ORIGINAL ARTICLE

EFFECTS OF ZOLEDRONIC ACID ON OSTEOBLAST PROLIFERATION AND IGF-1 EXPRESSIONS IN RATS *IN VITRO*

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Abstract

This study aimed to investigate the effects of different concentrations of zoledronic acid (ZA) on the proliferation of rat osteoblasts and the expression of insulin-like growth factor-1 (IGF-1) *in vitro*, and to provide a theoretical basis for its clinical use. Rat osteoblasts were divided in 5 groups: 1 control group cultured in normal culture medium and 4 experimental groups cultured in normal culture media with 10^{-4} mol/L, 10^{-5} mol/L, 10^{-6} mol/L, 10^{-7} mol/L ZA for 7 days. The proliferation of the primary cultured rat osteoblasts was evaluated by MTT (methyl thiazolyl tetrazolium) assay. The expression of IGF-1 was evaluated by ELISA method, and the ALP (alkaline phosphatase) was determined to detect the viability of basic phosphatase. ZA in concentrations above 10^{-5} mol/L inhibits the osteoblast proliferation. When the concentration of ZA was between 10^{-6} and 10^{-7} mol/L, no effect was observed on the cell proliferation. ZA inhibits osteoblasts ALP production at concentrations above 10^{-6} mol/L. At concentrations between 10^{-6} and 10^{-7} mol/L ZA have no effect on the expression of IGF-1. ZA is closely related to proliferation and IGF-1 expressions of rat osteoblasts.

Rezumat

Acest studiu a urmărit investigarea efectelor acidului zoledronic (ZA) în diferite concentrații asupra proliferării osteoblastelor de șobolan și asupra expresiei factorului de creștere *insulin-like* (IGF-1) și să furnizeze o bază teoretică pentru utilizarea sa clinică. Proliferarea osteoblastelor primare de șobolan a fost evaluată prin testul MTT (metil tiazolil tetrazoliu). Expresia IGF-1 a fost evaluată printr-o metodă ELISA, iar fosfataza alcalină (ALP) a fost determinată pentru a detecta viabilitatea fosfatazei bazice. ZA în concentrații mai mari de 10^{-5} moli/L inhibă proliferarea osteoblastelor. Când concentrația ZA a fost între 10^{-6} și 10^{-7}mol/L, nu s-au observat efecte asupra proliferării celulare. ZA inhibă sinteza de ALP a osteoblastelor la concentrații de peste 10^{-6} mol/L. La concentrații între 10^{-6} și 10^{-7} moli/L, ZA nu a prezentat nici un efect asupra expresiei IGF-1. Rezultatele obținute experimental demostrează că activitatea acidului zoledronic este strâns legată de proliferarea și expresia IGF-1 a osteoblastelor de șobolan.

Keywords: zoledronic acid, cell proliferation, insulin-like growth factor-1, osteoblasts

Introduction

Osteoporosis (OP) is a systemic metabolic skeletal disease [1]. Bisphosphonates (Bps) are important drugs for the treatment of osteoporosis. Zoledronic acid (ZA) is the third generation of Bps [2]. It has been reported that ZA inhibits bone metastases and anti-bone resorption by 100-850 times more than previous generations of bisphosphonates [3, 4]. Clinical trials have shown that ZA is the most pharmacologically active Bps [5, 6], being associated with increased absorption and high affinity for bone tissue that allow specific transport to bone formation location [7]. Moreover, the accumulation of ZA in osteoclasts leads to their loss of function and final apoptosis [8]. Part of ZA is also released through osteoclast apoptosis leading to increased concentration in bone microenvironment [9]. The activity of osteoclasts can be affected by the accumulation of ZA in the fracture sites.

Insulin-like growth factor-1 (IGF-1) is the most abundant growth factor in bone matrix [10, 11], being generated and stored in bone cells and released during bone resorption [12]. Studies showed that IGF-1 or the growth hormone determine the proliferation of osteoblast-like cells and bone marrow stromal cells by 49%-190% [13, 14]. This study aimed to investigate the effect of ZA on osteoblasts proliferation and IGF-1 expression on rats osteoblasts.

Materials and Methods

Animals

We used Sprague-Dawley (CD-SD) rats born within 24h, which were raised in the laboratory of The Affiliated Chenggong Hospital of Xiamen University (The 174 Hospital of PLA), China.

