Effects of clodronate combined with hydroxyapatite on multi-directional differentiation of mesenchymal stromal cells

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Abstract

Introduction: Bisphosphonates (BPs) can be locally used to improve the osteogenesis around hydroxyapatite (HA) implants. However, there are almost no reports discussing the effects of BPs in the bonding state with HA on bone mesenchymal stromal cells (BMSCs). Clodronate is a BP widely used in clinical practice. This study was designed to evaluate the effects of clodronate combined with HA on BMSCs' multi-directional differentiation.
Material and methods: The HA and clodronate-HA complex were prepared. BMSCs were isolated from Sprague-Dawley rat bone marrow and then the cells were cultured with both HA and clodronate-HA. The method of transcriptional and translational assay (MTT) and multi-directional induction (including osteogenic, adipogenic, and myogenic differentiation) were used to evaluate the effect of clodronate-HA on BMSC differentiation.
Results: Scanning electron microscopy indicated active proliferation of the cells on clodronate-HA and HA. MTT of BMSCs cultured on clodronate-HA and HA demonstrated no significant differences between the two groups. BMSCs differentiated into osteocytes, adipocytes, and myocytes after being cultured with both clodronate-HA and HA. This indicated that BMSCs still retained multi-directional capability. The alkaline phosphatase activity of osteogenic induced BMSCs of both groups had no significant difference. However, there was a significant difference in total protein found between them.
Conclusions: The results suggest that clodronate in the bonding state with HA has no obvious inhibition of the proliferation and activity of BMSCs on the complex, and there was no evidence of a negative effect on multi-directional capability of the BMSCs.

Key words: BMSC, bisphosphonate, hydroxyapatite, multi-directional differentiation.

Introduction

Bisphosphonates (BPs), synthetic analogues of pyrophosphate, are potent inhibitors of bone resorption and have been successfully used with increasing frequency in the treatment of osteoporosis, fibrous dysplasia, and osteoarthritis [1-3]. It has also been reported that BPs have positive effects on bone matrix synthesis [4], and improved bone formation and mineralization [5]. In fact, the local use of BPs to achieve rapid bone growth around biomaterials post-surgery has been suggested by various researchers [6, 7]. Especially, combining BPs with hydroxyapatite (HA)

would be interesting [8], given that HA is widely used in orthopedic and oral maxillofacial implants, and is used to restore hard tissue defects. Bisphosphonates display a common backbone structure of P-C-P, where C is carbon and P is a phosphonate group. Bisphosphonates are known to be easily combined with HA by chelation. The two phosphonate groups are both essential for binding to the calcium ion of HA by chelation and for the biochemical mechanism. When combined with HA, the line structure of BPs transfers to a six-membered ring structure bonding state. It is well known that the structural change of a chemical compound could modify its function. However, there are almost no reports discussing the effects of BPs combined with HA in the bonding state on bone mesenchymal stromal cells (BMSCs), especially on their multi-directional capability. Bone mesenchymal stromal cells are biologically important cells for tissue reconstitution, and can differentiate along multiple lineages such as chondrocytes, osteocytes, adipocytes, myocytes, and astrocytes [9-11]. Biomaterials combined with BMSCs implanted into the bone defect could help improve osteogenesis [12, 13]. Therefore, it is necessary to investigate the effect of the BP-HA complex on BMSCs *in vitro*, before it could be possible to combine both BMSCs and BPs together to improve HA restoring hard tissue defects *in vivo*.

Clodronate, one of the nitrogen-containing BPs used widely in clinical practice, has a high affinity for HA (calcium ions). This study was designed to compare pure HA with the clodronate-HA complex to evaluate whether clodronate would have a negative effect on BMSC proliferation, activities and multi-directional differentiation.

