Comparative Effects of Oral and Injectable Bisphosphonates in Primary Human Gingival Fibroblasts

OCTAVIA IULIA BALEAN1, ALIN DANIEL FLOARE1, ROXANNE FOCHT1, DANIELA JUMANCA1, DORINA CORICOVAC2*, IULIA PINZARU2, ATENA GALUSCAN2, DOINA CHIORAN1, ADELINA CHEVERESAN2, ANGELA CODRUTA PODARU2

1Victor Babes University of Medicine and Pharmacy Timisoara, Faculty of Dental Medicine, 2nd Eftimie Murgu Sq, 300041, Timisoara, Romania
2Victor Babes University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2nd Eftimie Murgu Sq, 300041, Timisoara, Romania

Bisphosphonates are effective antiresorptive agents frequently used in the treatment of different bone disorders, as osteoporosis, Paget’s disease and tumours that cause osteolysis. A major concern related to bisphosphonates therapy is represented by osteonecrosis of jaw, a serious, debilitating, and mostly, a therapy-resistant disease, reported as a frequent side effect of bisphosphonates. The present study was aimed to assess and compare the effect of four commercially available bisphosphonates, very frequently used as oral (Fosamax - F and Actonel - A) and injectable (Ossica - O and Zoledronic acid - Z) therapy on primary human gingival fibroblasts - HGF viability. Alamar blue cell viability assay was performed to assess the effect of test compounds (1.5; 2.5; 5 and 10 µM) on gingival fibroblasts viability after a 24 h interval. In these experimental conditions, injectable bisphosphonates (O and Z) proved to be safe for HGF cells, whereas oral compounds (F and A) were cytotoxic even at low concentrations.

Keywords: bisphosphonates, primary human gingival fibroblasts, cell viability, cell morphology.
Experimental part

Materials and methods

Cell line

The cell line used in the present study was human primary gingival fibroblast - HGF (ATCC® PCS-201-018®), purchased from ATCC (American Type Cell Collection) and received as frozen vial. These cells are adherent, bipolar and refractile, present a spindle-shape and were obtained from a Caucasian female.

Reagents

The culture specific medium - Fibroblast Basal Medium (ATCC PCS-201-030), and Fibroblast growth kit-low serum (ATCC PCS-201-041) were acquired from ATCC, whereas the other reagents used, as: trypsin -EDTA solution, PBS (phosphate saline buffer), Trypan blue, DMSO (dimethyl sulfoxide), Alamar blue (resazurin sodium salt) were bought from Sigma Aldrich (Germany) and Thermo Fisher Scientific (USA). The test compounds from the class of bisphosphonates: Actonel (risedronic acid -75 mg tablets, Actavis, Islanda), Fosamox (Alendronate sodium -70 mg tablets, Merck Sharp &Dohme, Romania), Ossica (ibandronic acid -1 mg/ mL solution for i.v administration, Gedeon Richter, Hungary) and Zoledronic acid (Actavis -4 mg/ 5 mL solution for parenteral use), were prepared as follows: Actonel and Fosamax were dissolved in DMSO, obtaining stock solutions of 10 mM, and Ossica and Zoledronic acid were diluted in culture medium.

Cell culture

During the experiment, the cells were grown in specific media - Fibroblast Basal Medium (ATCC PCS-201-030), supplemented with Fibroblast growth kit-low serum (ATCC PCS-201-041). HGF cells were maintained in a humidified incubator provided with 5% CO₂ at 37°C. The cells were numbered using a cell counting device - Countess II Automated Cell Counter (Thermo Fisher Scientific, USA), in the presence of Trypan blue.

