# Ozone saline solution counteracts bisphosphonates noxious effects in primary human gingival fibroblasts

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**Research article**

**Keywords:** bisphosphonates, ozone saline solution, primary human gingival fibroblasts, cell viability, cell morphology.

**Posted Date:** July 15th, 2019

**DOI:** https://doi.org/10.21203/rs.2.11340/v1

# Abstract

Background 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. In the present study were proposed two approaches: 1) to verify the impact 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 and 2) to evaluate the protective effect of an ozone saline solution on HGF cells pretreated with bisphosphonates. Methods 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. An ozone - O3 saline solution – 80 μg/mL was added to bisphosphonates pretreated fibroblasts for 24 h and cell viability and cell morphology changes were determined by the means of Alamar blue test and microscopic images. Results Fosamax and Actonel induced a significant reduction of HGF cells viability even at concentrations as low as 2.5 μM (82 and 79.33%) and changes in cells morphology (round and floating cells), effects that were reversed by O3 saline solution administration: an increased cell viability after F and A at 2.5 μM: 147.54 and 120.11%), no changes in cells morphology and an improved confluence. Ossica and Zoledronic acid exerted no cytotoxic effect. Conclusions In conclusion, 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, effects that were counteracted by O3 saline solution administration. Based on these data, ozone saline solution might represent a therapeutic alternative for bisphosphonates noxious effects on oral mucosa cells.


# Background

Bisphosphonates are a class of pharmaceutics applied as treatment for several bone disorders and cancers, as: Paget's disease, osteoporosis, multiple myeloma and hypercalcemia of malignancy, that act by suppressing osteoclast differentiation, impairing its activity and leading to early apoptosis. Their activity on osteoclast is also related to adverse events as damaged bone healing and remodeling processes that have as consequence an augmented risk of developing osteonecrosis of jaw, a serious and debilitating condition, in patients subjected to surgical dental procedures as extraction or implant placement [1, 2, 3, 4, 5, 6]. Since more than fifteen years ago, there were raised notable concerns regarding the liaison between the use of bisphosphonates and the increased risk to develop osteonecrosis of the jaw after invasive dental procedures [7]. A considerable number of studies were conceived to find strategies for dental management of patients at risk to develop osteonecrosis of the jaw following antiresorptive (bisphosphonates) and antiangiogenic compounds use [1, 7, 8, 9, 10, 11], still, there are no effective therapeutic alternatives to reverse bisphosphonates noxious effects, and to find an appropriate prevention/treatment method for osteonecrosis of the jaw is rather challenging [12].

Ozone ($O_3$) is an unstable gas, naturally produced by atmospheric air with a triatomic molecule and a reduced half-life (40 minutes at 20 ºC), that exerts multiple biological effects, as: antimicrobial (more efficient as compared to chloride), stimulator of blood circulation and immune response, anti-hypoxic, features that recommend it for medicine and dentistry applications [13, 14, 15]. Among the multiple dental applications of the ozone, there can be stated the following: prevention of dental caries, endodontic treatment, tooth extraction, periodontal pocket disinfection and osseous disinfection, accelerated healing, tissue regeneration, remineralization of tooth surface, teeth whitening, etc [13, 16, 17]. Moreover, medical ozone formulated as oil suspension or administered as gas insufflations to patients with bisphosphonates-induced osteonecrosis of the jaw lesions proved to be a promising therapeutic option in small (< 2.5 cm – oil suspension) and big lesions (> 2.5 cm – gas insufflations) [18, 19].

In the light of the data presented above, the present study was subjected: (i) to assess the impact of orally (sodium risedronate – Actonel - A and alendronate – Fosamax - F) and intravenously (ibandronic acid – Ossica - O and zoledronic acid - Z) bisphosphonates on human primary gingival fibroblasts (HGF) and (ii) to verify if an ozone saline solution counteracts/reverses bisphosphonates noxious effects induced in HGF cells.

## 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), Fosamax (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% $CO_2$ at 37°C. The cells were numbered using a cell counting device - Countess$^{TM}$ 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 ($1x10^4$ cells/well/ 200 µL) and let to grow until the appropriate confluence was reached (24 - 48 h). 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 37ºC and measured the absorbance values at 570 and 600 nm by the means of xMark™ Microplate Spectrophotometer (Biorad).

# Ozone impact on human primary gingival fibroblasts

To determine the effect of ozone ($O_3$ saline solution – 80 µg/mL) on cells viability and its impact on cells capacity to recover after test compounds toxicity, HGF cells were stimulated with Actonel and Fosamax (compounds that proved to be cytotoxic) (1.5; 2.5; 5 and 10 µM) for 24 h. The old media was removed, and it was added 100 µL of fresh medium and 100 µL of ozone saline solution/well for 24 h according to the protocol described by Perez *et al.* [20], followed by application of Alamar blue assay, as described above.

# Cell morphology

The effects of test compounds (A, F, O and Z) ± ozone saline solution on cells morphology were assessed by taking pictures before addition of test compounds and after the stimulation period (24 h). The pictures were acquired by using the Olympus IX73 inverted microscope provided with DP74 camera photo and documented with the CellSens V1.15 software (Olympus, Tokyo, Japan).

# 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 and by unpaired

t test with Welch's correction (GraphPad Prism v. 6.0 Software, SUA). The difference between groups was considered statistically significant if $p < 0.05$.

## Results

# Bisphosphonates decrease human primary gingival fibroblasts – HGF viability

In the present study, it was evaluated the effect induced by several bisphosphonates (A, F, O and Z) frequently used as treatment for osteoporosis, on HGF cells 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 1A) had no toxic effects on cells viability, moreover a stimulatory effect was noticed; in the case of F (Figure 1B) it was observed a dose-dependent toxicity, the lowest percentage of viable cells being recorded at 10 μM (67.47 % viable cells), and A (Figure 1B) proved to be toxic even at the lowest concentrations tested (1.5 and 2.5 μM: 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%).

Figure 1. *In vitro* viability evaluation of Zoledronic acid – Z, Ossica – O, Fosamax – F and Actonel – A (1.5, 2.5, 5 and 10 μM) on HGF – human primary gingival fibroblasts at 24 h post-stimulation by Alamar blue assay. The results are expressed as cell viability percentage (%) normalized to control (for Z and O - stimulated cells) and to DMSO (for F and A-stimulated cells). The data represent the mean values ± SD of three independent experiments. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control/DMSO followed by Dunnett's multiple comparisons post-test (** p<0.01 and **** p< 0.0001).

