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Effect of zoledronate, a third-generation bisphosphonate, on proliferation and apoptosis of human dental pulp stem cells

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Abstract:

Clinical use of zoledronate is accompanied with osteonecrosis of the jaw but the pathogenesis is not well understood. We assumed that zoledronate may have cytotoxicity against stem cells of the oral cavity, and in this way helps to initiate or promoting osteonecrosis.

Dental pulp stem cells (DPSCs) and gingival fibroblasts (GFs) were isolated from volunteers who were undergoing third molar extraction. The proliferation of DPSCs and GFs was evaluated using thiazolyl blue tetrazolium bromide assay. Effect of zoledronate on apoptosis was determined by propidium iodide staining and western blotting analysis.

Incubation with zoledronate for 72 h and 7 days, significantly decreased proliferation of DPSCs and GFs at concentrations more than 0.4 μM ($P < 0.001$). The IC_{50} of zoledronate was lower for DPSCs than GFs (0.92 μM versus 3.5 μM for 7 days treatment). After 72 h treatment with zoledronate, percent of apoptotic DPSCs significantly increased which was accompanied by increased level of pro-apoptotic proteins caspase-3 and Bax, and decreased the level of anti-apoptotic protein Bcl-2.

In conclusion, zoledronate has antiproliferative and pro-apoptotic effects in DPSCs. These effects may involve in promoting zoledronate-induced osteonecrosis and suggest an unfavorable impact of this drug on regenerative potentials of the body stem cells.

Keywords: Apoptosis; Bisphosphonates; Dental pulp; Proliferation; Stem cells; Zoledronate

Introduction

Bisphosphonates are synthetic analogs of naturally occurring pyrophosphate but resistant to degradation by phosphatases. Bisphosphonates bind to the hydroxyapatite crystals and prevent osteoclast-mediated bone resorption. Zoledronate is a third-generation bisphosphonate, and the presence of nitrogen in its structure made it more potent inhibitor of osteoclast activity than earlier bisphosphonates (Green 2005; Li and Davis 2003) It is being increasingly prescribed for the treatment of patients with abnormal bone metabolism including osteoporosis, Paget's disease, and tumor-induced bone loss (Green 2005; Wellington and Goa 2003). There is also evidence that zoledronate reduces proliferation and viability of tumor cells and increases overall survival of cancerous patients beyond prevention of bone resorption (Coleman 2010; Morgan et al. 2010; Koto et al. 2010; Almubarak et al. 2011).

Although zoledronate is effective in the prevention of skeletal-related complications in patients with malignant bone diseases, its clinical uses are accompanied with some side effects such as ocular inflammation, gastrointestinal disorders, and osteonecrosis (Wessel et al. 2008 ; Colucci, et al. 2009 ; Tadrous et al. 2014). Bisphosphonate-related osteonecrosis is characterized by a non-healing hole in the oral mucosa that finally results in an exposed necrotic zone in the mandibular or maxillary bone (Ruggiero 2007). Several mechanisms have been proposed for the pathogenesis of bisphosphonate-induced jaw osteonecrosis including a decrease of angiogenesis, inhibition of bone remodeling, infection, and cytotoxicity (Allen and Burr 2009). For example, the cytotoxic effect of zoledronate has been shown on gingival fibroblast cell line and oral epithelial cell (Scheper et al. 2009; Basso et al. 2013). In addition to fibroblasts and epithelial cells, it is rational to hypothesize that if zoledronate is toxic for stem cells of the orofacial region, tissue repair process is impaired and this also help to initiate or promoting osteonecrosis. The

critical roles of stem cells in tissue repair, postnatal tissue development, and disease modification are well known (Sloan and Smith 2007; Wu et al. 2014).

Dental pulp is one of the main sources of mesenchymal stem cells (MSCs) in the orofacial region. The dental pulp stem cells (DPSCs) have high proliferative potential and multi-lineage differentiation capability, and therefore play important role in dentine regeneration and repair (Sloan and Smith 2007). It has been found that bisphosphonates are taken up by osteoclasts and other cells in the dentine microenvironment and therefore may accumulate in the dentine (Coxon et al. 2008). This raises the possibility that zoledronate may change the functions of DPSCs. Therefore, the aim of the present in vitro study was to evaluate the possible effects of zoledronate on the proliferation and apoptosis of human DPSCs. The effect of zoledronate on cell proliferation was also examined on primary cultured human gingival fibroblasts (GFs) to examine if its effects are selective for DPSCs.

Materials and Methods

Chemicals and Reagents

Alizarin Red, ascorbic acid, DAPI, dexamethasone, β -glycerophosphate, penicillin-streptomycin solution, 3-isobutyl-1-methylxanthine (IBMX), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide, and bicinchoninic acid protein assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). CM-DiI fluorescence dye, fetal bovine serum (FBS), and Dulbecco's Modified Eagles Medium (DMEM) were obtained from Invitrogen (Grand Island, NY, USA). Dimethyl sulfoxide and Oil Red O were purchased from Merck (Darmstadt, Germany). Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD29, CD34, CD44, and CD45 were obtained from AbD Serotec (Raleigh, USA). Phycoerythrin (PE)-

conjugated antibodies against CD90 and CD105 were purchased from Novus Biologicals (Littleton, CO, USA) and Exbio (Czech Republic), respectively. Zoledronate from Novartis Pharmaceuticals Corporation was kindly provided by Negin Daroo Noavar Com. (Iran). Antibodies against β -actin, cleaved caspase-3, Bcl-2, Bax, and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, USA).

