cells and gingival fibroblasts with bisphosphonates led to significant morphological alterations in these cells, especially for zoledronic acid (Fig. 1a, b). Further, images revealed a significant impact on the adhesion of these cells to titanium surfaces after the addition of bisphosphonates, and this effect seemed to increase over time.

Expression of cell adhesion molecules

The expression of HCAM by epithelial cells was selected for qualitative demonstration of the adhesion and spreading of these cells onto the titanium surfaces. The results showed that, for SA, decreased cell adhesion was observed starting at 5 μM, while for ZA, similar results were detected for lower concentrations, starting at 0.5 μM (Fig. 2a), for both periods, but with more intense effects at 48 h. Adhesion of gingival fibroblasts was also qualitatively assessed by the immuno-identification of actin filaments, which demonstrated results similar to those for epithelial

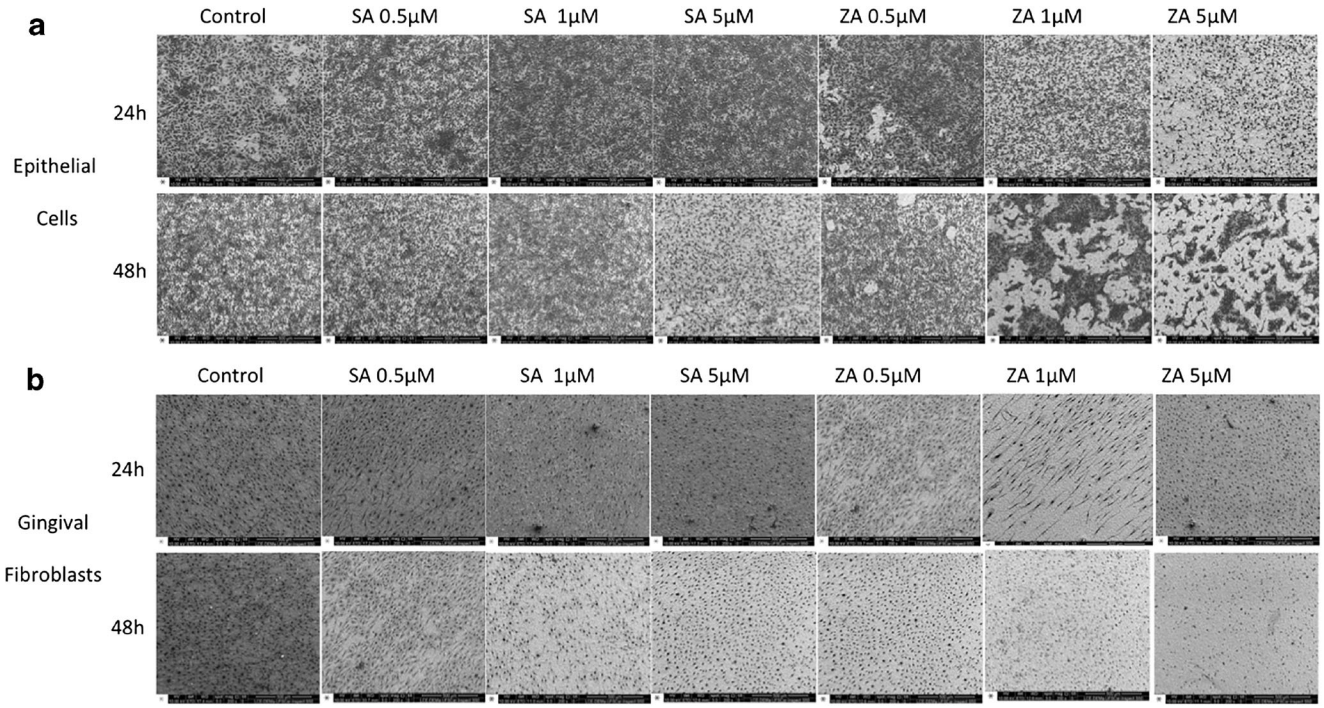

Fig. 1 Morphology of epithelial cells (**a**) and gingival fibroblasts (**b**) adhered to titanium surfaces and treated with sodium alendronate (SA) and zoledronic acid (ZA) at different concentrations for 24 and 48 h. Scanning electron microscopy (SEM), ×200

cells, with extensive cell damage for ZA-treated fibroblasts, but also decreased adhesion of SA-treated cells, especially for 5 μM (Fig. 2a). Gingival fibroblasts seemed to be more negatively affected by bisphosphonates when compared with the epithelial cells. For both cell lines, effects were concentration- and time-dependent (Fig. 2b).

