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Research paper

Evaluation of the bisphosphonate effect on stem cells derived from jaw bone and long bone rabbit models: A pilot study

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ABSTRACT

Background and objective: Bisphosphonates have been widely used and the number of patients experiencing medication-related osteonecrosis of the jaw (MRONJ) has been increasing. This study was designed to evaluate the effect of zoledronate on stem cells derived from different tissues.

Design: Stem cells derived from four different tissues were compared using rabbit models (JPO: periosteum from the jaw bone (mandible), JBM: bone marrow from the jaw bone, LPO: periosteum from long bone (tibia), and LBM: bone marrow from long bone). Stem cells were grown in the presence of zoledronate at final concentrations ranging from 10^{-6} M to 10^{-10} M. Morphology was viewed under an inverted microscope, and the analysis of cell proliferation was performed using a Cell Counting Kit-8 (CCK-8) on days 1, 2, 4, and 7.

Results: The CCK-8 results for LBM showed that the increase of CCK-8 values was correlated with a longer incubation time. Compared to the untreated control, growth in the presence of zoledronate at 10^{-10} M and 10^{-8} M resulted in decreased CCK-8 values for LBM on day 7 ($P < 0.05$). The CCK-8 results for JBM, LPO, and JPO on days 1, 2, 4, and 7 showed that the presence of zoledronate did not produce statistically significant changes compared with the untreated control.

Conclusion: Zoledronate in the tested concentrations from JBM, LPO, and JPO did not produce noticeable alterations in the viability of mesenchymal stem cells. This *in vitro* experiment suggests that the occurrence of MRONJ solely in the oral cavity is not due to differences in the cellular proliferation of stem cells in the response to zoledronate.

1. Introduction

Bisphosphonates are pyrophosphate analogues with a high affinity for hydroxyapatite crystals. These compounds can be classified into nitrogen-containing bisphosphonates (including alendronate, ibandronate, risedronate, and zoledronate) and non-nitrogen containing bisphosphonates (including etidronate and clodronate) (Casado-Diaz, Santiago-Mora, Dorado, & Quesada-Gomez, 2013; Kim, Ko, & Park, 2015). Bisphosphonates have been widely used for their multimodal bone-sparing action and their ability to prevent the development of osteolytic lesions in various cancers (Sharma, Hamlet, Petcu, & Ivanovski, 2016). Intravenous zoledronate increases bone mineral density while decreasing bone turnover and formation (Doggrell, 2002). Zoledronate has also been applied in combination with chemotherapy and surgery to treat osteosarcomas (Piperno-Neumann et al., 2016).

Mesenchymal stem cells are characterized by their osteogenic, adipogenic, and chondrogenic differentiation capabilities (Jin et al., 2015). Previously, stem cells derived from periosteum and the bone marrow of jaw bone (mandible) and long bone (tibia) were compared to determine a suitable cell source (Park, Bae et al., 2012). Harvesting stem cells from the intraoral area is more feasible and less invasive because it can be performed under local anesthesia, and is more accessible. This procedure has fewer complications associated with paresthesia and pain, with more places from which to harvest (Park, Kim, Lee, Kim, & Kim, 2015). Bisphosphonates have been shown to exert a variety of actions on mesenchymal stem cells (Kim et al., 2015). One study showed that bisphosphonates suppressed osteogenic differentiation, while another

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study showed positive effects on the osteogenic differentiation of mesenchymal stem cells (Casado-Diaz et al., 2013; Fujita et al., 2011). As a result of the wide use of bisphosphonates, the number of patients experiencing medication-related osteonecrosis of the jaw is increasing (Katsarelis, Shah, Dhariwal, & Pazianas, 2015). Irreversible osteonecrosis was reported only in the oral cavity, and this experiment was performed to evaluate whether this phenomenon was due to a difference in stem cell responses. According to the authors' knowledge, there is no known study that evaluated the effects of zoledronate on different sources of stem cells. Therefore, this study was designed to evaluate the effect of zoledronate on stem cells derived from the periosteum and bone marrow of jaw and long bones. Additionally, the evaluation of the possible explanation of MRONJ occurring only in the oral cavity and the application of stem cells obtained from the intraoral area for the treatment of MRONJ was performed.

2. Material and methods

2.1. Harvest of tissue and isolation of cells

Thirty healthy female New Zealand white rabbits (aged 2–3 months and weighting 2.5–3.5 kg) were purchased from Koatech (Kyeonggi, Korea). The animals were all bred in the same regulated environment in the Clinical Research Institute of Uijeongbu St. Mary's Hospital, the Catholic University of Korea. All animal experiments were performed in strict accordance with a protocol approved by the Ethics Committee for Animal Experiments of Uijeongbu St. Mary's Hospital, the Catholic University of Korea (UJA2013-23A). The tissues were harvested under anesthesia by administering a mixture of 15 mg/kg of Tiletamine/Zolazepam (Zoletil 50, Virbac Laboratories, Carros, France) and 5 mg/kg of Xylazine (Xylazine Hydrochloride, Bayer Korea, Seoul, Korea) intramuscularly to the femoral muscle.