Isolation of human DPSCs and GFs

The DPSCs were isolated from teeth of healthy subjects who were undergoing oral surgery (third molar extraction) in the Clinic of Dentistry, Mashhad University of Medical Sciences (Iran). The study procedures were reviewed and approved by the Ethics Committee of the Mashhad University of Medical Sciences. The collected teeth were cleaned and cut to expose the pulp chamber. The pulp tissues were cut into small pieces (\approx 2-3 mm) and stem cells were obtained by explant culture method (Ghorbani et al. 2014). Briefly, the tissue pieces were explanted into culture flasks and their surfaces were covered with FBS. The explants were incubated overnight at 37 °C in an atmosphere of 5% CO₂. Then, FBS was replaced by high glucose DMEM supplemented with 20% FBS, penicillin (100 units/ml) and streptomycin (100 μ g/ml), and the cultures were monitored until spindle cells were appeared and expanded around the tissue pieces. In the subconfluent state, the cells were harvested and expanded further through 3 passages.

Periodontal fibroblasts were obtained from a gingival specimen of volunteers who were undergoing third molar extraction. The tissue specimens were washed with sterile PBS and cut into small pieces. Then, the pieces were explanted into culture flasks as described above. After the appearance of fibroblast cells, they were further cultured until confluent.

Flow cytometric analysis of DPSCs

The expression of stem cell-associated surface markers was assessed by flow cytometric analysis. The isolated DPSCs at passage 4 were detached from culture flask by trypsin-EDTA, centrifuged at 2000 rpm for 5 min, and resuspended in phosphate buffer solution containing 2% FBS. Then, the cells were incubated with antibodies against CD29, CD34, CD44, CD45, CD90, and CD105 for 30 min at 4°C. After washing with phosphate buffer, the cells were suspended in 500 µl of the buffer containing 2% FBS and analysis were performed using FACSCalibur flow cytometer (BD Biosciences).

Evaluation of the multipotency of DPSCs

The multipotency of DPSCs was tested by evaluating their ability to differentiate into adipocyte and osteoblast lineages. To induce differentiation, DPSCs were seeded in 6 wells plate containing DMEM supplemented with 10% FBS and penicillin/streptomycin and cultured to reach 80% confluent. Then the culture medium was changed with adipogenic or osteogenic differentiation media. Adipogenic medium consisted of DMEM supplemented with 3% FBS, 1 µM dexamethasone, and 0.2 µM insulin (Feizpour 2014). The adipogenic medium was exchanged every 3 days and the cells were maintained in this medium for 3 weeks. Adipogenesis was confirmed by Oil Red O which stains intracellular triglyceride droplets. The cells were fixed with 10% formalin and then incubated for 20 min with Oil Red O solution (Alinejad et al. 2015). Then, the cells were washed three times with distilled water and photographed using an inverted microscope.

Osteoblastic differentiation medium consisted of DMEM supplemented with 10% FBS, 10 µg/ml ascorbic acid, 5 mM β-glycerol phosphate, and 0.1 µM dexamethasone (Ghorbani et al. 2014). This medium was exchanged every 3 days and the cells were maintained for 4 weeks.

Differentiation was confirmed by Alizarin Red (which stains extracellular calcium deposits) and alkaline phosphatase staining. For Alizarin Red staining, the cells were fixed with 10% formalin and then incubated for 5 min with 2% Alizarin Red solution, or alkaline phosphatase reagent. Then, the cells were washed three times with distilled water and photographed using an inverted microscope.

Effect of zoledronate on proliferation of DPSCs and GFs

The isolated DPSCs (at passage 4) and GFs (at passage 2) were seeded in 96-well plates overnight and then cultured for 24 h, 48 h, 72 h or one week in DMEM containing 10% FBS and different concentrations of zoledronate (0.187-100 μ M). Then, MTT reagent was added to each well (at the final concentration of 0.05%) and the cells were maintained in an atmosphere of 5% CO₂ at 37°C. After 3 h, the supernatant was removed and the formazan crystals were dissolved in 100 μ L dimethyl sulfoxide. The optical density of formazan dye was read at 540 nm (background 630 nm) using a StatFAX303 plate reader. The MTT assay was performed three times in triplicate.

Cell fluorescence staining by CM-DiI and DAPI

Fluorescence staining of plasma membrane and nucleus of DPSC was performed using CM-DiI and DAPI, respectively. After 72 h treatment with zoledronate (1.5, 25 and 100 μ M), the DPSCs were incubated with CM-DiI (2 mM) for 5 min at 37°C followed by 15 min at 4°C. Then, the cells were washed with PBS and fixed using 4% paraformaldehyde. The fixed cells were incubated with DAPI (10 μ g/mL) for 20 min at 37°C and washed again with PBS prior to observing by inverted fluorescence microscope.