# 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 2, 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.

Figure 2. The aspect of HGF – human primary gingival fibroblasts in culture: Control – unstimulated cells and cells stimulated with different concentrations of DMSO (1.5; 2.5; 5 and 10 μM) for 24 h. Pictures were taken using the 20x objective.

Fosamax stimulation induced several slight changes of HGF cells shape, changes that become more evident with increasing the concentration (Figure 3). 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.

Figure 3. The aspect of HGF – human primary gingival fibroblasts in culture: Control – unstimulated cells and cells stimulated with different concentrations of Fosamax - F (1.5; 2.5; 5 and 10 μM) for 24 h. Pictures were taken using the 20x objective.

Similar results as the ones described for Fosamax were observed in the cells stimulated with Actonel (Figure 4), with the difference that changes in cells shape (round cells), a decreased adherence and the presence of cell debris appeared even at the lowest concentration (1.5 μM) tested.

Figure 4. 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.

In the case of cells stimulated with Zoledronic acid (Figure 5) and Ossica (Figure 6), 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 1A).

Figure 5. 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.

Figure 6. 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.

# Ozone suppresses bisphosphonates cytotoxicity on HGF cells

In this study it was also verified if ozone saline solution can reduce the cytotoxic effects induced by test compounds. For this experiment were chosen only F and A, compounds that proved to decrease the percentage of viable cells.

After a 24 h stimulation with F and A (1.5, 2.5, 5 and 10 μM), the medium was removed and replaced with 100 μL new medium + 100 μL ozone saline solution (80 μg/mL)/ well for other 24 h. The impact of test compounds stimulation followed by ozone administration was evaluated by the means of Alamar blue assay.

As it can be seen in Figure 7A, stimulation with ozone ($O_3$) solution led to a significant increase of cells viability percentage. Similar results were observed in the case of the cells stimulated with DMSO (data not shown). The images presented in Figure 7B confirm the viability results since the cells that were in contact with $O_3$ solution show a higher confluence, no presence of round and floating cells, what indicates a beneficial effect of ozone on human primary gingival fibroblasts growth and proliferation.

Figure 7. Impact of ozone ($O_3$) saline solution on HGF- human primary gingival fibroblasts: A. viability assessment and B. morphology after stimulation with $O_3$ solution for 24 h. Data obtained for viability test (A) represent the mean values ± SD of three independent experiments. Unpaired t test with Welch's correction was applied to determine the statistical differences in rapport with control cells (**** $p < 0.0001$).

A significant augmentation of the viable cells percentages was also noticed in the cells stimulated with test compounds F and A followed by $O_3$ solution addition for 24 h as compared to the ones that did not received $O_3$ solution (Figure 8). These results show that $O_3$ solution, not only suppressed the toxic effects of test compounds (F and A), but also improved their capacity to recover and stimulated their growth and proliferation.

Figure 8. Assessment of ozone ($O_3$) solution on HGF - human primary gingival fibroblasts viability stimulated previously with Fosamax – F and Actonel – A (1.5, 2.5, 5 and 10 µM). Data represent the mean values ± SD of three independent experiments. Unpaired t test with Welch's correction was applied to determine the statistical differences in rapport with $O_3$ unstimulated cells (**$p<0.01$, ***$p<0.001$ and **** $p < 0.0001$).

Ozone ($O_3$) solution determined several changes in the morphology of HGF cells stimulated with F and A (see Figures 9 and 10), as follows: the cells present spindle and elongated shapes as the control cell, are very adherent to the cell culture plate, their confluence is higher as compared to - $O_3$ group and there were no floating cells or debris within the culture plate.

Figure 9. The aspect of HGF – human primary gingival fibroblasts in culture stimulated with different concentrations of Fosamax - F (1.5; 2.5; 5 and 10 µM) for 24 h: without (- $O_3$) and with (+ $O_3$) ozone stimulation. Pictures were taken using the 20x objective at a scale bar of 50 µm.

Figure 10. The aspect of HGF – human primary gingival fibroblasts in culture stimulated with different concentrations of Actonel -A (1.5; 2.5; 5 and 10 µM) for 24 h: without (- $O_3$) and with (+ $O_3$) ozone stimulation. Pictures were taken using the 20x objective at a scale bar of 50 µm.

# Discussions

Bisphosphonates are synthetic analogues of pyrophosphates known to strongly bind to hydroxyapatite (a mineral from bone structure) and modify bone resorption by decreasing bone metabolism and

remodeling processes [7]. There were described two different classes of bisphosphonates: non-nitrogen (risedronate) and nitrogen containing (alendronate, ibandonic acid and zoledronic acid) bisphosphonates, that present a disparate mechanism of action, as follows: non-nitrogen compounds are taken up by the osteoclast and metabolized to adenosine triphosphate analogues with noxious effects that induce osteoclast apoptosis or cell death, whereas nitrogen containing bisphosphonates are interiorized by osteoclasts during resorption process and impair the mevalonate pathway (involved in the synthesis of cholesterol) leading to suppression of bone resorption [21]. 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, 10, 11]. 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 bisphosphonates – alendronate (the most frequently used worldwide) [2].

Taking into consideration that the category of patients that use bisphosphonates therapy becomes wider day by day (approximatively 75 million people are affected by osteoporosis in Europe, U.S. and Japan) [22], it is 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 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.

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 compared to immortalized cell lines [23, 24]. In addition, previous studies proved that primary human gingival fibroblasts are valuable and reliable *in vitro* models for screening toxicity/cytocompatibility of dental materials [23, 25, 26].

Our results showed that F and A become cytotoxic even at low concentrations as 1.5 μM (see Figure 1B) and also cells morphology was altered (see Figures 3 and 4), whereas O and Z had no cytotoxic effects at the concentrations tested (1.5; 2.5; 5 and 10 μM) (see Figure 1A). The cytotoxic effect of alendronate – F on human primary gingival fibroblasts was also shown by other groups of research [27, 28, 29], 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 [30], 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 [31]. The results obtained in the present study showed that Z (zoledronic acid) and O (ibandronic acid) are not cytotoxic for primary gingival fibroblasts at concentrations ≤ 10 μM.

Another study conducted by Soydan *et al.* [28] demonstrated the toxic effects of Alendronate (Fosamax) and PAM (pamidronate) 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 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 [14, 32].