Bone fragments and marrow were obtained from the tibia (LBM) and mandible (JBM) of the rabbits. The tissues in the DMEM media were vigorously shaken and filtered with a 70 μm strainer. The isolated cells were centrifuged and resuspended in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sijiqing, China) and antibiotics (100 U/mL of penicillin and streptomycin 100 μg/mL). An approximately 1 × 1 cm section of periosteum was harvested from the tibia (LPO) and mandible (JPO) of the rabbits. After rinsing the periosteum thoroughly with PBS containing 100 U/mL penicillin and 100 μg/mL streptomycin, the biopsies were minced into small pieces and digested in 0.06% collagenase type II (Invitrogen Corporation, Carlsbad, CA) for 4 h with 5% CO2 and 95% air at 37 °C. The isolated cells were then centrifuged and resuspended in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sijiqing, China) and antibiotics (100 U/mL of penicillin and streptomycin 100 μg/mL). Cells from four groups (LBM, JBM, LPO, and JPO) were cultured in high-glucose DMEM containing FBS and antibiotics, and the medium was changed every two days. When the cultures reached 90% confluence, the cells were subcultured with coenzyme solution, which was composed of 0.125% trypsin (Sigma, St. Louis, MO) and 0.2% ethylenediaminetetraacetic acid (EDTA, Invitrogen). The characteristics of the stem cells were evaluated using a colony-forming assay, osteogenic differentiation, adipogenic differentiation, and chondrogenic differentiation (Park, Bae et al., 2012). For colony-forming assay, the cells were plated and the number of colonies was counted. For osteogenic, adipogenic, and chondrogenic differentiation, the cells were cultured in differentiation media and cultures were stained with Alizarin red solution, oil red O solution, and toluidine blue staining, respectively. The morphology of the LBM, LPO, JBM, and JPO cells was viewed under an inverted microscope (Leica DM IRM, Leica Microsystems, Wetzlar, Germany) and the images were saved as JPEG files.

2.2. Cell proliferation

Cells from four groups were plated at a density of 2×10^3 cells/100 μl in 96-well plates and cultured in a monolayer. The cells were incubated in the presence of zoledronic acid (Zometa, Novartis, Stein, Switzerland) at final concentrations of 0 (untreated control), 10^{-6}M, 10^{-8}M, and 10^{-10}M. The proliferation was quantitatively analyzed with a cell counting kit-8 (CCK-8, Dojindo Molecular Technologies Inc., Rockville, MD) (Jung et al., 2012). At days 1, 2, 4, and 7, 10 μl of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazoium, monosodium salt] solution was added to the culture plates and incubated for 2 h. The absorbance was then read at 450 nm using a microplate reader. Ten experiments were performed for each groups and the number of results included in this study was nine for LBM and the number of results for the other groups (JBM, LPO, and JPO) was ten. In every experiment, 3 rabbits were used and 12 wells were prepared for each group. Three wells of each group were used for each evaluation. Therefore the total numbers of measured samples were 27 for LBM and 30 for the others each day.

2.3. The effects of zoledronic acid concentration on the proliferation of different types of cells

The effects of different concentration of 10^{-6} M, 10^{-8} M, and 10^{-10} M on the proliferation of four types of cells (LBM, JBM, LPO, and JPO) were tested.

2.4. Relative rate of proliferation depending on the concentration of zoledronic acid

The relative rate of proliferation was the CCK-8 value divided by the value of day 1.

2.5. Statistical analysis

Results were presented as mean ± S.D. Two-way analysis of variance (ANOVA) was performed to evaluate the effects of zoledronic acid concentration and culturing period using a commercially available program (PASW Statistics 18; SPSS Inc., Chicago, IL). The effects of cell type (LBM, JBM, LPO, and JPO) and the culturing time (days 1, 2, 4, and 7) on relative proliferation rate were determined. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Cell proliferation

No obvious differences in morphology or cell size were noted among the four groups (Fig. 1). Visual evaluation showed that LBM, JBM, LPO, and JPO mainly consisted of spindle cells.

3.2. The effects of zoledronic acid concentration on the proliferation of different cell types

The CCK-8 results of LBM on days 1, 2, 4, and 7 are shown in Fig. 2. An increase in CCK-8 values was noted with longer incubation times. Compared to the untreated control, growth in the presence of zoledronic acid at 10^{-10}M and 10^{-8}M resulted in a decrease in the LBM CCK-8 values on day 7 ($P < 0.05$).

The CCK-8 results for JBM on days 1, 2, 4, and 7 indicated an increase in CCK-8 values was noted with longer incubation times (Fig. 2). No statistically significant changes were noted due to the presence of zoledronic acid when compared to the control ($P > 0.05$).

The CCK-8 results for LPO on days 1, 2, 4, and 7 are shown in Fig. 2. An increase in CCK-8 values was noted with longer incubation times. No statistically significant changes were noted due to the presence of