Effect of zoledronate on apoptosis of DPSCs

The DPSCs at passage 4 were seeded in 12-well plates overnight and then cultured for 72 h in DMEM containing 10% FBS and different concentrations of zoledronate (0.75-50 μ M). Then, the cells were permeabilized and stained with 500 μ L propidium iodide reagent (5 mg propidium iodide, 100 mg sodium citrate, and 100 μ l triton-X 100 in 100 ml distilled water) was added to each well and the plates were maintained 30 min at 37°C (Mortazavian et al. 2013). To detect apoptotic cells, the propidium iodide nuclear fluorescence intensity of the cells was determined by flow cytometry.

Western blotting analysis

After treatment with zoledronate, the DPSCs were harvested and suspended in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% triton-X 100, 1 mM EDTA, 0.2% SDS, 1% protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. After 30 min, the cell lysate was centrifuged at 10000 rpm for 20 min at 4 °C, and its protein concentration was determined using bicinchoninic acid protein assay kit. Equal amounts of proteins from each sample were subjected to 12.5% SDS-PAGE (w/v). The proteins were transferred to a polyvinylidene fluoride membrane and subjected to immunoblotting using primary antibodies against β -actin, caspase-3, Bcl-2, and Bax. The bounds were made visible using a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody and an enhanced chemiluminescence system. Results were analyzed by Gel Pro Analyzer Software (Media Cybernetics) and normalized with respect to the corresponding β -actin band.

Statistical Analysis

Data were analyzed with IBM SPSS Statistics version 20 software using one-way analysis of variance and post hoc Dunnett multiple comparison tests. Results are expressed as mean \pm SEM and probability level of $p < 0.05$ was considered statistically significant.

Results

Characterization of DPSCs

Flow cytometric analysis showed that DPSCs were positive for the MSCs markers CD29, CD44, CD90, and CD105, while were negative for hematologic markers CD34 and CD45 (Fig. 1). To test whether the cells have the pluripotent capacity, they were cultured in adipocyte and osteocyte differentiating media. Oil Red O staining displayed intracellular lipid droplets and confirmed the capability of isolated DPSCs to differentiate into adipocyte lineage. Also, alkaline phosphatase and Alizarin Red staining showed osteogenic differentiation ability of DPSCs.

Effect of zoledronate on DPSCs proliferation

As shown in Figure 2, up to 24 h none of the zoledronate concentrations decreased proliferation of DPSCs. When the cells were incubated for 48 h in the presence of zoledronate, only concentration of 100 μM significantly decreased proliferation of DPSCs ($p < 0.05$). Regarding 72 h, the proliferation of DPSCs significantly decreased at concentrations more than 0.4 μM . In the presence of 0.8, 1.5, 3, 6, 12, 25, 50, and 100 μM of zoledronate DPSCs proliferation decreased from $100 \pm 4\%$ (control) to $68 \pm 3\%$, $67 \pm 5\%$, $67 \pm 6\%$, $68 \pm 4.5\%$, $67 \pm 5\%$, 61 ± 4 , 51 ± 5 , and $33 \pm 4\%$, respectively ($P < 0.001$). Similarly, after 7 days treatment with this drug, proliferation of DPSCs significantly decreased at concentrations more than 0.4 μM and compare to control reached to $8 \pm 2\%$ ($p < 0.001$) at a concentration of 100 μM . The IC_{50} value for 7 days

treatment with zoledronate was 0.92 μ M. Figure 3 shows that untreated DPSCs were confluent and exhibited spindle shape. When the cells were treated for 72 h with zoledronate, a significant reduction in the number of viable cells was observed.

Effect of zoledronate on GF proliferation

Figure 4 shows the effects of zoledronate on the proliferation of GFs. After 72 h treatment with this drug, proliferation of GFs decreased from $100 \pm 2\%$ (control) to $84 \pm 1\%$, $80 \pm 2\%$, $77 \pm 1\%$, $71 \pm 2.5\%$, 62 ± 1.2 , 58 ± 2 , and $42 \pm 1\%$ at concentrations of 1.5, 3, 6, 12, 25, 50, and 100 μ M, respectively ($P < 0.001$). Similarly, after 7 days treatment, the proliferation of GFs significantly decreased at concentrations more than 0.4 μ M and the IC₅₀ value was 3.5 μ M.

Effect of zoledronate on DPSCs apoptosis

Figure 5 demonstrates the effect of 72 h incubation with different concentrations of zoledronate on apoptosis of DPSCs. As shown in the histogram of fluorescence intensity of propidium iodide-stained DPSCs, the sub-G1 region made by cells with reduced DNA content corresponded to apoptotic DPSCs (Fig. 5A). The addition of increasing concentrations of zoledronate to the cell culture medium increased the percent of DPSCs in Sub-G1 phase in a concentration-dependent manner. In the presence of 0.8, 1.5, 3, 6, 12, 25 and 50 μ M of zoledronate, the percent of apoptotic DPSCs was $27 \pm 8\%$, $43 \pm 8\%$, $46 \pm 6.5\%$, $60 \pm 2\%$, $70 \pm 6.5\%$, $84 \pm 2\%$, and $88 \pm 1\%$, respectively, which was significantly ($p < 0.05$ - $p < 0.001$) higher than that of untreated cells $16 \pm 2\%$ (Fig. 5B).