Looking for new ways to reduce the side effects of bisphosphonates, ozone was described as a viable choice for healing and restoring oral cells viability. Besides the benefits of ozone in dentistry (carious lesions treatment, endodontics, reduction of dental hypersensitivity, periodontics, oral surgery, pedodontics, orthodontics) [15, 17], were also mentioned other biological effects, as: (a) antimicrobial activity against aerobic and anaerobic bacteria (especially *Staphylococcus aureus*), fungi and viruses; (b) stimulator of the circulatory system, increasing haemoglobin synthesis and the production of red blood cells, thereby producing tissue oxygenation; (c) modulator of immune cells, acting as a cytokine, and increasing their phagocytosis and diapedesis; (d) stimulator of angiogenesis as well as the proliferation of fibroblasts; and (e) pain reduction capacity [15, 33].

Addition of the ozone saline solution (stock concentration - $O_3$ saline solution – 80 μg/mL; 100 μL/well) to pretreated HGF cells with F and A for 24 h led to an increase of cells viable percentages as compared to the ones stimulated only with F and A (see Figure 8) and it was also noticed an improvement of cells confluence and a cells morphology similar to the control ones (unstimulated cells) (see Figures 9 and 10), what demonstrates that ozone solution in these experimental conditions was able to reverse the toxic effects induced by F and A. Our data are in line with other data from the literature that confirm the protective effects of ozone on gingival fibroblasts [32, 34, 35].

Moreover, daily application of an ozonated water treatment accelerated the rate of physiological healing. In a study comparing the use of ozonated oil in an experimental group with a control group using antibiotic therapy in the treatment of alveolitis, it was found that patients treated with ozonated oil healed more quickly [36, 37]. Ozone was also used in the treatment of avascular osteonecrosis of the maxilla (ONJ). Complete healing of lesions with disappearance of symptoms has been noticed [38, 39].

# Conclusions

In the view of 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. Ozone saline solution administration to

bisphosphonates - treated cells reversed the toxic effects of test compounds (F and A) and improved cells capacity to recover by stimulating their growth and proliferation. These *in vitro* results represent a valuable background that endorse the hypothesis that ozone saline solution could be considered a therapeutic alternative for bisphosphonates noxious effects on oral mucosa cells.

# List Of Abbreviations

A – Actonel

ATCC – American Type Cell Collection

bFGF - basic fibroblast growth factor

CTGF - connective tissue growth factor

DMSO – dimethyl sulfoxide

EDTA - Ethylenediaminetetraacetic acid

F – Fosamax

HGF – primary human gingival fibroblasts

HHOB-c - human osteogenic cells

HOK - human oral keratinocytes

HUVEC - human umbilical cord vein endothelial cell

i.v. – intravenous

O – Ossica

$O_3$ – ozone

ONJ - osteonecrosis of the jaw

PAM - pamidronate

PBS – phosphate saline buffer

TGF-Beta - transforming growth factor beta

Z – Zoledronic acid

# Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Conflict of interest

The authors declare that they have no competing interests.

## Authors' contributions

OIB, AG, ACP concepted and designed the study, contributed to the interpretation of data, drafting the article and revising it critically. DC, IP, CD were responsible with acquisition of data, analysis and interpretation of data and critically revision of the manuscript. ADF, RF, DJ, DC contributed to the design of the study and by revising critically the manuscript. The final form of the manuscript was read and received the approval of all the authors.

## Acknowledgements

Not applicable.

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# Figures

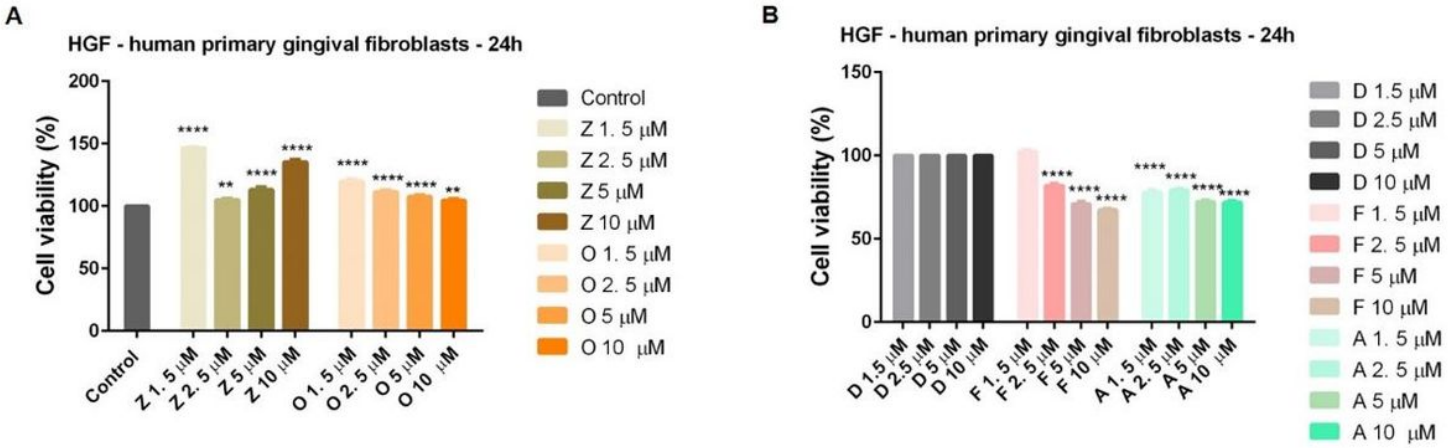
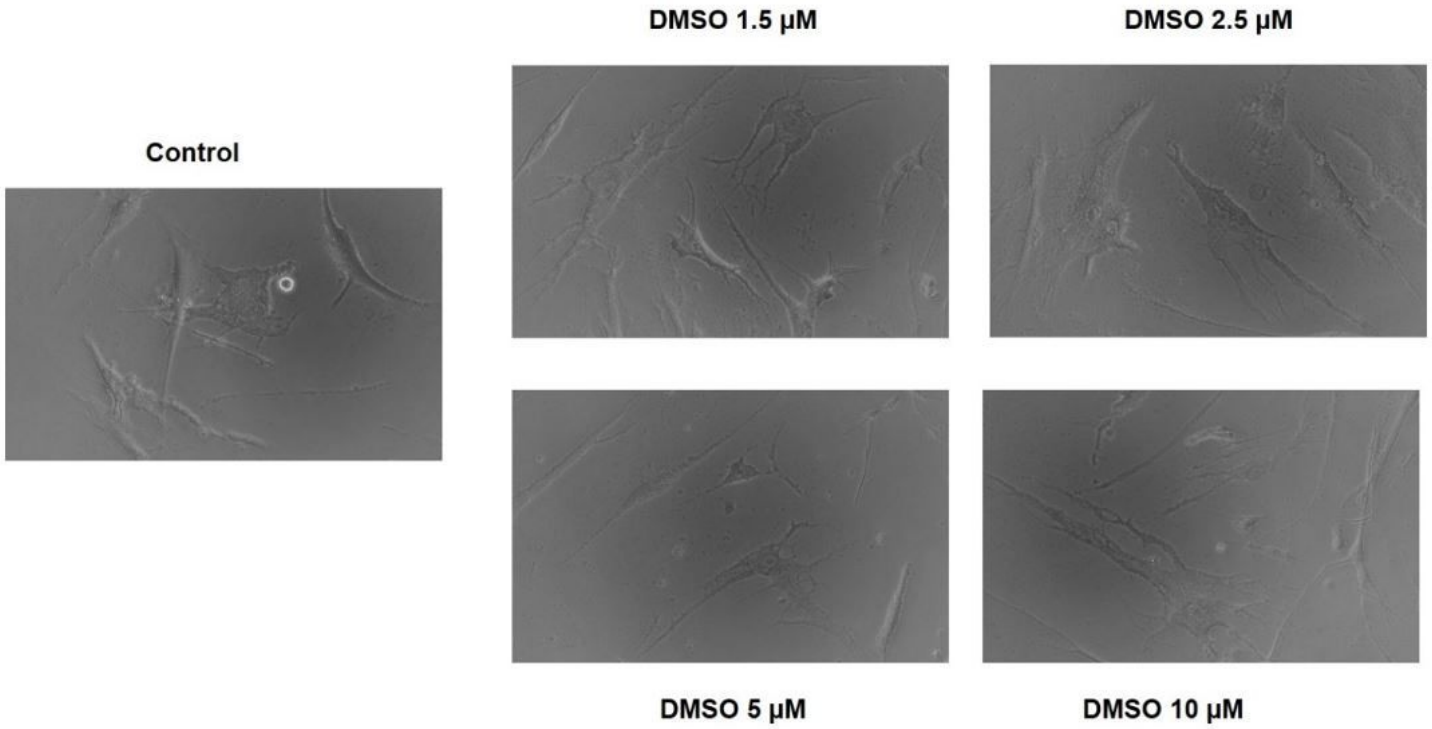