Cell viability assessment

To verify the potential toxicity of the test compounds on human primary gingival fibroblasts - HGF it was applied Alamar blue assay. In brief, HGF cells were seeded in 96-wells plates (1x10⁴ cells/well/ 200µL) and let to grow until the appropriate confluence was reached (24 - 48 h) [15]. Different concentrations (1.5; 2.5, 5 and 10 µM) of the test compounds (Actonel - A; Fosamax - F; Ossica - O, Zoledronic acid - Z) were added in fresh culture medium and maintained for 24 h in contact with HGF cells. After 24 h, it was added 20 µL of Alamar blue, incubated for 3 h at 37°C and measured the absorbance values at 570 and 600 nm by the means of xMark™ Microplate Spectrophotometer (Biorad).

Cell morphology

The effects of test compounds (A, F, O and Z) ± ozone toxicity, the lowest percentage of viable cells being calculated for the highest concentration (5 and 10 µM: 77.93% and 79.33%), respectively. No. 9 (20193326).

Results and discussions

Bisphosphonates are synthetic analogues of pyrophosphates known to strongly bind to hydroxypatite, a mineral from bone structure and modify bone resorption by decreasing bone metabolism and remodeling processes [8]. The side effects of bisphosphonates include irritation of the oesophagus, dysphagia, migraines, intestinal obstruction, joint and bone pain, but most importantly osteonecrosis of the jaws - a main dental adverse event [3, 5, 11, 12]. Osteonecrosis of the jaw has become a major concern in the field of dentistry, since the risk of development to patients under the treatment with these agents that suffer invasive dental procedures is quite elevated and treating this kind of patients requires a complex management of the pathology. The most cases of osteonecrosis of jaw were recorded after administration of orally bisphosphonate -alendronate (the most frequently used worldwide) [2].

Taking into consideration that the category of patients that use bisphosphonates therapy becomes wider day by day (approximately 75 million people are affected by osteoporosis in Europe, U.S. and Japan) [16], it’s important to know the impact of these compounds in the organism, especially, since this kind of medication is for long-term.

In this study, it was verified and compared the effect of several types of bisphosphonates (Fosamax - F, Actonel - A, Ossica - O and Zoledronic acid - Z) on human primary gingival fibroblasts - HGF viability and morphology after a 24 h stimulation.

Bisphosphonates decrease human primary gingival fibroblasts - HGF viability

Stimulation for 24 h of HGF cells with different compounds from the bisphosphonates family led to distinct results regarding the percentage of viable cells, as follows: Z and O (Figure 2A) had no toxic effects on cells viability, moreover a stimulatory effect was noticed; in the case of Actonel and Fosamax it was observed a dose-dependent toxicity, the lowest percentage of viable cells being recorded at 10µM (67.47 % viable cells), and Actonel -A (fig. 2B) proved to be toxic even at the lowest concentrations tested (1.5 and 2.5µM: 77.93% and 79.33%), percentage of viable cells that was close to the ones calculated for the highest concentration (5 and 10 µM: 72.37 and 71.99%).

Statistical analysis

The results obtained were expressed as means ± SD, and the difference between means was compared by one-way ANOVA, using the post-hoc Tukey’s and Dunnett’s multiple comparison tests (GraphPad Prism v. 6.0 Software, SUA). The difference between groups was considered statistically significant if p < 0.05.
Bisphosphonates impair human primary gingival fibroblasts - HGF morphology

Since the viability results indicated signs of toxicity after test compounds stimulation (F and A), it was verified if there were induced some changes in cells morphology. DMSO was used as vehicle for Actonel and Fosamax. As it can be seen in Figure 3, DMSO-stimulated cells present similar characteristics as control (unstimulated) cells, spindle-shape, bipolar, adherence to the plate, what indicates no toxicity signs induced by different concentrations of DMSO.

Fosamax -F stimulation induced several slight changes of HGF cells shape, changes that become more evident with increasing the concentration (Figure 4). Most of the cells kept their spindle shape and their adherence was not affected, still there were also noticed some cells that were round and floating, and cells debris was also present (mainly at 5 and 10 µM), data that are in line with cell viability results.

Similar results as the ones described for Fosamax -F were observed in the cells stimulated with Actonel -A (Figure 5), with the difference that changes in cells shape (round cells), their adherence and the presence of cell debris appeared even at the lowest concentration (1.5 µM).