Effect of zoledronate on pro-apoptotic and anti-apoptotic proteins

Effects of zoledronate on the level of proteins involved in apoptosis are shown in Figure 6 and Figure 7. The level of pro-apoptotic proteins caspase-3 and Bax and anti-apoptotic protein Bcl-2 were determined by Western blotting analysis. Treatment of DPSCs for 72 h with zoledronate

significantly increased the level of caspase-3 and Bax, while decreased the level of Bcl-2 in comparison with untreated cells. The ratio of Bax/Bcl-2 proteins, an important factor for apoptotic signal, significantly increased in the presence of zoledronate.

Discussion

The effects of zoledronate on proliferation and viability of different cell types have been examined in previous studies (Basso et al. 2013; Agis et al. 2010; Rodriguez-Lozano et al. 2015; Gong and Su 2015). However, no study has yet examined the possible cytotoxic effects of zoledronate on DPSCs. In the present study, we isolated human DPSCs from normal subjects and characterized them by evaluating the expression of stem cell surface markers, and by assessing their multipotency for differentiating into adipocytes and osteocytes. Then, the effects of zoledronate on DPSCs proliferation and apoptosis were investigated. The results revealed that zoledronate, when incubated for long time periods (more than 48 h), can reduce proliferation of DPSCs. The exact intracellular mechanisms responsible for the antiproliferative action of zoledronate remain to be revealed in future studies. However, results of propidium iodide staining showed that this reduced proliferation was accompanied by an increased cell-cycle blockage in G1 phase. Also, data of Western blotting demonstrated that the level of anti-apoptotic protein Bcl-2 was reduced but pro-apoptotic proteins Bax and caspase 3 were increased in the presence of zoledronate, which supports a pro-apoptotic property for this drug against DPSCs.

The effects of zoledronate on proliferation and apoptosis of DPSCs were started from concentrations as low as 0.8 and 3 μ M, respectively. In agreement with the present observation, it has been reported that zoledronate at the concentration of $\geq 3 \mu$ M inhibited the proliferation of

mandible derived MSCs (Gong and Su 2015). These concentrations are clinically relevant in patients receiving zoledronate since its plasma level is approximately 1 μM following a 15-min infusion of 4 mg drug (Chen et al. 2002; Skerjavec et al. 2003). In our work, the antiproliferative effect of zoledronate (for 7 days treatment) was observed with the IC_{50} values of 0.92 μM and 3.5 μM for DPSCs and GFs, respectively. This observation suggests that DPSCs are more vulnerable to cytotoxic effects of zoledronate.

There are contradictory reports on the effects of zoledronate on functions of stem cells isolated from different tissues. A number of studies have shown that zoledronate decreases proliferation of MSCs isolated from the mandible, periodontal ligament, and bone marrow (Rodriguez-Lozano et al. 2015; Gong and Su 2015), inhibits differentiation of rat mandible MSCs (Gong and Su 2015) and reduces the ability of bone marrow MSCs to migrate (Gallo et al. 2012). However, other studies have indicated that it extends the life span of bone marrow MSCs (Misra et al. 2015; Heino et al. 2016) and protects their ability to proliferate and differentiate following exposure to irradiation (Misra et al. 2015). Results of the present work are in line with those studies showing that zoledronate inhibits proliferation of MSCs derived from mandible and periodontal ligament (Rodriguez-Lozano et al. 2015; Gong and Su 2015). Also, our data are consistent with those of other investigators indicating zoledronate is toxic to cells of the soft tissue lining of the mouth (Basso et al. 2013; Agis et al. 2010).

The cytotoxicity of zoledronate against stem cells of dental pulp, as one of the main sources of MSCs in the oral cavity, supports the hypothesis that this drug impairs tissue repair process in the orofacial region and in this way helps to the development of osteonecrosis in patients receiving it for long periods. In consistent with this hypothesis, Yamashita et al. (2011) reported that zoledronate blunts oral wound healing in rats. Also, the antiproliferative and pro-apoptotic

effects of zoledronate on DPSCs raise this possibility that this drug also may affect the behavior of stem cells in other parts of the body. Considering the important roles of MSCs in postnatal tissue development, tissue repair, and disease modification (Sloan and Smith 2007; Wu et al. 2014), the unfavorable effects of zoledronate on these roles should be taken into account.

In conclusion, the present results demonstrated that zoledronate has antiproliferative and proapoptotic effects in DPSCs. These effects may involve in initiating or promoting zoledronate-induced osteonecrosis, and also suggest an unfavorable impact of this drug on regenerative potentials of the stem cells.

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Figures legends:

Figure 1. Flow cytometric analysis of cell-surface markers in DPSCs derived from human third molar teeth. White and gray areas demonstrate control and specific antibody staining, respectively. This analysis showed that DPSCs at passage 4 expressed MSCs markers CD29, CD44, CD90, and CD105, and were negative for hematologic markers, CD34 and CD45.

Figure 2. Effect of zoledronate on proliferation of human DPSCs. The percent of cell proliferation was normalized against untreated control cells (concentration of 0). Data are mean \pm SEM (n = 9). *p < 0.05 versus concentration of 0; ***p < 0.001 versus control (concentration of 0).