**Figure 1**

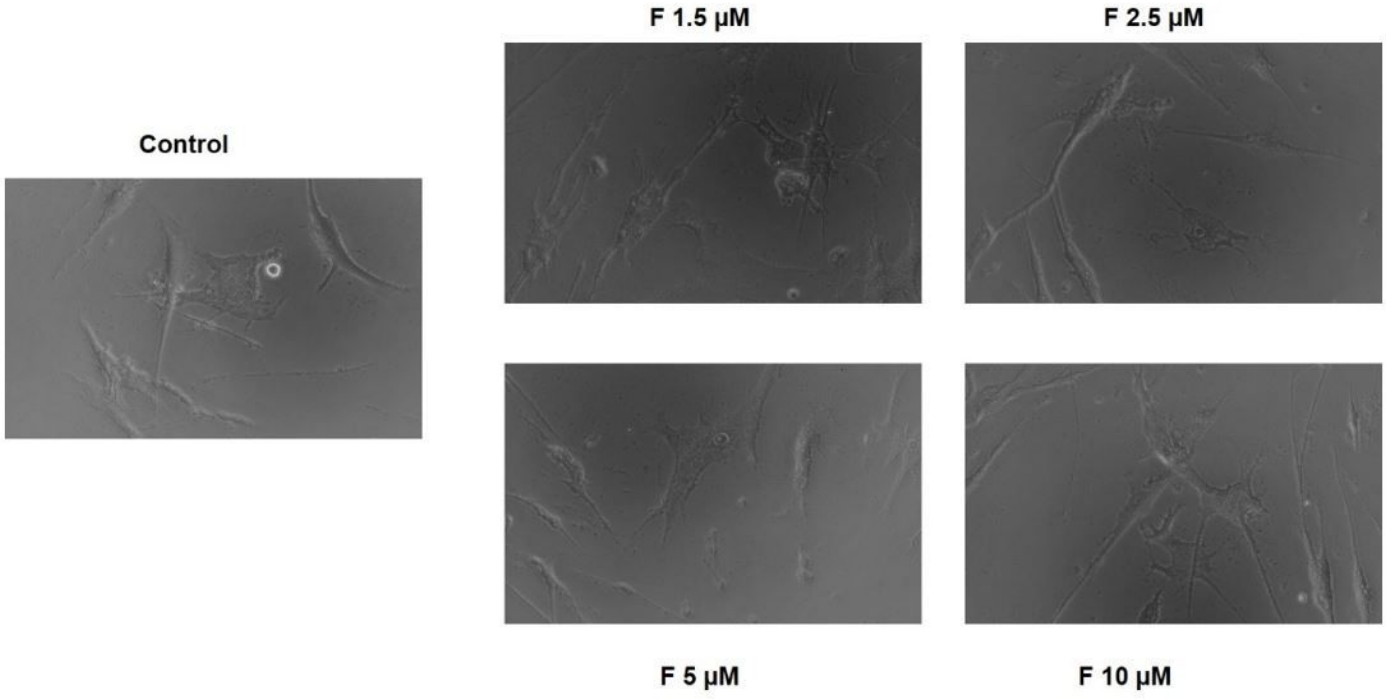
In vitro viability evaluation of Zoledronic acid – Z, Ossica – O, Fosamax – F and Actonel – A (1.5, 2.5, 5 and 10 µM) on HGF – human primary gingival fibroblasts at 24 h post-stimulation by Alamar blue assay. The results are expressed as cell viability percentage (%) normalized to control (for Z and O - stimulated cells) and to DMSO (for F and A-stimulated cells). The data represent the mean values ± SD of three independent experiments. One-way ANOVA analysis was applied to determine the statistical differences

in rapport with control/DMSO followed by Dunnett's multiple comparisons post-test (** p<0.01 and **** p< 0.0001).