Reagents: zoledronic acid (Novartis, Switzerland); DMEM (dulbecco's modified eagle medium, Shanghai Huiying Biotechnology Co., Ltd., China); FCS (foetal calf serum, Thermo Fisher Scientific, USA); paraformaldehyde (Zibo Qixing Chemical Technology Co., Ltd., China); type II collagen (Shanghai Pureone Biotechnology Co., Ltd., China); trypsin (Shanghai Yaxin Biotechnology Co., Ltd., China); beta-glycerine sodium phosphonate (Beijing Gaoke Henghui Technology Development Co., Ltd., China); MTT (methyl thiazolyl tetrazolium, Dongren Chemical Technology (Shanghai) Co., Ltd., China); IGF-1 kit (Beijing Keruimei Technology Co., Ltd., China); ALP (alkaline phosphatase) determination kit (Shenzhen Mairui Biomedical Electronics Co., Ltd., China).

Apparatus/Devices: inverted microscope (Shunyu Optical Technology (Group) Co., Ltd., China); DH5000A constant temperature incubator (Hangzhou Jutong Electronics Co., Ltd., China); 721E VIS spectrophotometer (Beijing Lianhe Keli Technology Co., Ltd., China); SW-CJ-1FD super clean worktable (Shanghai Haozhuang Instrument Co., Ltd., China); electrothermal constant-temperature drying box (Dongguan Huatai Testing Instrument Co., Ltd., China); carbon dioxide incubator (Shanghai Jiehan Experimental Equipment Co., Ltd., China); enzyme labelling instrument (DG5033A) (Shanghai Precision Instruments Co., Ltd., China).

Osteoblast identification

According to Vicky *et al*. [15], SD rats were killed by neck-clipping and then immersed for 5 minutes in 75% alcohol solution. From each rat the head was used to collect the skull. For removing the surrounding connective tissues, the skull was rinsed in PBS for 3 times and then kept for 20 minutes at 37 in 0.25% trypsin solution. After removing the surrounding connective tissues, the skull was again rinsed with PBS for 3 times, cut with ophthalmic scissors and digested for 1 h at 37, with 0.1% collagenase II solution and then by shaking for 1 h 37. After the enzyme reaction the solution was filtered and centrifuges for 5 minutes at 1000 r/min. The medium was re-suspended and inoculated; the liquid was changed every 2 days. 80% of the cells were fused and passaged. Fibroblasts were removed by repeated adherence method in each passage to achieve the purpose of purification. Purified and cultured fourth generation osteoblasts were inoculated into a 35 mm diameter culture dish for co-culture experiment.

Osteoblasts were first cultured and passaged, and the growth of osteoblasts was observed by using an inverted phase contrast microscope. The fourth-generation primary cultured calvarial osteoblasts of the SD rats were inoculated on a six-well plate with cover glass, which were removed when cells covered about 90% of the cover glass. Afterwards the cells were washed with PBS rinsing and fixed for 10 min in 95% ethanol. Then the cells were coloured with alkaline phosphatase staining and observed under the microscope. The cytoplasm of osteoblasts showed black and gray granules or lumpy deposits.

The fourth-generation primary cultured rat calvarial osteoblasts were seeded onto a six-well plate with cover glass and cultured in DMEM nutrient solution containing 10% new-born foetal bovine serum supplemented with 50 μg/mL vitamin C and 10 mol/L beta-glycerine sodium phosphonate. The nutrient solution was changed every two days and alizarin red-calcium nodules were stained and observed under the microscope on days 15 - 20 when there were more opaque mineralized nodules. The cells were observed under the microscope, and orange-red opaque nodules suggested the positive result.

Detection of MTT proliferation ability

The fourth-generation cultured cells were digested with 0.25% trypsin. DMEM was added to terminate the digestion. The cells were seeded in 48-well plates at 5×10^3 cells *per* well. 8 samples were made for each group, and in each well was added 200 μL DMEM solution and incubated for 24 h at 37°C in 5% CO_2 incubator. Then, the cells were cultured with DMEM medium containing 10^{-4} mol/L, 10^{-5} mol/L, 10^{-6} mol/L, 10^{-7} mol/L, and 0 mol/L ZA for 7 days, during which the nutrient solution was changed every 3 days. 20 μL of MTT (5g/L) was added 4 h before the end of cell culture. Then, the supernatant was discarded and 100 μL of DMSO was added to each well and shaken for 10 min. The absorbance (OD) of each well was measured at a wavelength of 490 nm.