Cell number

Quantitative analysis of the number of cells that remained attached to the titanium substrate after bisphosphonate treatment revealed that, for epithelial cells, there was a significant decrease in the number of cells that remained attached to the

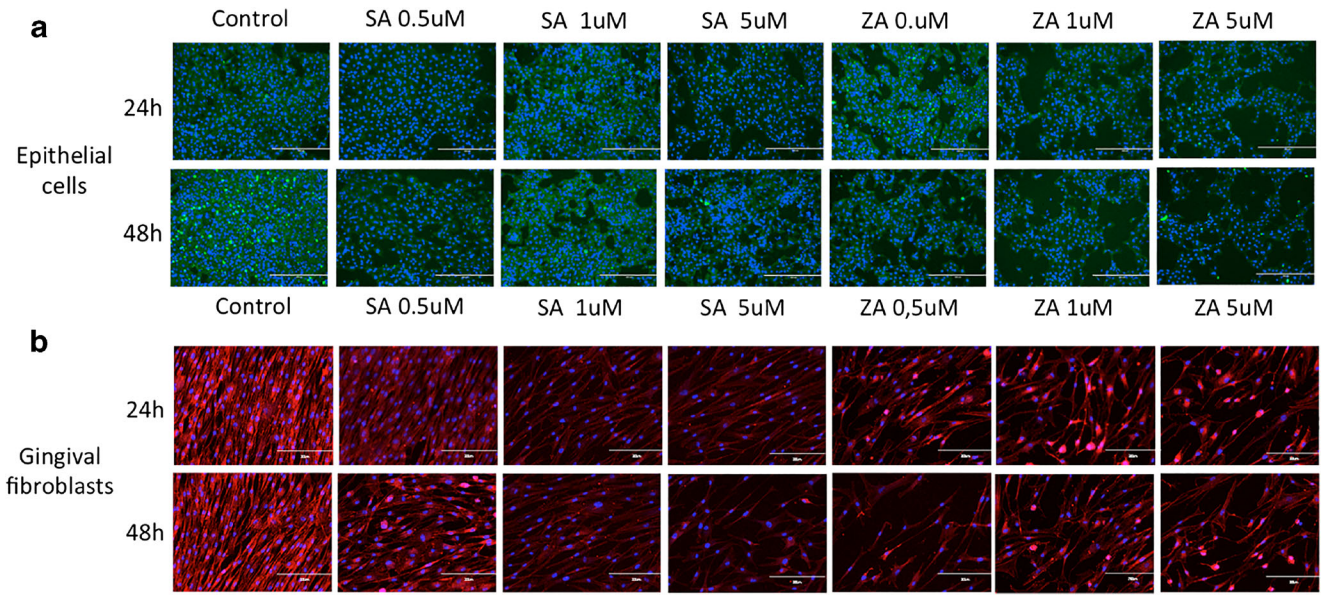

Fig. 2 Photomicrographs of membrane expression of HCAM (*green*) by epithelial cells adhered to titanium surfaces and treated with ZA or SA for 24 or 48 h (**a**). Nuclei were stained with Hoescht (×10); Photomicrographs of cytoskeletal identification of actin (*red*) of gingival fibroblasts adhered to titanium surfaces and treated with ZA or SA for 24 or 48 h (**b**). Nuclei were stained with Hoescht (×10)

discs for cells treated with SA at 5 μM and with ZA at all concentrations (Figs. 2 a and 3a). At 48 h, both bisphosphonates at all concentrations decreased the number of cells adhering to the substrate (Figs. 2 b and 3b).