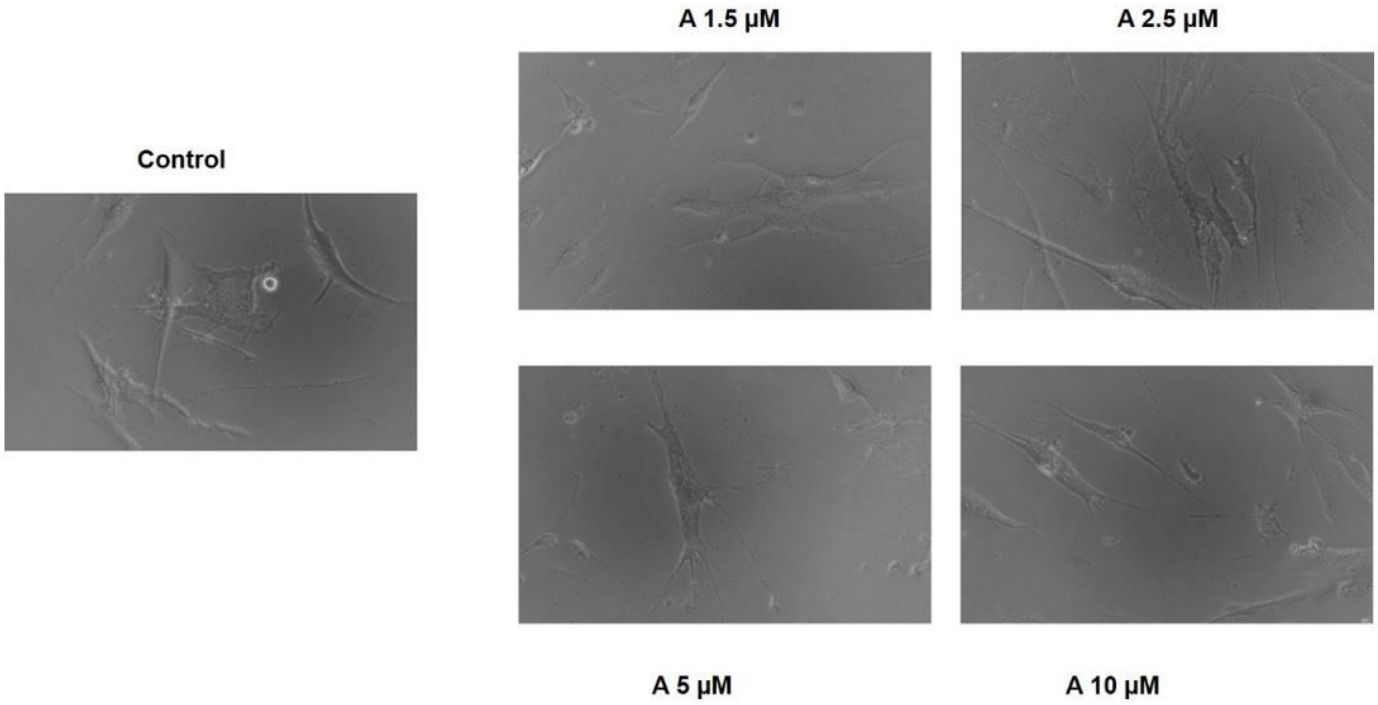

## Figure 2

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



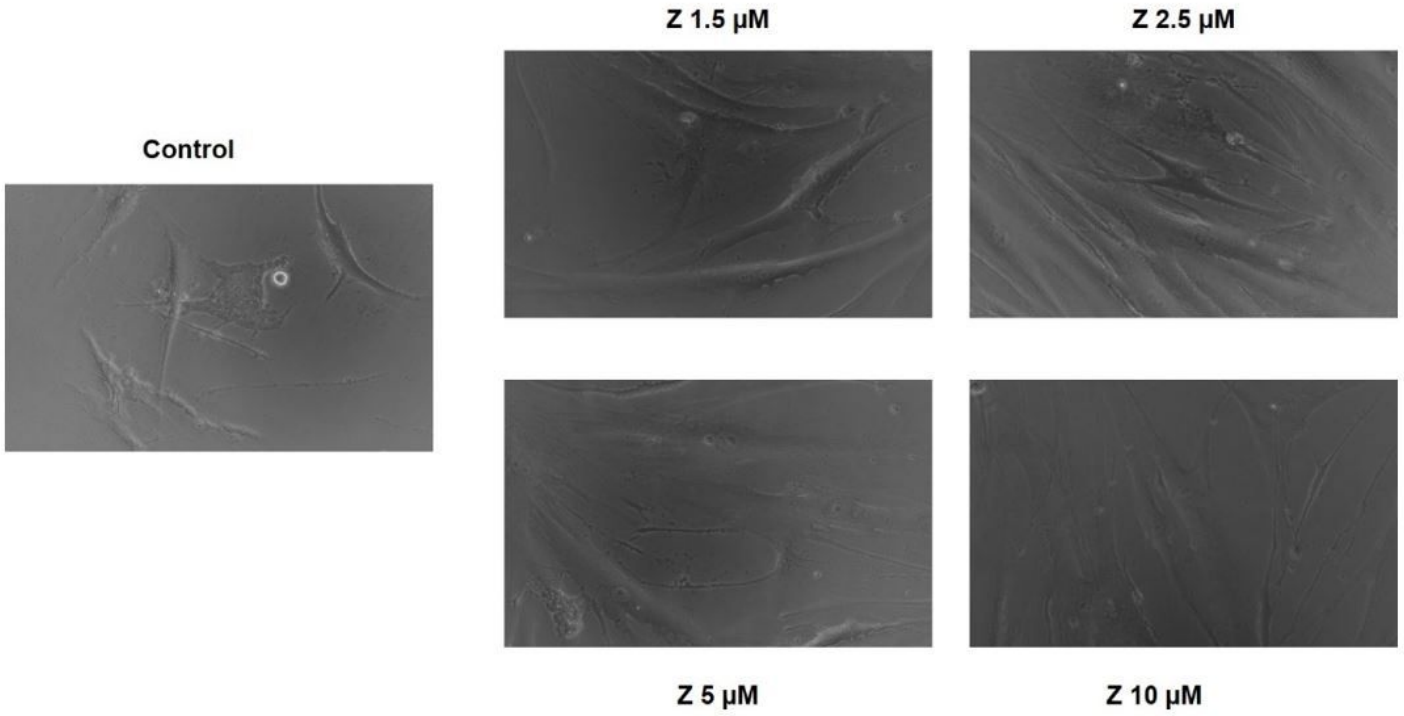
## Figure 3

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