Material and methods

Material preparation

The preparation of the clodronate-HA complex (the HA bioceramics for bone scaffolds were prepared by Sichuan University Research Center for Nano-Biomaterials, the compressive strength was 40.0 MPa, 10 mm × 10 mm × 1.0 mm) was carried out using a method previously described [14]. Briefly, the scaffolds were immersed in a solution of 4 mg/ml dichloromethylene-BP clodronate disodium (Shering, Germany) with a pH of 6.5 to form clodronate-HA chelate for 48 h at 37°C. The specimens were cleaned ultrasonically with distilled water for 30 min and stored in constant temperature desiccators (as cleaned specimens). The release of clodronate in medium was tested by reverse phase liquid chromatography at 220 nm, using a Waters 1525 binary HPLC pump (Waters Co., USA), a Rheodyne (Cotati, CA, USA) model 7725i injection valve fitted with a 20 µl loop, and a Waters 2487 dual λ absorbance detector (Waters Co., USA). The Phenomenex C_{18} column (USA) was used to separate the clodronate in the samples at 25°C using a mixture of acetonitrile and n-amylamine, and the flow rate was 1 ml/min. Generally, clodronate-HA specimens were rinsed in 1 ml of an eluting agent for 24 h, then the solutions were collected, and the concentration of clodronate was measured. The procedures were performed once a day over 21 days.

In this study, HA bioceramics without clodronate (10 mm × 10 mm × 1.0 mm) were sterilized as a control.

Isolation and culture of BMSCs

Bone mesenchymal stromal cells were obtained from Sprague-Dawley (SD) rats according to the established methods of our lab [15]. BMSCs were cultured in an incubator at 37°C, with 5% CO_2 for about 48 h, until cell adherence was observed.

Bone mesenchymal stromal cells were cultured on slides and stained according to the manufacturer's recommendations with the rabbit anti-mouse monoclonal antibody of CH3 (SANTA CRUZ, USA; working dilution was 1 : 100). Positive cells were detected with Biotin-conjugated goat anti-mouse IgG (ZhongShan, Beijing, China). CD45 (SANTA CRUZ, USA) were also stained as above.

Bone mesenchymal stromal cells were digested in 0.25% trypsin with 0.02% ethylenediaminetetraacetic acid (EDTA, Sigma, Germany) and blown into cell suspension, where density was adjusted to 2 × 10^5 cells/ml after cell counting, for pre-emergency. The materials above were bathed in α-modified Eagle's medium (α-MEM) for 24 h, then in calf serum for more than 4 h before being put into use. Then, the BMSCs were seeded respectively on the clodronate-HA and HA discs placed in 12-well plates and incubated with fresh medium at 37°C with 5% CO_2. The culture mediums were changed three times a week. A phase contrast microscope (OLYMPUS, Japan) and a scanning electron microscope (SEM, KYKY-2800) were used to observe the cell growth on the scaffolds. The cell proliferation rate on the scaffolds was measured using the method of transcriptional and translational assay (MTT) test on day 1, 2, 3, 4, 5, 6, and 7 after incubation. The absorbance was measured using a Beckman DU7400 spectrophotometer at a wavelength of 570 nm.

The MTT data were analyzed by a paired t-test with SPSS 10.0 (SPSS, Chicago, IL, USA). Differences at $p < 0.05$ were statistically significant.

Identification of BMSCs' multi-directional differentiation

The cells were cultured in a 12-well plate for 48 h, then the cells were collected using 0.25%

trypsin with 0.02% EDTA (Sigma, Germany) and recultured with specific media for osteogenic, adipogenic, and myogenic differentiation.

Osteogenic differentiation

According to the method of Conget PA [16], low glucose Dulbecco's modified Eagle's medium (LG-DMEM, GIBCO, USA; DMEM containing 10% FBS, 100 mM dexamethasone, 10 nM β-glycerophosphate, and 0.25 mM L-ascorbic acids) was added to the plate. The cells were cultured for 2 weeks with media changed twice a week. Induced cells were collected using 0.25% trypsin with 0.02% EDTA and recultured on the surface of a cover glass which contained polylysine in the 12-well plate. After 2 weeks, staining for alkaline phosphates (ALP) and calcium with Alizarin Red S (10%, pH 4.2) was carried out. Cells were collected on day 2, 6, 10, and 14, to measure the ALP and total protein content. Discs were first washed three times with phosphate buffer saline (PBS), and then the cells were collected by using 0.25% trypsin with 0.02% EDTA and centrifuging. Before determination, the cell suspension was put into a –80°C freezer for at least 12 h for the following analysis.