Figure 3. Morphological observation of human DPSCs treated for 72 h with zoledronate. Fluorescence staining of plasma membranes and nuclei was performed using CM-DiI and DAPI dyes, respectively. Untreated DPSCs are confluent and exhibit spindle shape, while a significant reduction in the number of viable cells can be seen in treated groups (Original magnification \times 100).

Figure 4. Effect of zoledronate on the proliferation of human GFs. The percent of cell proliferation was normalized against untreated control cells (concentration of 0). Data are mean \pm SEM (n = 6). ***p < 0.001 versus control (concentration of 0).

Figure 5. Effect of zoledronate on apoptosis of human DPSCs. The cells were treated for 72 h with different concentrations of zoledronate and then stained with propidium iodide. The sub-G1 region made by cells with reduced DNA content corresponded to apoptotic cells. A: Representative histogram of the fluorescence intensity of propidium iodide-stained DPSCs; B: Quantitative analysis of DPSCs apoptosis. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

Figure 6. Effect of zoledronate on the levels of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins in human DPSCs. The cells were treated for 72 h with different concentrations of zoledronate. * $p < 0.05$ and *** $p < 0.001$ versus control.

Figure 7. Effect of zoledronate on the level of pro-apoptotic protein caspase-3 in human DPSCs. The cells were treated for 72 h with different concentrations of zoledronate. * $p < 0.05$ and ** $p < 0.01$ versus control.