At 24 h, only groups of gingival fibroblasts treated with SA and ZA at 5 μM demonstrated a decrease in the number of cells attached to the titanium discs (Figs. 2c and 3c). All experimental groups demonstrated decreased numbers of gingival fibroblasts attached to titanium surfaces after 48 h of contact with bisphosphonates (Figs. 2d and 3d).

Cell viability

Treatment of epithelial cells with SA and ZA for 24 h had no significant effect on the viability of epithelial cells, except for ZA at 5 μM (Fig. 4a). At 48 h, however, both bisphosphonates at all concentrations promoted a significant decrease in the viability of these cells (Fig. 4b).

For gingival fibroblasts, after 24 h of contact with the bisphosphonates, only the groups treated with ZA at the three selected concentrations showed reduced cell viability, while cells treated with SA demonstrated viability similar to that of the control group (Fig. 4c). At 48 h, a similar result was observed; however, the group treated with SA at 5 μM also showed decreased cell viability, similar to that caused by ZA treatment (Fig. 4d).

Collagen synthesis

Collagen synthesis by epithelial cells was not affected by bisphosphonate treatment for 24 h; however, at 48 h, decreased synthesis of this protein was observed when cells were exposed to ZA at 1 and 5 μM (Fig. 5a). For gingival fibroblasts, this synthesis was not affected by bisphosphonates at both periods of analysis (Fig. 5b). Results of collagen synthesis were normalized by cell viability for each sample of the experimental and control groups.

EGF and VEGF synthesis

Results also demonstrated that EGF synthesis by epithelial cells was decreased for ZA-treated cells at 24 h, while at 48 h, a negative effect on this synthesis was observed for SA at 1 and 5 μM and for ZA at all concentrations (Fig. 6a, b).

VEGF synthesis by gingival fibroblasts was increased by SA at 5 μM for the 24-h period, while ZA did not significantly affect this synthesis (Fig. 6c). At 48 h, as observed for the former period, VEGF synthesis was positively affected by SA, while cells treated with ZA showed results similar to those for the control group (Fig. 6d). Results of EGF and VEFG synthesis were normalized by cell viability for each sample of the experimental and control groups.

Synthesis of IgG

Evaluation of the synthesis of immunoglobulin G by epithelial cells demonstrated that the exposure to both types of bisphosphonates at all concentrations resulted in significant increases of this synthesis when compared with that of the control group, with no difference among treated groups (Fig. 7). IgG concentration of each sample was normalized by the corresponding cell viability rate.

Discussion

Several studies have demonstrated that bisphosphonates are associated with the development of oral osteonecrosis (15, 16). This effect seems to be related to a direct toxicity of these