To measure the ALP level, each sample was added to the wells of a 24-well plate with 100 µl paranitrophenyl phosphate (PNP, Sigma, The Netherlands) solution. The well plate was protected from light and incubated at 37°C for 1 h. ALP activity was quantified by absorbance measurements at 405 nm. Finally, the ALP content of cells was counted through the column diagram.

The total protein concentration of the cells on the materials on day 2, 6, 10, and 14 was determined with a Micro BCA Protein Assay Kit (Pierce, USA), using bovine serum albumin (BSA), (Gibco BRL, USA) as a standard.

The data of ALP activity and total protein concentration were analyzed by a paired *t*-test with SPSS 10.0 (SPSS, Chicago, IL, USA). Differences at $p < 0.05$ were statistically significant.

Adipogenic differentiation

Adipogenic differentiation was achieved by adding α-MEM supplemented with 10% fetal bovine serum (FBS), 10% normal rabbit serum, 10 nM dexamethasone, 5 µg/mL insulin, and 50 µM 5,8,11,14-eicosatetraynoic acid into plates. A week later, the induced cells were collected and recultured as above. Finally, lipid droplets were stained with Oil Red O (0.3% in isopropanol with 0.4% dextrin) [17].

Myogenic differentiation

BMSCs were first induced into myocardial cells by adding LG-DMEM (containing 15% [v/v] FBS, 7.5 µmol/l 5-aza). 24 h later, the medium was replaced with LG-DMEM which contained 15% (v/v) FBS and incubated at 37°C with 5% CO_2 for 5 days. After being circulated three times, the induced cells were collected and recultured as before. Immunocytology of desmin (Sigma, USA) and connexin-43 (Sigma, USA) was used to verify myocytes.

Results

Clodronate combined with HA

Clodronate combined with the HA by chelation according to x-ray photoelectron spectrometry (XPS) and Fourier transform infrared spectroscopy (FT-IR) analyses (Figure 1 A and B) [14]. In the clodronate release test, the amount of clodronate was monitored over 21 days. During the first 6 days a large amount of clodronate was released; the first 3 days showed a sharp decline (Figure 1C).

Isolation and culture of bone mesenchymal stromal cells

The adherent cells appeared round or polygonal, growing on the inner wall of the culture flask 24 h after the initial plating. After replating, the cells expanded approximately and were fibroblast-like, appearing polygonal or spindle-shaped with long processes. The BMSCs' cell-surface antigen profile showed a strong positive for SH3 (Figure 2A) and negative for CD45 (Figure 2B).

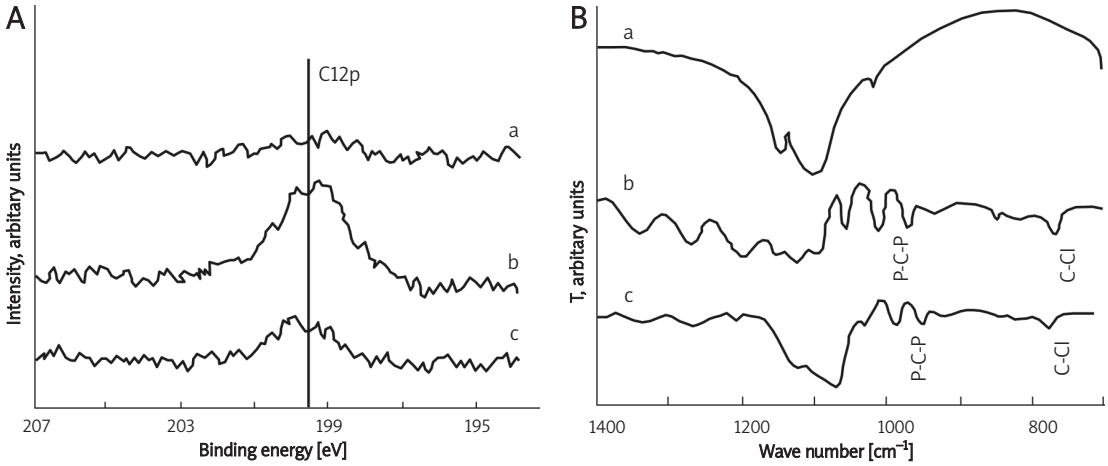
The SEM demonstrated adherence of BMSCs on clodronate-HA. Morphologically, 7 days after culture, the cells with several pseudopodia extended to the surface of both clodronate-HA and HA. The neighboring cells almost formed a continuous multilayered conformation (Figures 3A and B).