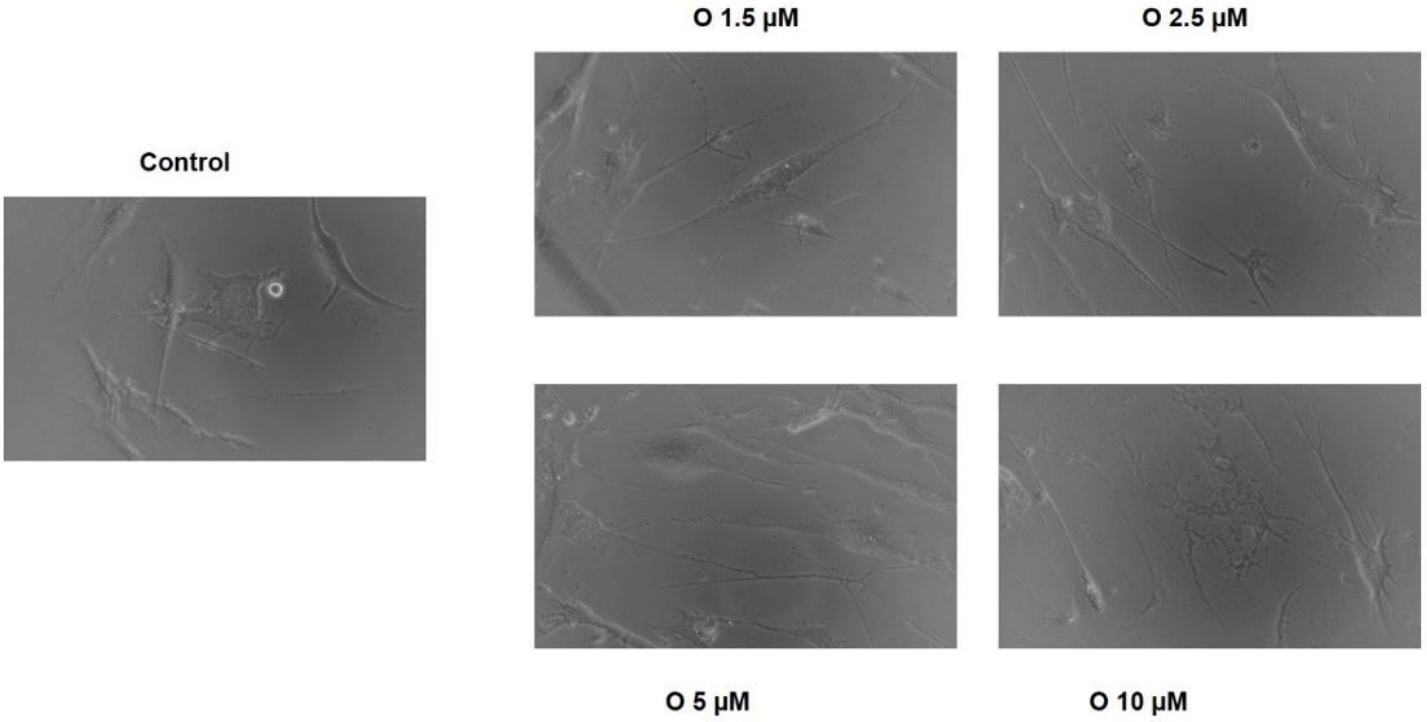

## Figure 4

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.



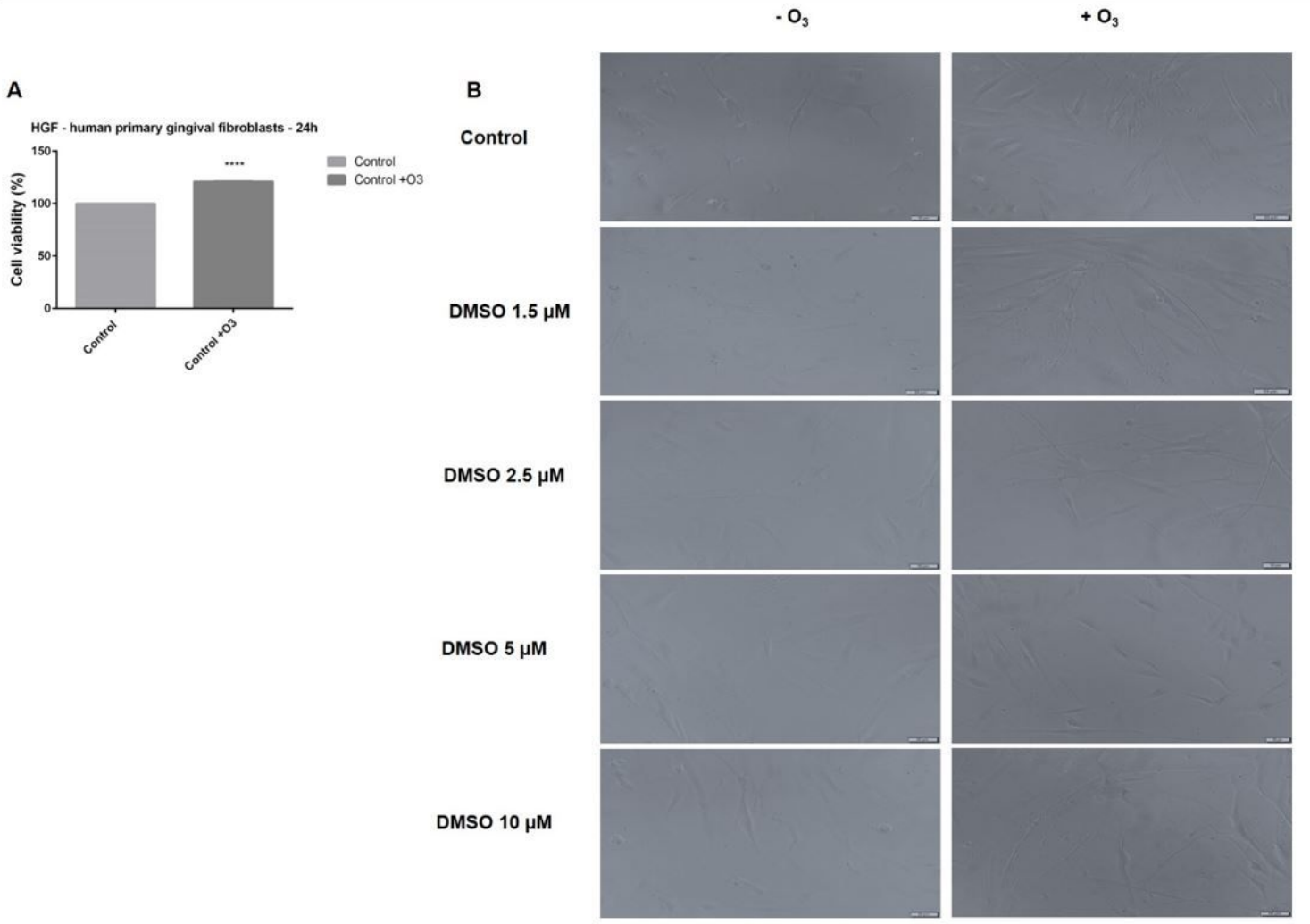

## Figure 5

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.