Draft

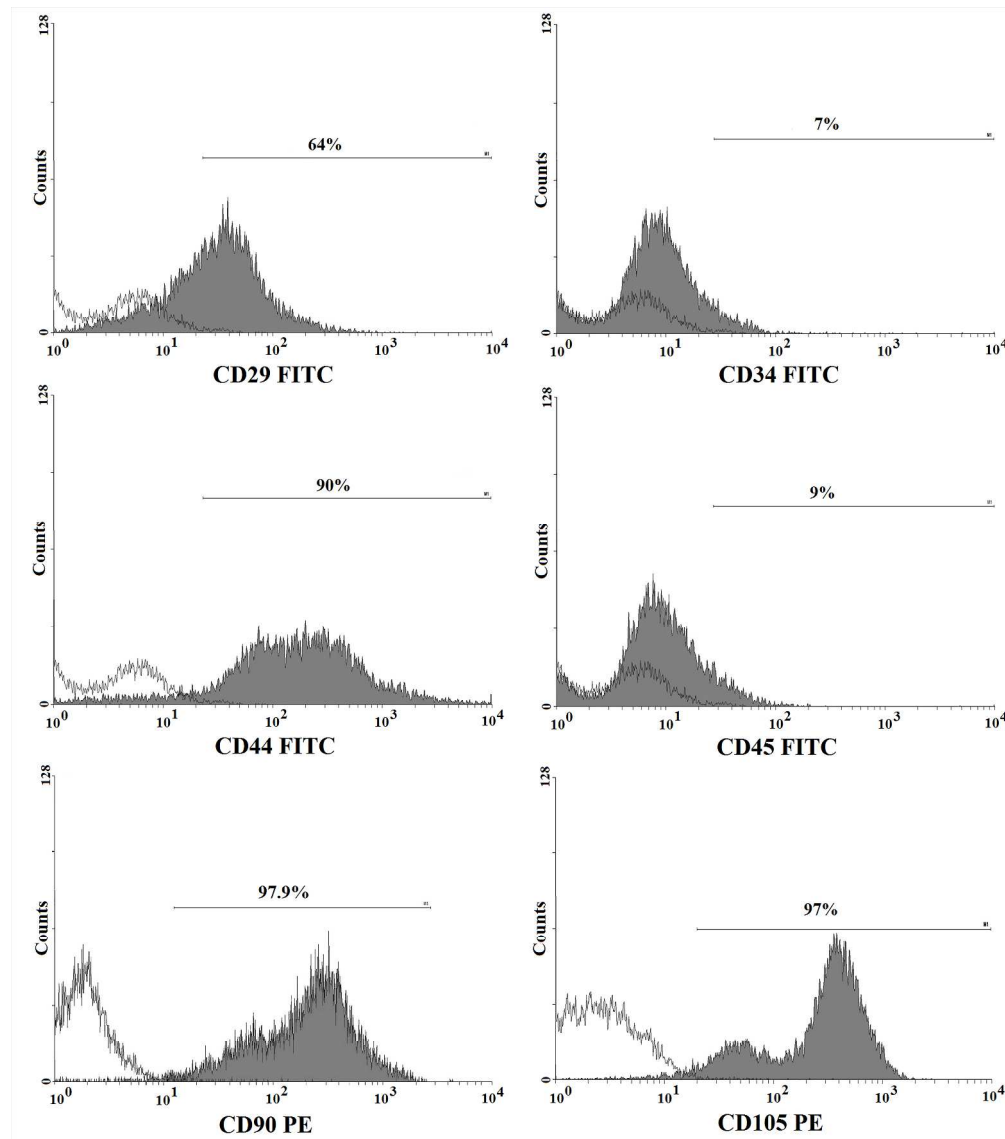


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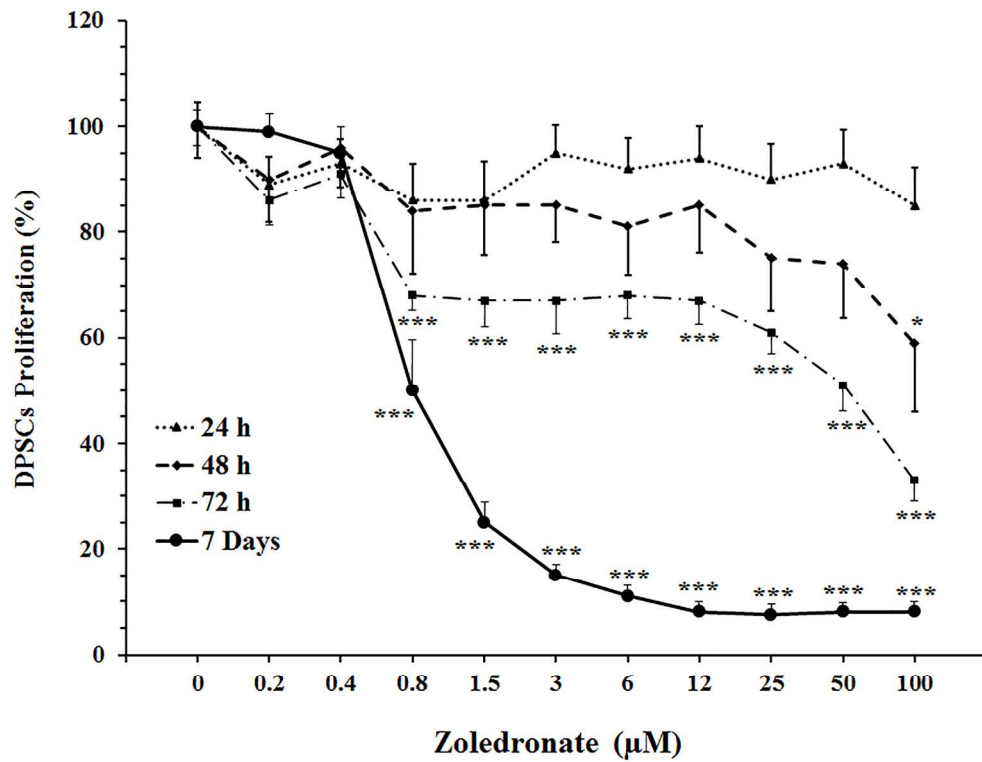


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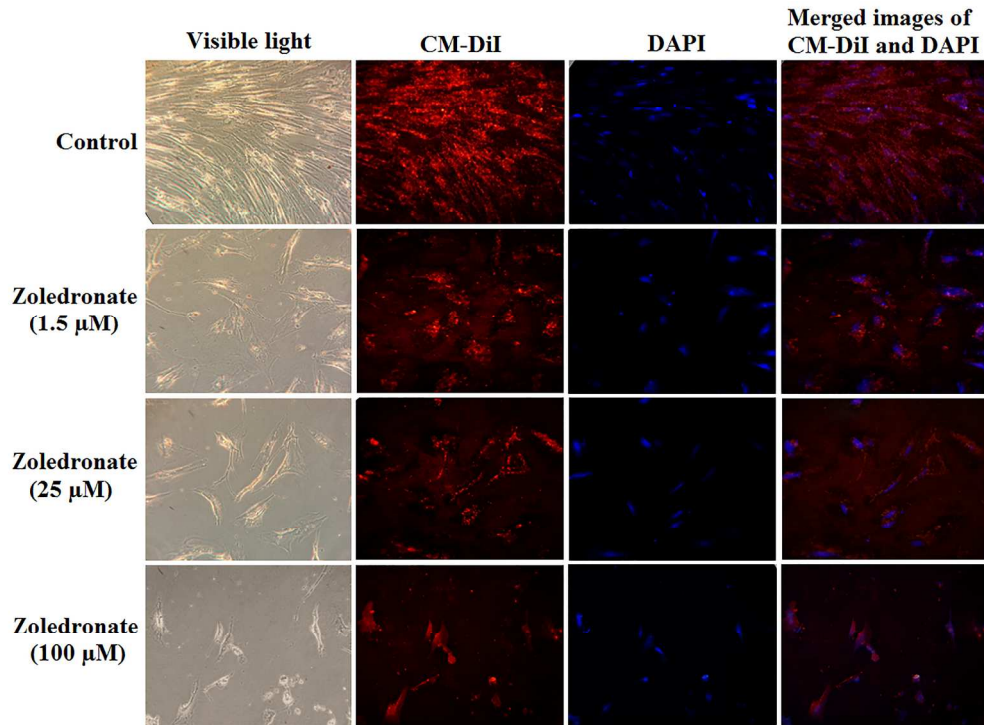