The MTT assay reflected a parallel growth tendency of the adherent cells on both materials and the levels of proliferation were close at the same time point (Figure 4). According to the statistical analysis, there was no significant difference ($p > 0.05$).

Multi-directional differentiation of bone mesenchymal stromal cells

Approximately 2 weeks after the osteogenic-specific induction, the cells grew tightly packed and spindle-shaped. Then clusters of round calcium-producing cells formed and calcified nodules could be seen (Figure 5A). Alizarin Red S also demonstrated the calcium formation (Figure 5B). ALP staining showed a strong positive (Figure 5C).

Figure 6A demonstrates that on day 2, 6, 10, and 14 of incubation, the ALP activity of the osteogenic-specific induced BMSCs on the clodronate-HA appeared slightly higher than that on the HA,



although the difference was not significant ($p > 0.05$). However, Figure 6B shows a significant difference in protein content between the two groups ($p < 0.05$).

One week after adipogenic-specific inductions, lipid vacuoles were observed in the cells of both groups. As time passed, the lipid vacuoles coalesced and eventually filled the cells (Figure 7A). The lipid vacuoles were stained positively with Oil Red O (Figure 7B).

After myogenic-specific inductions, the cells of both groups showed positive staining of desmin (Figure 8A) and connexin-43 (Figure 8B).

Discussion

In the present study, we investigated the effect of the clodronate-HA complex on BMSC proliferation, activities and multi-directional differentiation *in vitro*. The results demonstrated that clodronate in the bonding state had no obvious inhibition of proliferation and multi-directional capability of the BMSCs, except for a slightly significant difference in the total protein content. This study might provide experimental evidence for further studies regarding the application of BMSCs to improve BP-HA restoration of hard tissue defects *in vivo*.

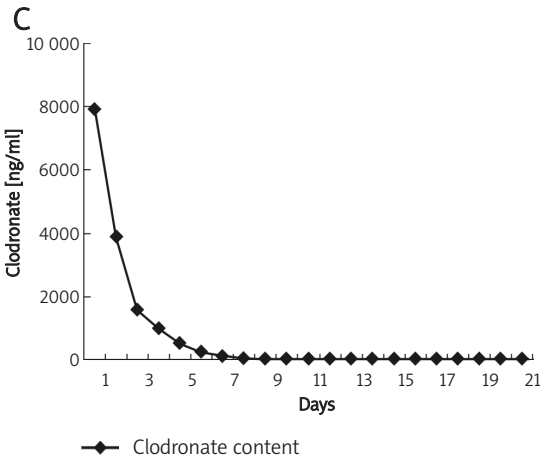


Figure 1. XPS and FT-IR analyses. **A** – XPS spectra of HA (a), clodronate-HA (b) and 30 s-sputtered clodronate-HA (c); **B** – FT-IR spectra of HA (a), clodronate (b) and clodronate-HA; **C** – *in vitro* release of clodronate

BPs inhibit bone resorption by binding to bone mineral and also inhibit the function of osteoclasts that take up the drug. BPs have been developed to treat several diseases related to bones, teeth, and calcium metabolism. Clodronate is a kind of BP, which is a well known potent inhibitor of osteoclast