In the case of cells stimulated with Zoledronic acid -Z (Figure 6) and Ossica - O (Figure 7), it was observed a higher confluence of the cells that presented similar shapes with control cells (spindle shape with a high adherence to the plate), data that are in agreement with cell viability findings (Figure 2A).

Human primary gingival fibroblasts were selected for this study based on the following considerations: (i) these cells are the main constituents of the gingival connective tissue and the most abundant residents of oral mucosa; (ii) play key roles in scarless wound healing process by releasing multiple growth factors (transforming growth factor beta - TGF-Beta, connective tissue growth factor - CTGF, and basic fibroblast growth factor - bFGF); and iii) primary cells offer more reliable data as compare to immortalized cell lines [17, 18]. In addition, previous studies proved that primary human gingival fibroblasts are valuable and reliable in vitro models for screening toxicity/cytocompatibility of dental materials [17, 19, 20].

Our results showed that F and A become cytotoxic even at low concentrations as 1.5 µM (Figure 2B) and also cells morphology was altered (Figures 4 and 5), whereas O and Z had no cytotoxic effects at the concentrations tested (1.5; 2.5; 5 and 10 µM) (Figure 2A). The cytotoxic effect of
Alendronate -F on human primary gingival fibroblasts was also shown by other groups of research [21-23], data that are in agreement with these results. Stimulation of human stem cells derived from the gingiva with risedronate in the range of 1-10 µM led to changes in cells morphology and a reduced viability [24], findings that confirm our results.

An in vitro study developed on human umbilical cord vein endothelial cells (HUVEC), human gingival fibroblasts (HGF), human osteogenic cells (HHOB-c) and human oral keratinocytes (HOK) stimulated with 50 µM ibandronate, pamidronate or zoledronate for 24 h showed that bisphosphonates used reduced all cells viability [25]. The results obtained in the present study showed that Z (zoledronic acid) and O (ibandronic acid) are nontoxic for primary gingival fibroblasts at concentrations ≤10 µM.

Another study conducted by Soydan et al. [22] demonstrated the toxic effects of Alendronate (Fosamax) and PAM on human gingival fibroblasts, characterized by significant changes in the apoptotic and proliferative indices leading to an in vitro faulty epithelisation of the oral mucosa. The apoptotic index of the PAM group was significantly higher than the alendronate group for all concentrations, especially at 24 h of incubation (p < 0.05). The decrease in the proliferation index was similar for both drugs; however, after 72 h of incubation, the decrease in the PAM group proliferation index was significantly higher (p < 0.05). The change of these indices is an important factor in the management of delayed healing of the oral mucosa, secondary to surgery in patients under bisphosphonate treatment, and is a problem that reduces the success rate of healing in cases of bisphosphonate-induced maxillary osteonecrosis [26, 27].

Conclusions

In the view of all these findings, it could be stated that oral bisphosphonates - Fosamax and Actonel exert a concentration-dependent toxicity on human primary gingival fibroblasts by decreasing the percentage of viable cells and modifying cells morphology. In contrast, injectable bisphosphonates - Zoledronic acid and Ossica did not affect the viability or HGF cells morphology.

Fig. 5 The aspect of HGF - human primary gingival fibroblasts in culture: Control - unstimulated cells and cells stimulated with different concentrations of Actonel - A (1.5; 2.5; 5 and 10 µM) for 24 h. Pictures were taken using the 20x objective.

Fig. 6 The aspect of HGF - human primary gingival fibroblasts in culture: Control - unstimulated cells and cells stimulated with different concentrations of Zoledronic acid - Z (1.5; 2.5; 5 and 10 µM) for 24 h. Pictures were taken using the 20x objective.

Fig. 7. The aspect of HGF - human primary gingival fibroblasts in culture: Control - unstimulated cells and cells stimulated with different concentrations of Ossica - O (1.5; 2.5; 5 and 10 µM) for 24 h. Pictures were taken using the 20x objective.
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