ALP activity assay

The fourth-generation rat osteoblasts in nutrient solution were grouped according to drug concentrations: the experimental group (10^{-4} mol/L, 10^{-5} mol/L, 10^{-6} mol/L, 10^{-7} mol/L), and the control group (0 mol/L). Every 3 days, the nutrient solution was replaced with the ZA solutions in the experimental groups and the nutrient solution was replaced with the normal culture medium in the control group. After culturing for 7 days, cells were digested with 0.25% trypsin and the supernatant was discarded by centrifugation. 400 μL of 1% SDS cell lysate was added and lysed for 30 min at

4°C. After centrifugation, 50μL of the supernatant was extracted and added into a 48-well culture plate, 8 replicates *per* sample. The instructions for the alkaline phosphatase assay kit were strictly followed. Blank and standard control wells were set up at a wavelength of 490 nm, the absorbance of each well was measured with a microplate reader, and the alkaline phosphatase activity was calculated according to the kit's instruction.

IGF-1 expression assay
The fourth generation rat osteoblasts were cultured in 48-well plates, 3×10^4 cells *per* plate. The cultured cells were divided in 5 groups exposed to media with 10^{-4} mol/L, 10^{-5} mol/L, 10^{-6} mol/L, 10^{-7} mol/L, and 0 mol/L ZA from the second day, 8 replicates *per* group. The medium was changed every two and a half days, and the supernatant was collected after 7 days of culturing. The IGF-1 ELISA kit was used to determine the expression IGF-1 according to manufacturer instructions.

Statistical methods
The data obtained from the experiments were all expressed as mean ± standard deviations. SPSS 20.0 software was used for statistical analysis. The difference among multiple groups was analysed by using one-way ANOVA. The comparison between two groups was performed using the LSD (Least-Significant Difference) method and $p < 0.05$ was considered statistically significant.

Results and Discussion

Cell Growth Status
The osteoblasts obtained after enzymatic digestion showed a spherical shape before adherence. After 48-hour culture, it was found that the cells adhered to the wall and protruded, and the cells became fusiform or irregular polygons (Figure 1A). The nucleus was round or oval and centered or biased to one side. After 3 to 4 days (Figure 1B), osteoblasts grew and covered the bottom of the flask, and some proliferated into colonies. The cells in the center of the colony were closely arranged, and their morphology was consistent with the characteristics of osteoblasts.
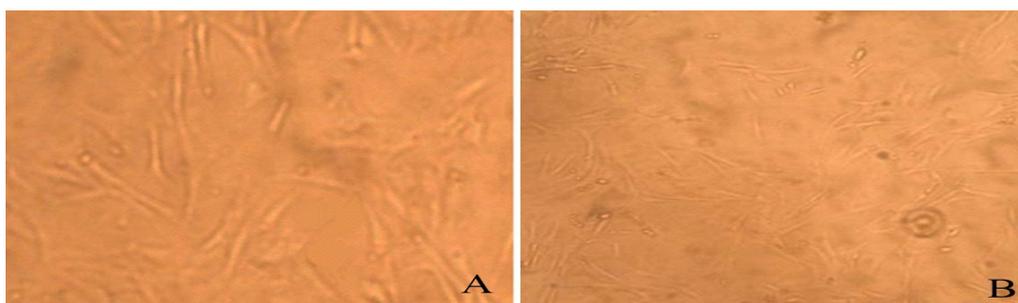

Figure 1.
Growth of primary osteoblasts (A: 48h after culture; B: after 3-days culture) (×200)

After ALP staining, most of the cells showed dark gray or black granular or massive deposits in the cytoplasm, which was consistent with the characteristics of osteoblasts. The cells cultured in this study were identified as osteoblasts (Figure 2A).
Microscopically there were observed mineralized nodules with varying sizes of orange-red centers with alizarin red staining (Figure 2B).
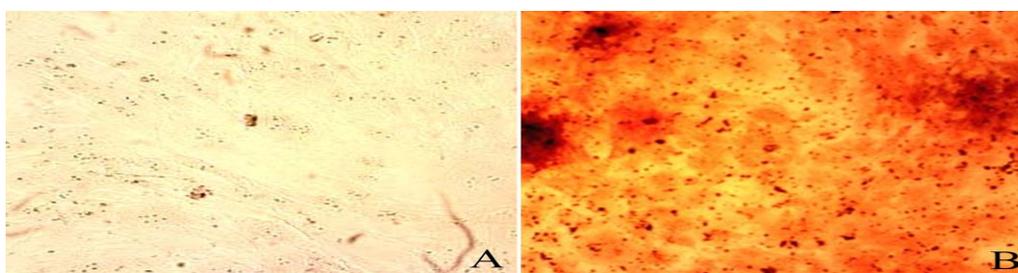

Figure 2.
Osteoblast staining (A: alkaline phosphatase staining; B: alizarin red staining) (×100)

The effect of ZA on proliferation and expressions of IGF-1
Seven days after osteoblast exposure to different concentrations of ZA it was observed a dose dependent decrease of osteoblast number with the increase of ZA concentration. When the concentration was above 10^{-5} mol/L, the difference between the experimental group and the control

group was statistically significant ($p < 0.01$). At lower concentrations ($\leq 10^{-6}$ mol/L), there was no significant difference ($p > 0.05$, compared with the control group).