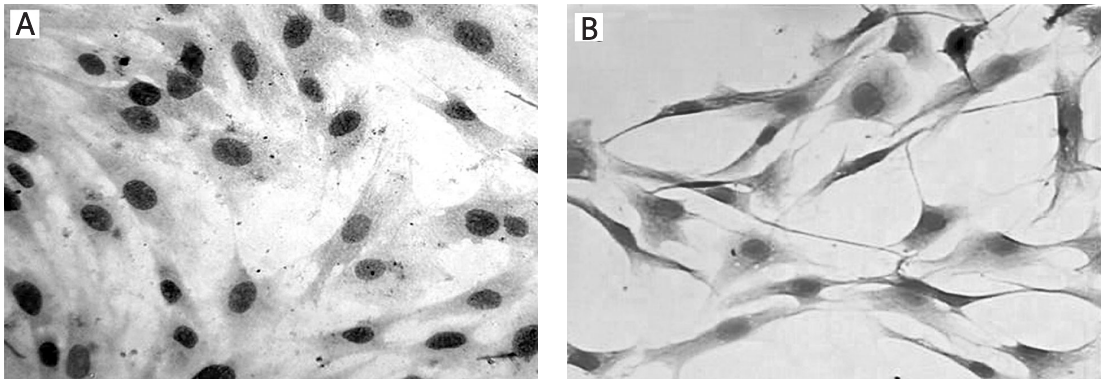


Figure 2. Immunocytology of BMSCs. **A** – SH3 positive staining of BMSCs; **B** – CD45 negative staining of BMSCs (original magnification of all images 200×)

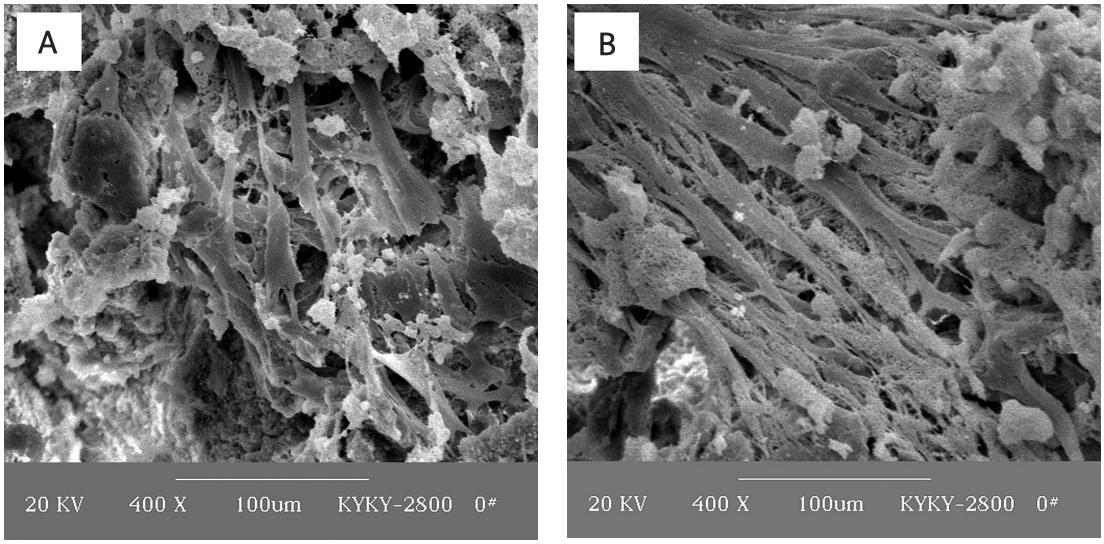


Figure 3. SEM images of BMSCs on clodronate-HA (**A**) and SEM images of BMSCs on HA after 7 days of culturing (**B**)