## Figure 6

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.




## Figure 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.

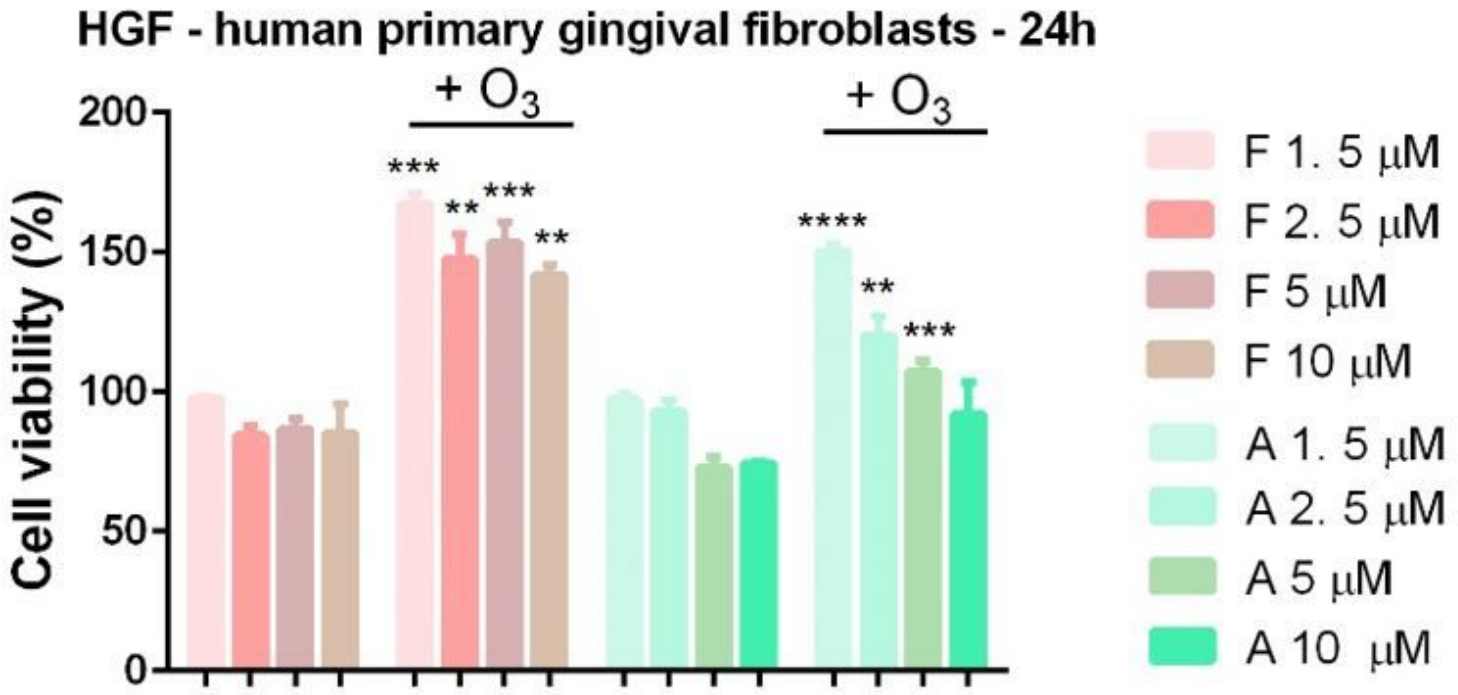
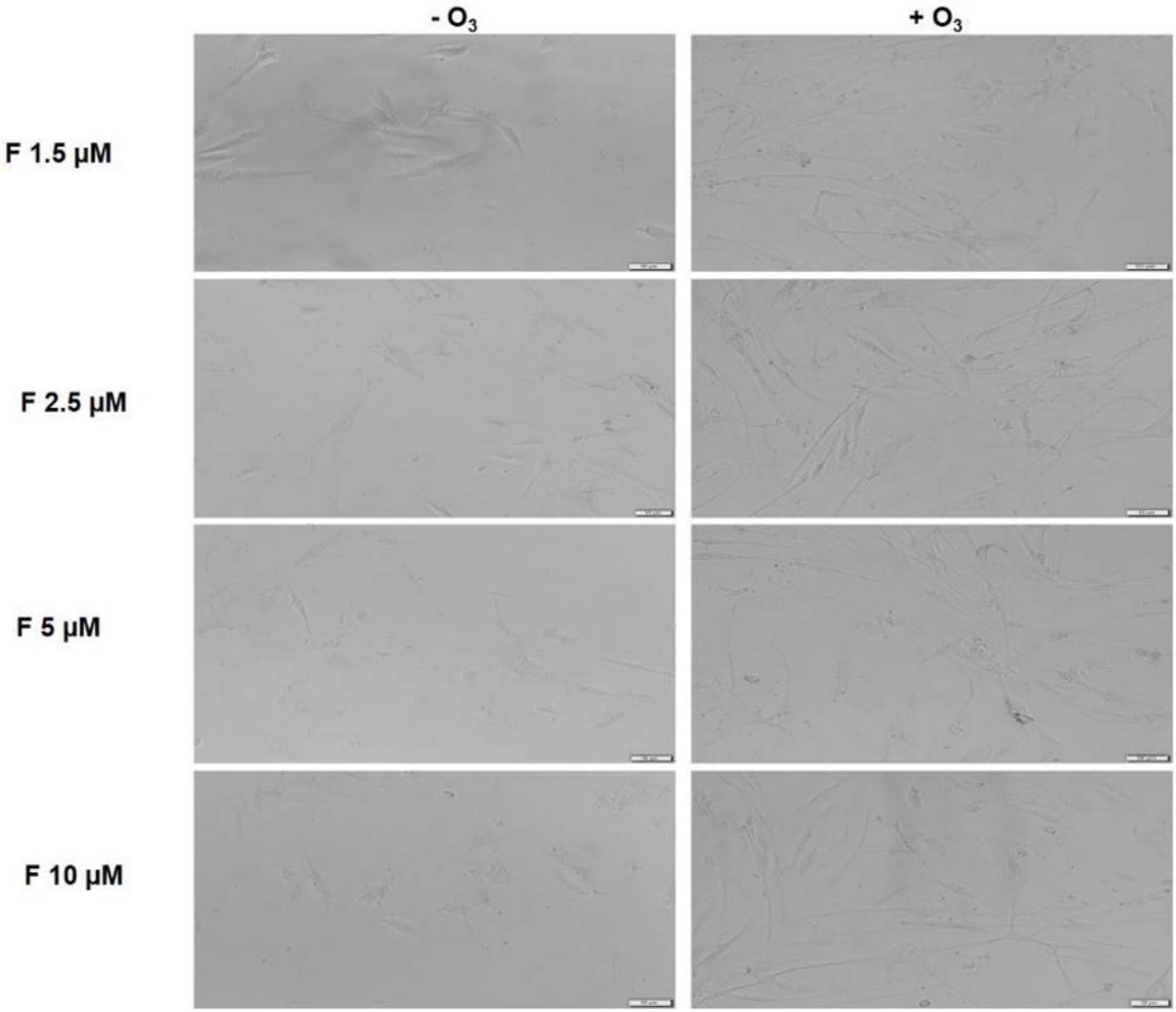