The ELISA quantitative detection of IGF-1 expressions in osteoblasts showed that when ZA concentration was $\geq 10^{-5}$ mol/L, the level of IGF-1 expression in the experimental group was lower than that in the control group ($p < 0.01$), but no significant differences were observed at concentrations below 10^{-6} mol/L ($p > 0.05$, compared with the control group). The specific data are shown in Table I.

Table I

Effects of ZA on proliferation (OD Value) and IGF-1 expressions of primary osteoblasts of the newly-born rats

Concentration (mol/L)	0	10^{-4}	10^{-5}	10^{-6}	10^{-7}
OD Value	1.27 ± 0.11	$0.05 \pm 0.01^{**}$	$0.53 \pm 0.06^{**}$	1.31 ± 0.12	1.30 ± 0.15
IGF-1Content (µg/L)	161.46 ± 16.27	$11.65 \pm 3.08^{**}$	$20.17 \pm 4.72^{**}$	153.82 ± 20.13	164.25 ± 19.92

Note: ** $p < 0.01$, compared with the control group

The effect of ZA on ALP activity

ZA inhibited the expression of ALP at concentrations above 10^{-6} mol/L. It was observed no inhibitory effect at concentration of 10^{-7} mol/L. The specific data are shown in Table II.

Table II

Effects of ZA on ALP activity of primary osteoblasts of the newly-born rats (units/100 mL)

Concentration (mol/L)	0	10^{-4}	10^{-5}	10^{-6}	10^{-7}
ALP	20.98 ± 2.14	$1.24 \pm 0.18^{**}$	$1.95 \pm 0.26^{**}$	$12.23 \pm 1.21^{**}$	19.35 ± 2.24

Note: ** $p < 0.01$, compared with the control group

Osteoblasts and osteoclasts are the core cells in bone metabolism. In some genetic bone diseases the osteoclasts and osteoblasts have an excessive activity, leading to a disorganized bone appearance with severe clinical manifestations such us bone pain and ostheoarthritis [16, 17]. Osteoblasts cultured in this study showed a good adherence, and were identified as positive by alkaline phosphatase histochemical staining. Alizarin red staining showed a positive reaction, demonstrating that the cells had mineralization ability *in vitro*.

In vivo studies have shown that bisphosphonates inhibit bone remodelling [18, 19] and osteoblasts play an important role in this process. ALP is an early indicator to identify the differentiation degree of osteoblasts and it is a prerequisite for the initiation of mineralization [20, 21]. The results of this study showed that at concentrations above 10^{-5} mol/L, AZ significantly inhibits the proliferation of osteoblasts after 7 days of exposure. The inhibitory effect was dose depending, increasing with the concentration of ZA used, being associated with the inhibition of ALP activity. At concentrations below 10^{-6} mol/L, ZA did not show any significant effect on the proliferation of osteoblasts, suggesting that high levels of ZA inhibit osteoblasts ALP synthesis more than their proliferation. The high concentration of ZA was unfavourable for both the maturation and differentiation of osteoblasts, and the results of this study were consistent with those of Orriss and Pan *et al*. [22, 23]. Therefore, it was hypothesized that ZA promoted bone mineralization through the ALP pathway only at low concentrations, when the ZA concentration in the bone microenvironment was below 10^{-6} mol/L.

A long period is required for healing the osteoporotic fractures, and the activity of osteoblasts plays an important role in this process [24]. IGF-1 is the most abundant growth factor in bones [25]. Osteoblasts produce IGF-1 and promote the synthesis of bone matrix and osteoblasts in an autocrine and paracrine manner [26, 27]. Hock *et al*. [28] found that the collagen synthesis increased after IGF-1 action on the skull, and it was hence speculated that IGF-1 participated in bone formation through two mechanisms. First, it promoted the proliferation of mesenchymal cells to osteoblasts; besides, it promoted the maturation of osteoblasts and the secretion of cell matrix.

Conclusions

The study show that ZA decrease osteoblast number and IGF-1 expression in osteoblasts in a dose dependent manner, but increased the formation of osteoblast mineralized nodules. Therefore, high concentration of ZA can simultaneously increase bone mineralization and decrease osteoblast activity. As a result, the brittleness of newly formed bones may increase.

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