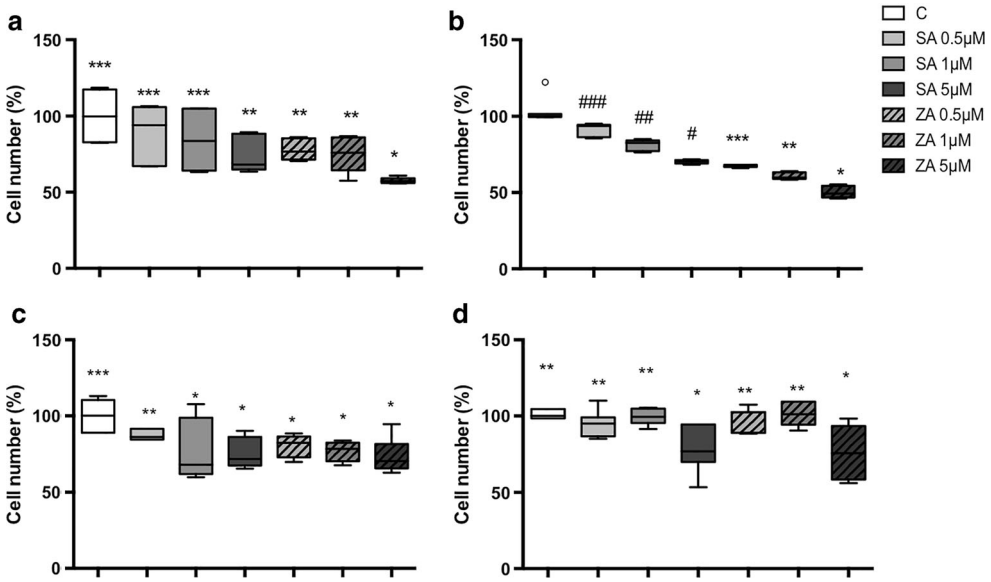

Fig. 3 Number of epithelial cells adhered to the titanium surface after treatment with bisphosphonates for 24 h (**a**) and 48 h (**b**) and number of gingival fibroblasts adhered to the titanium discs after treatment with bisphosphonates for 24 (**c**) and 48 h (**d**). *Bars* represent medium and standard deviation; groups identified by *different symbols* indicate statistical difference (Mann-Whitney, $p < 0.05$)

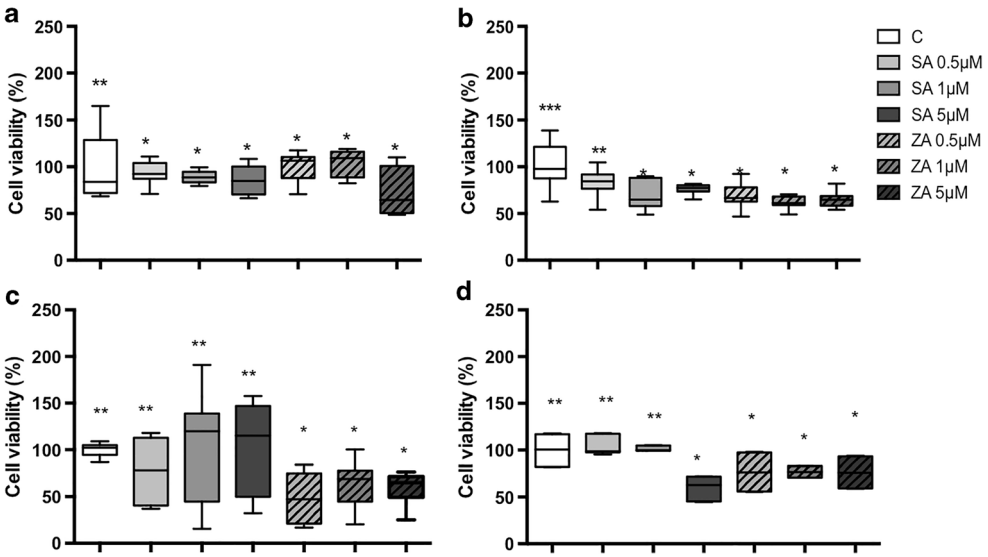

Fig. 4 Viability of epithelial cells adhered to the titanium surface after treatment with bisphosphonates for 24 h (**a**) and 48 h (**b**) and viability of gingival fibroblasts adhered to the titanium surface after treatment with bisphosphonates for 24 h (**c**) and 48 h (**d**). Bars represent medium and standard deviation; groups identified by different symbols indicate statistical difference (Mann-Whitney, $p < 0.05$)

drugs to the bone and oral mucosal cells, as well as to other local factors such as trauma and oral microbiota (7, 15-18). According to clinical studies, the use of bisphosphonates, especially zoledronic acid, has also been related to instances of failure of oral implants, while these failures seem to be significantly lower for patients treated with less potent or orally administered bisphosphonates, such as sodium alendronate (24–26), and these adverse events can occur as a short-term or even as a late effect more than 24 months after implant insertion (19).

The different outcomes for patients undergoing bisphosphonate treatment highlight the hypothesis that these effects might have a direct relationship to the types and regimens of these drugs (19, 27).

Bisphosphonates are indicated for the treatment of neoplastic and metabolic bone diseases (28). Over the decades, the evolution of these drugs has resulted in the development of more potent drugs (2). However, along with high potency, the effects of toxicity seem to be higher for these medications (3–6). Bisphosphonates can be prescribed for oral or intravenous intake, according to the type of drug, and can be indicated for different periods and intervals (2). These factors seem to be directly associated with the adverse effects described for the oral cavity (19, 27).