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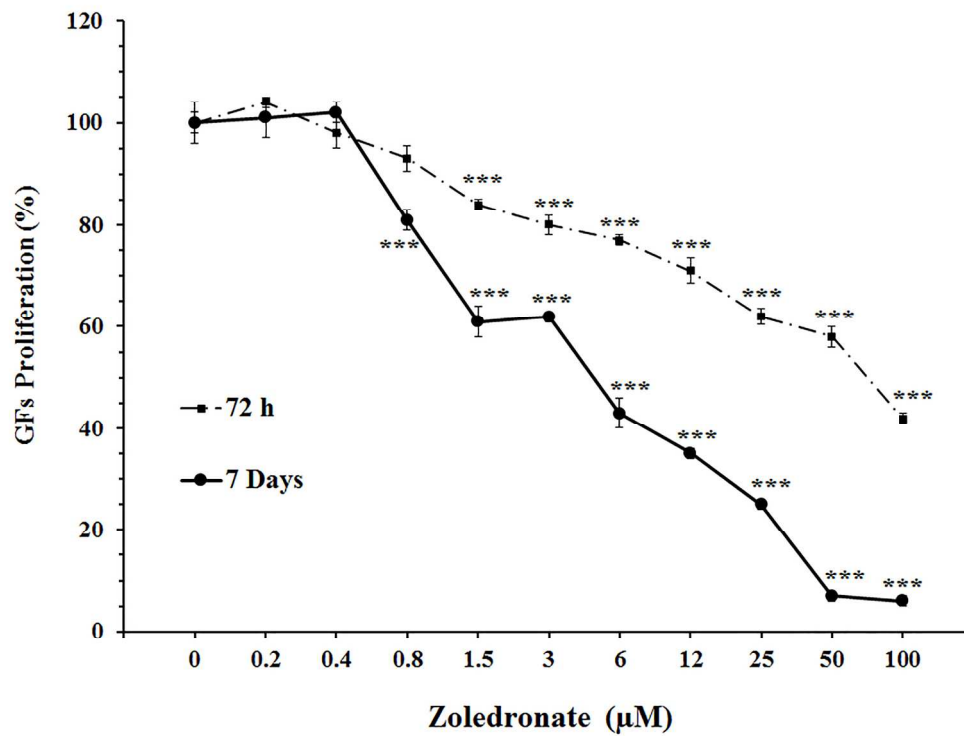


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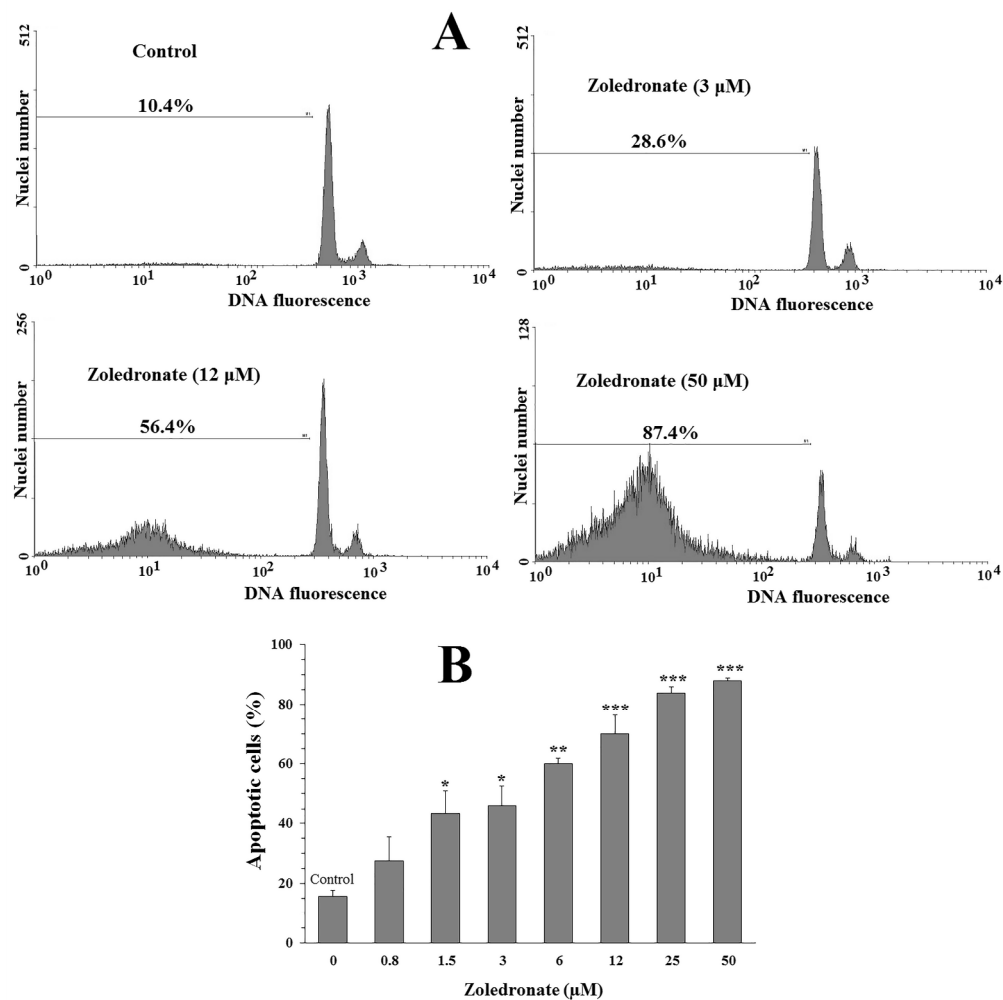