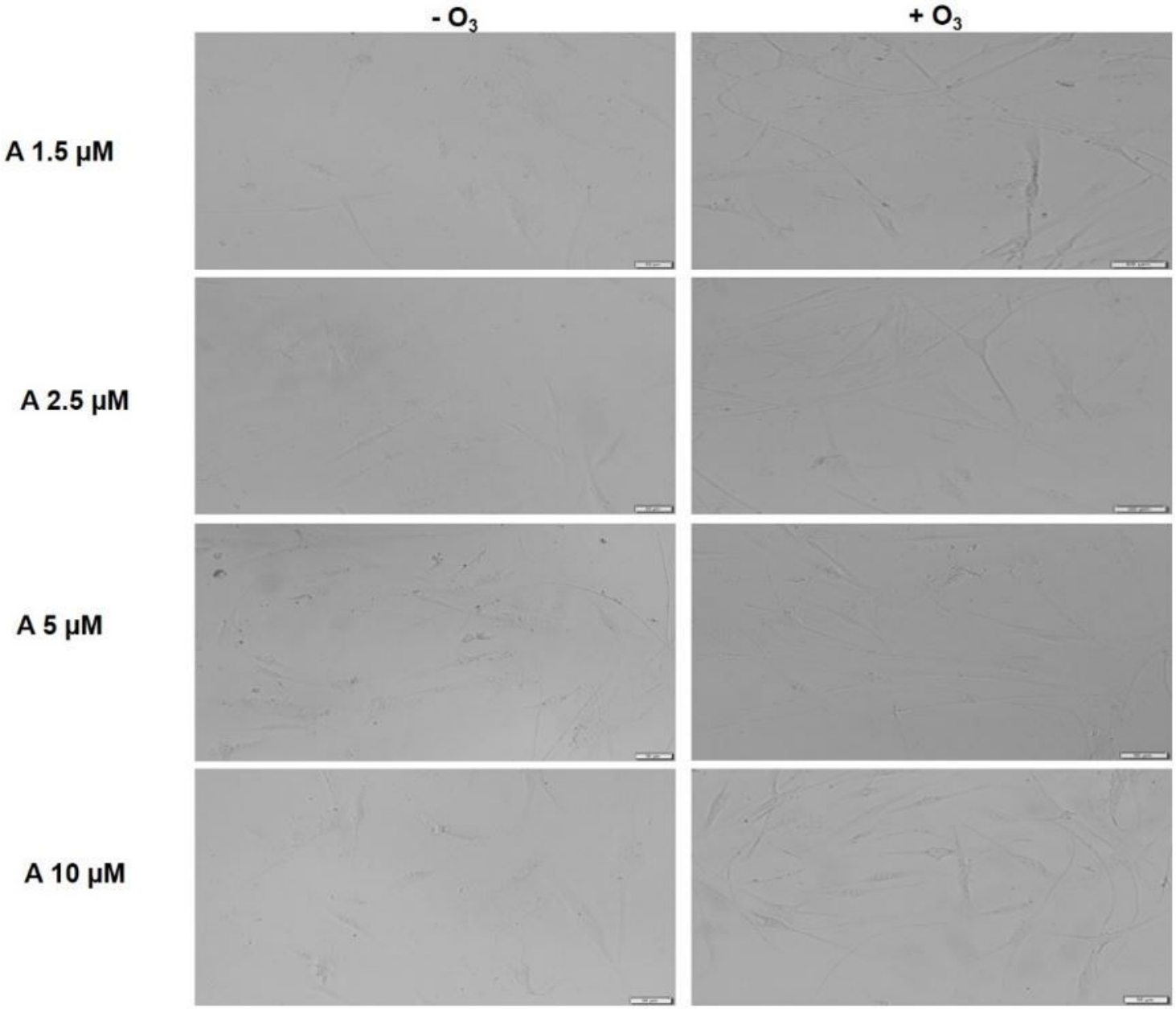

**Figure 8**

Assessment of ozone (O3) solution on HGF - human primary gingival fibroblasts viability stimulated previously with Fosamax – F and Actonel – A (1.5, 2.5, 5 and 10 µM). Data represent the mean values ± SD of three independent experiments. Unpaired t test with Welch's correction was applied to determine the statistical differences in rapport with O3 unstimulated cells (**p<0.01, ***p<0.001 and **** p <0.0001).




## Figure 9

The aspect of HGF – human primary gingival fibroblasts in culture stimulated with different concentrations of Fosamax - F (1.5; 2.5; 5 and 10 µM) for 24 h: without (- O3) and with (+ O3) ozone stimulation. Pictures were taken using the 20x objective at a scale bar of 50 µm.




## Figure 10

The aspect of HGF – human primary gingival fibroblasts in culture stimulated with different concentrations of Actonel -A (1.5; 2.5; 5 and 10 µM) for 24 h: without (- O3) and with (+ O3) ozone stimulation. Pictures were taken using the 20x objective at a scale bar of 50 µm.