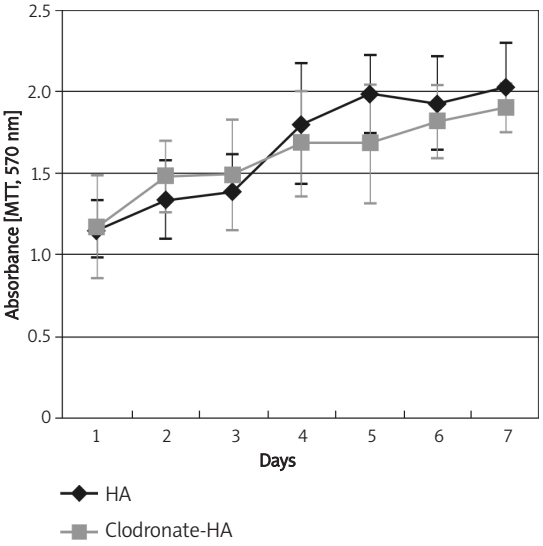


Figure 4. MTT test of BMSCs cultured on clodronate-HA and HA

activity and is widely used to treat metabolic bone diseases. In our previous study [14], clodronate was used to modify the surface of HA. The results of XPS and FT-IR analyses indicated that clodronate is immobilized on the surface of HA. It is generally accepted that the strong chelate binding of the P-C-P moiety to calcium ions is responsible for the high affinity of clodronate for HA [18].

Recently, there has been an increased amount of evidence that BPs also interact with osteoblasts and BMSCs. Almost all the research on BPs involve their unbound state, whereas little evidence involves their bonding state.

Some studies have indicated that BPs enhance proliferation and maturation of osteoblasts [19-20] and inhibit apoptosis [21]. The effects of BPs on early stages of osteoblastic differentiation are not yet well understood, but the effects of BPs stimulate the formation of osteoblast progenitors to promote early osteoblastogenesis [22]. Osteoblast progenitors derived mainly from BMSCs are critically involved in maintaining the dynamic equilibrium of bone turnover. BMSCs are able to differentiate along multiple lineages and can be used as precursor cells for tissue reconstitution or treatment [23-25]. Bone mesenchymal stromal cells play a fundamental role in bone regeneration. It has been reported that BMSCs are also the targets of BPs [18, 27]. BPs used in treatment promote differentiation and maturation of pluripotent BMSCs towards the osteoblastic phenotype by upregulating bone morphogenetic protein-2 (BMP-2) and core binding factor α1 (cbfa-1), which are considered to be the key transcription factors for osteoblastic differentiation [28].

BPs also upregulate ALP activity and stimulate fibroblastic colony formation to influence osteogenic differentiation of BMSCs [29]. ALP is the typical marker for osteoblast characterization and its activity is an early marker of osteogenic differentiation, with its increased expression associated with progressive differentiation of osteoblasts [30]. In this experiment, the ALP activity of osteogenic induced BMSCs in the two groups had no significant difference, showing that the presence of clodronate did not inhibit cell osteogenic differentiation. There was a significantly higher protein content observed in the clodronate-HA group, which was an interesting phenomenon. Further studies, however, are necessary to clarify what kinds of proteins were increasing, such as BMP-2 and cbfa-1, which were reported by previous studies [28].

Bisphosphonates in an unbound state could inhibit adipogenic differentiation of BMSCs [32]. BP-treated cells showed some changes in