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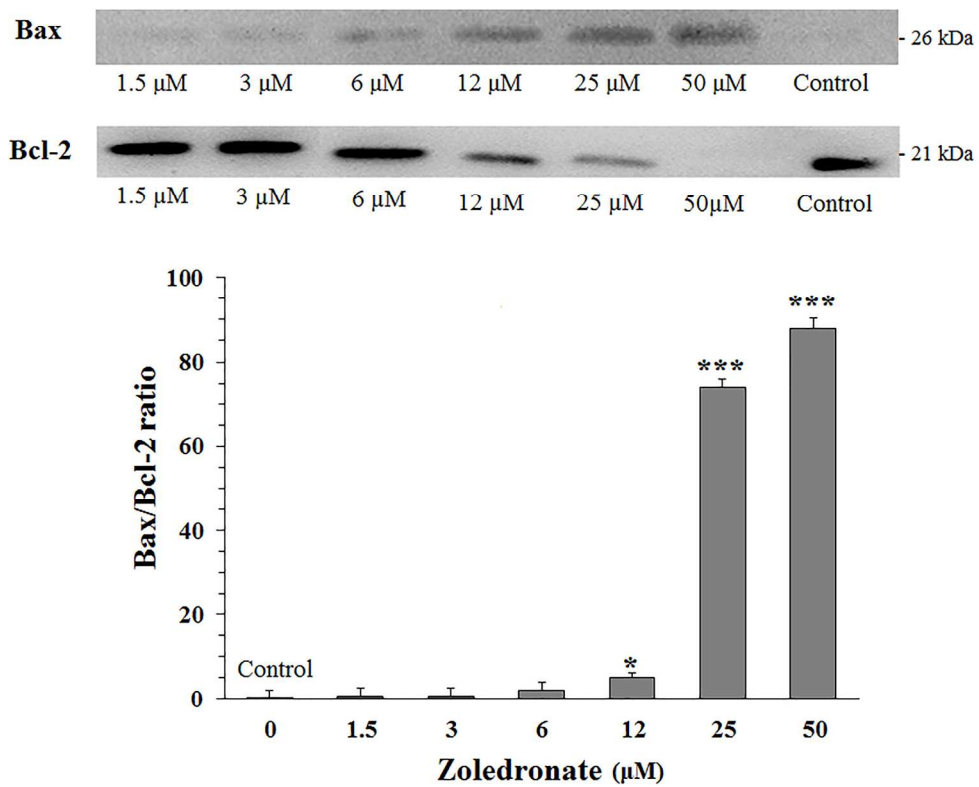


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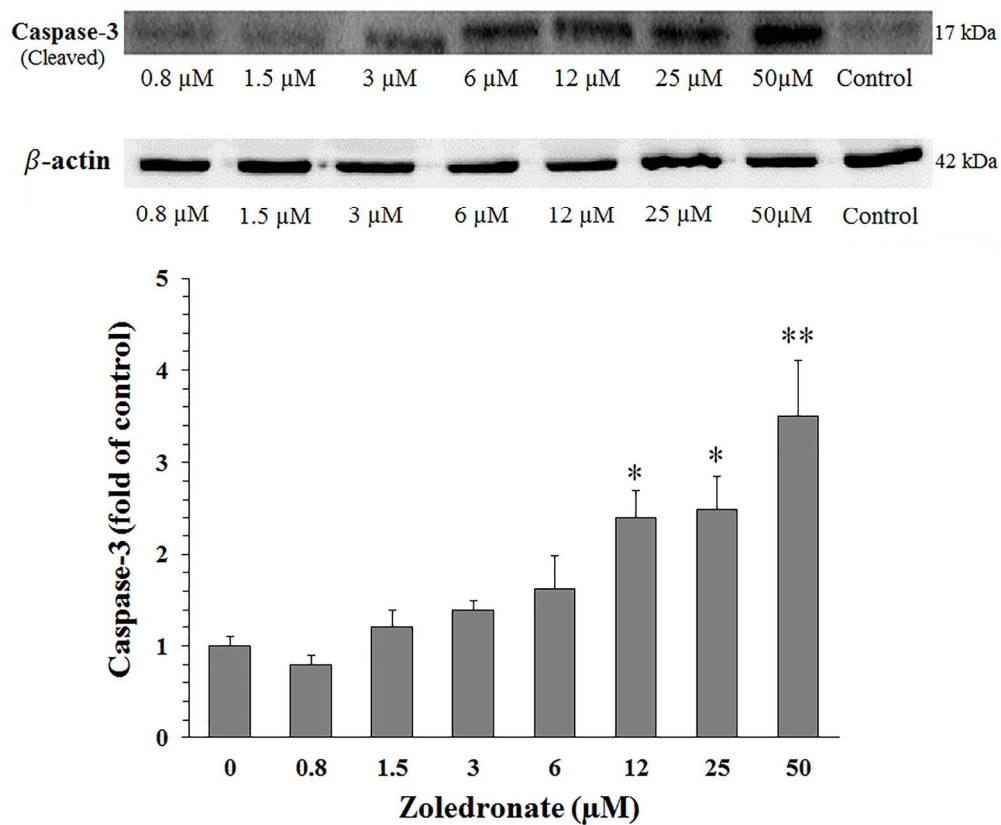


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