Results of this study demonstrated that the type of bisphosphonate is related to the toxicity of these drugs in oral mucosal cells, as is the concentration of bisphosphonate that is in contact with these cells. In a correlation to clinical events, these are the concentrations that can be released from the bone tissue at the time of the insertion of osseointegrated implants. Sodium alendronate has been reported to promote lower toxicity to oral mucosal cells when compared with ZA, and these results have been correlated to the failures of oral implants (19, 27).

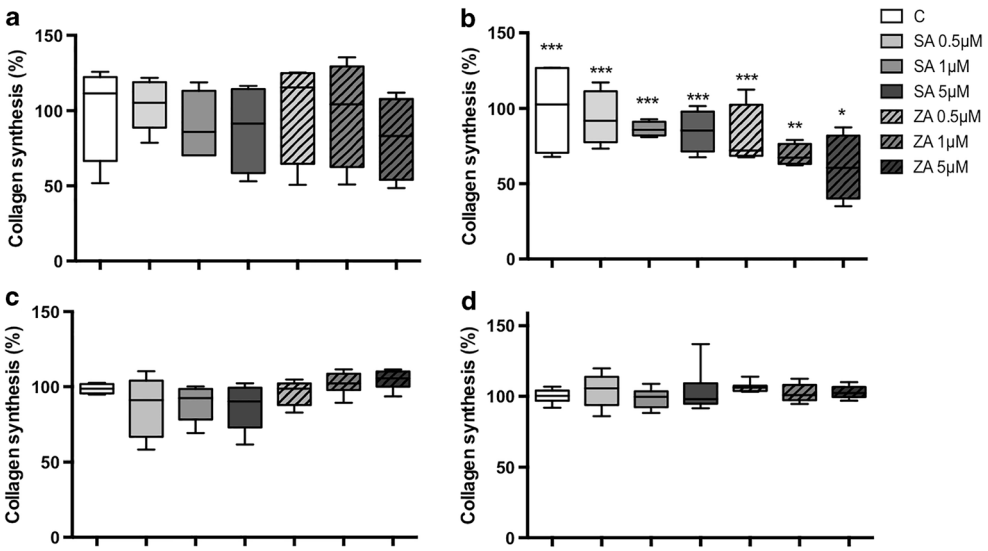

Fig. 5 Collagen synthesis by epithelial cells seeded on titanium surface after treatment with bisphosphonates for 24 h (**a**) and 48 h (**b**) and collagen synthesis by gingival fibroblasts seeded on titanium surface after treatment with bisphosphonates for 24 h (**c**) and 48 h (**d**). *Bars* represent medium and standard deviation; groups identified by *different symbols* indicate statistical difference (Mann-Whitney, $p < 0.05$)