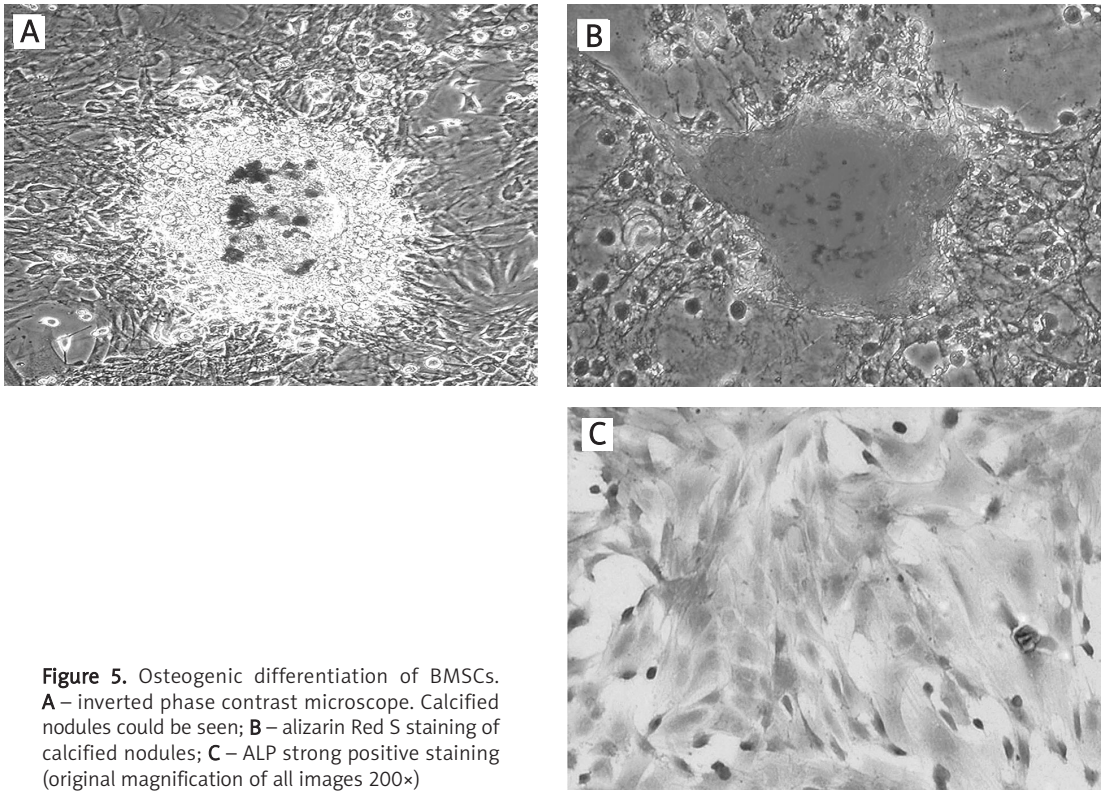


Figure 5. Osteogenic differentiation of BMSCs. **A** – inverted phase contrast microscope. Calcified nodules could be seen; **B** – alizarin Red S staining of calcified nodules; **C** – ALP strong positive staining (original magnification of all images 200×)

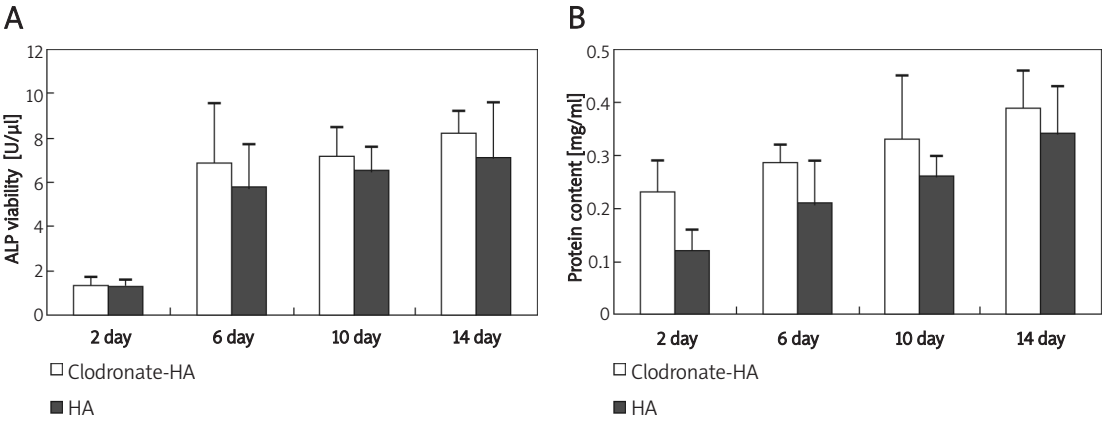


Figure 6. ALP viability and total protein of the cells on clodronate-HA and HA on inducing day 2, 6, 10, and 14. **A** – ALP activity; **B** – total protein content