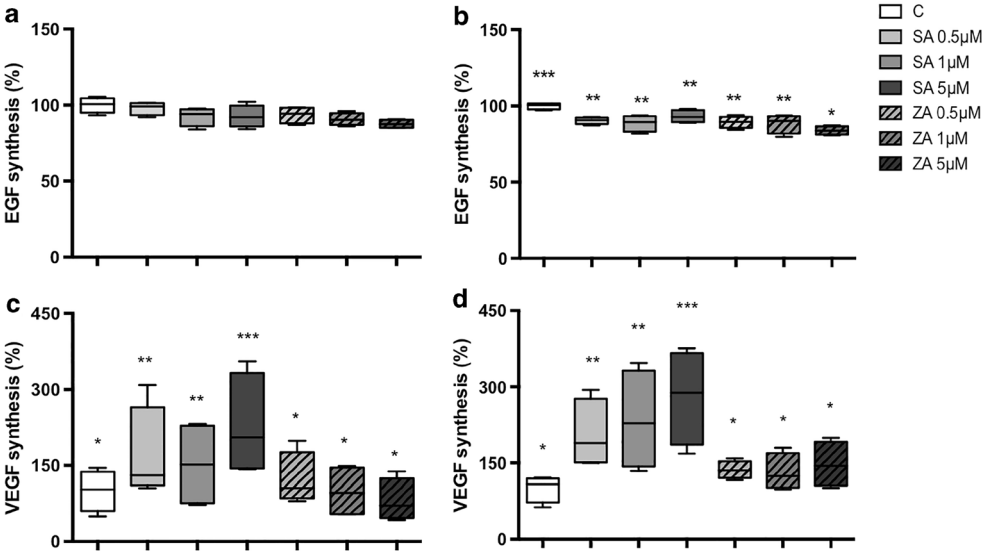

Fig. 6 EGF synthesis by epithelial cells seeded on titanium surface after treatment with bisphosphonates for 24 h (**a**) and 48 h (**b**) and EGF synthesis by epithelial cells seeded on titanium surface after treatment with bisphosphonates for 24 h (**c**) and 48 h (**d**). *Bars* represent medium and standard deviation; groups identified by *different symbols* indicate statistical difference (Mann-Whitney, $p < 0.05$)

The interface between gingival tissue and the dental surface is formed by the intimate relation of the junctional epithelium and connective tissue to enamel and cementum (29). This interface provides a physical and a biological barrier that protects the periodontal tissues from the invasion of pathogens (29). Therefore, the achievement of an effective biological seal is crucial for the maintenance of implant components and tissue homeostasis.

Several factors can affect the establishment of a biological seal, such as poor adhesion of oral mucosal cells to the prosthetic components (23). After the insertion of oral implants, these cells may adhere to the abutment, and this attachment must be followed by the expression of phenotypic characteristics related to the synthesis of adhesion molecules, in addition to extracellular molecules, growth factors, inflammatory cytokines, and immunoglobulins (23, 29).

The first and more evident effect of the presence of bisphosphonates was the lack of adhesion of these cells to the titanium surface. Since the attachment of cells could not be achieved, the physical barrier created by the interaction of gingival tissue and abutments was not effective, thus jeopardizing the other cell functions responsible for creating a biological barrier and mucosal healing.

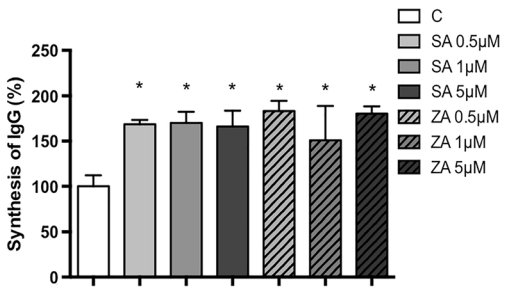

Fig. 7 IgG synthesis by epithelial cells seeded on titanium surface after treatment with bisphosphonates for 48 h. *Bars* represent medium and standard deviation; groups identified by *different symbols* indicate statistical difference (Tukey, $p < 0.05$)

Treatment with bisphosphonates negatively affected the cellular functions associated with adhesion of these cells to the titanium surfaces—viability and synthesis of growth factors—mainly when cells were exposed to ZA. However, at high concentrations, SA also caused a disturbance in the adhesion and metabolism of epithelial cells and gingival fibroblasts.

When the synthesis of immunoglobulin G was evaluated, a significant increase in the amount of this protein was detected for both bisphosphonates at all concentrations. This result can be related to an inflammatory response triggered by the presence of these drugs (30). In addition, this effect can also result in increased collagen synthesis, characteristic of inflammatory fibrous conditions (31), which could explain the increased collagen amount observed for bisphosphonate-treated cells.

Taken together, the results of this study illustrate the cellular events that may be involved in the failure of an efficient biological seal to be established when oral mucosal cells are exposed to bisphosphonates, leading to the failure of oral implants. ZA showed more intense effects on these cells, highlighting the importance of a full case-study and evaluation of risks and benefits for the insertion of oral implants in patients under treatment with this drug. Even for patients treated with SA, careful planning should not be dismissed, to avoid the development of osteonecrotic lesions and other adverse effects.

Moreover, these results also highlight the need for new strategies for the improvement of dental care for patients under bisphosphonate treatment, such as the development and improvement of implant components that could provide a better biological environment for cell adhesion, even in the presence of these medications.

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Compliance with ethical standards

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

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Ethical approval All procedures were performed in accordance to Ethics Committee of Araraquara School of Dentistry, Unesp, Brazil.

Informed consent Gingival fibroblasts were isolated after patients agreement by signing of informed consent.

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