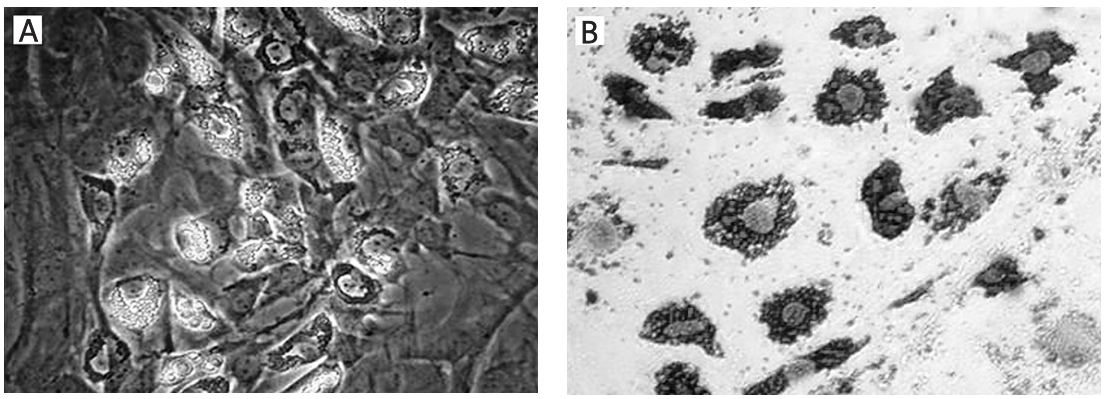


Figure 7. Adipogenic differentiation. **A** – inverted phase contrast microscope. **B** – Oil Red O positive staining (original magnification of all images 200×)

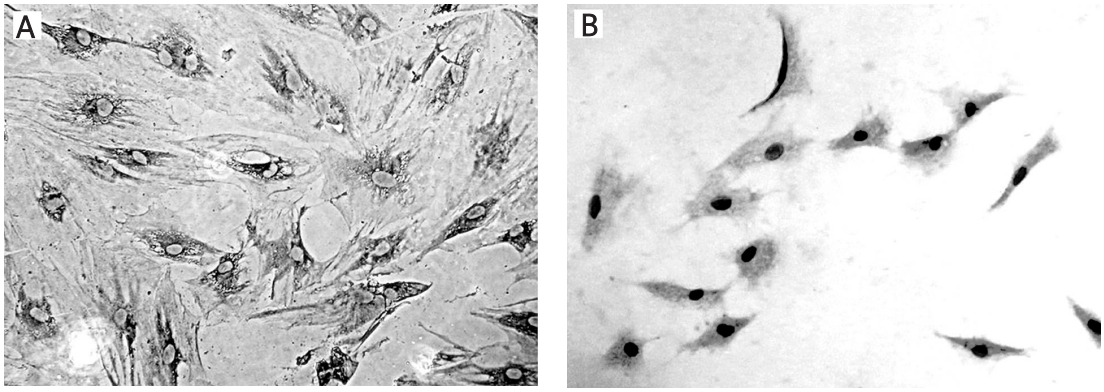


Figure 8. Myogenic differentiation. **A** – desmin positive staining. **B** – connexin-43 positive staining (original magnification of all images 200×)

phenotype and limitation in their capacity to produce fat droplets. This limitation could be due to the activation of extracellular signal-related kinases (ERK) and/or Jun amino-terminal kinases (JNK) followed by a decrease in peroxisome proliferator activated receptor gamma 2 (PPARγ2) transcription activity. In this study, the capability of adipogenic differentiation was shown after BMSCs were cultured with clodronate-HA. There were no obvious differences in morphology. One possible reason might lie in the structural change of clodronate. However, the quantitative analyses, such as PPARγ2, are out of testing, and further studies should be pursued.

In the current study, we compared clodronate-HA complex with HA. From the results, it could be concluded that clodronate-HA shows no obvious inhibition of the proliferation and activities of BMSCs, and has no evidently negative effect on the multi-directional capability of BMSCs.

Acknowledgements

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