Int J Clin Exp Med 2018;11(2):528-538
www.ijcem.com /ISSN:1940-5901/IJCEM0065217

Original Article

Taraxasterol as a proliferation-enhancing agent to enhance osteogenic differentiation of rat osteoblastic cells

Ru-Yin Hu[1,2*], Bo Li[2*], Li Sun[2*], Fu-Ben Xu[4], Xian-Teng Yang[2], Rui Luo[2], Xiao-Bin Tian[2], Jin-Min Zhao[1,3]

[1]Guangxi Medical University, Nanning 530021, China; [2]Department of Orthopedics, Guizhou Provincial People's Hospital, Guiyang 550002, China; [3]The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, China; [4]Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning 530003, China. [*]Equal contributors and co-first authors.

Received September 8, 2017; Accepted January 8, 2018; Epub February 15, 2018; Published February 28, 2018

Abstract: Osteoporosis is a disease characterized by decreased bone strength, decreased bone mass, and bone deterioration. The proliferation and osteogenic capacity of osteoblastic cells needs to be improved, for their use in cell-based therapy, and in the treatment of osteoporosis. Taraxasterol, the main constituent of the traditional Chinese medicinal herb *Taraxacum*, has been demonstrated to have anti-inflammatory, cholesterol-lowering, anti-rheumatic, anti-cancer, and protective effects. However, the protective effects of taraxasterol, on osteoblastic cells and osteogenic differentiation, have not been reported. Based on the hypothesis that taraxasterol may have an effect on osteoblastic cell growth and differentiation, we studied the effect of taraxasterol on rat osteoblastic cells by detecting cell proliferation, alkaline phosphatase (ALP) activity, and the expression of relevant osteogenic markers. Various concentrations of taraxasterol (0 μM, 6.25 μM, 12.5 μM, and 25 μM) were used for treatment of rat osteoblastic cells, with the cell proliferation to be detected. Bone morphogenetic protein-2 (BMP-2), runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), bone sialoprotein (BSP), type I collagen (COL-I), and osteocalcin (OCN) expression levels were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) using isolated RNA. Our results demonstrated that taraxasterol has a strong stimulatory effect on osteoblastic cell proliferation/enhancement, and on maintaining the phenotype. Taraxasterol also regulated osteoblast differentiation through the upregulation of ALP expression in rat osteoblastic cells. Also, treatment with taraxasterol enhanced the transcript levels of BMP-2, RUNX2, ALP, BSP, COL-I, and OCN. The underlying mechanism might be associated with taraxasterol's antioxidative effect, leading to favorable amounts of reactive oxygen species in the cellular environment. Thus, our study indicated that taraxasterol can be used as an osteogenic proliferation-enhancing agent for the therapy of osteoporosis.

Keywords: Osteoporosis, taraxasterol, rat osteoblastic cells, proliferation-enhancing, differentiation

Introduction

Osteoporosis is becoming a major public health problem that severely affects the quality of life for the elderly, as their life expectancy increases. An imbalance between bone resorption and bone formation is the dominant mechanism causing osteoporosis [1, 2]. Increased reactive oxygen species (ROS) levels greatly contribute to the pathogenesis of osteoporosis [3]. Even though therapies have been proposed to neutralize their damaging effects on bone, the molecular mechanisms involved in these processes remain ill-defined. With bone injury, osteoblasts migrate to the injury site, and contribute to bone formation by synthesizing various bone extracellular matrix molecules that are then mineralized [4-6]. It is known that MAPKs such as ERK1/2, p38, and JNK are activated in response to oxidant injury, and mediate changes in osteoblast survival, proliferation, and differentiation [7, 8]. Previously, it was generally accepted that the immune system exerts positive immunomodulatory effects on tissue regeneration [9, 10]. Macrophages are capable of engulfing tissue debris and secreting anti-inflammatory cytokines, to maintain the stability of the implant site [11], rendering the local tissue environment more conducive to bone regeneration [12, 13]. Accumulating evi-

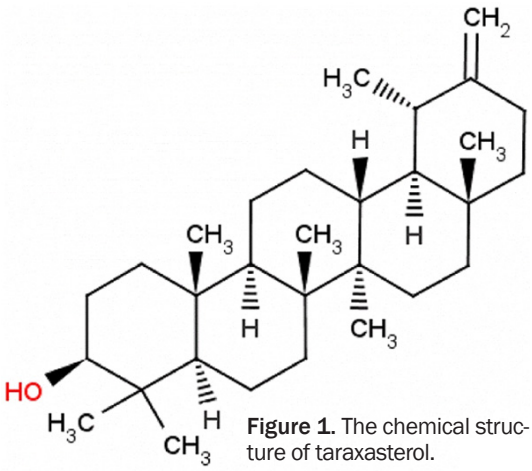

Figure 1. The chemical structure of taraxasterol.

dence suggests that natural compounds may have the ability to treat osteoporosis and have minimal side effects [14, 15]. Therefore, contemporary studies have focused on the use of appropriate agents to utilize the immune response in order to enhance the differentiation of bone forming cells toward a more regenerative and less inflammatory phenotype [16].

Taraxacum officinale, an Asian medicinal herb (Fa.: Asteraceae/Compositae), popularly known as the common dandelion, has been used as a food material based on its high nutritive value. Taraxasterol (**Figure 1**), a pentacyclic-triterpene isolated from *T. officinale*, has been reported to have anti-inflammatory effects [17]. Taraxasterol was found to inhibit the IL-1β-induced inflammatory response in human osteoarthritic chondrocytes [18]. Furthermore, studies have shown that taraxasterol had protective effects against LPS-induced acute lung injury [19], and endotoxic shock *in vivo* [20]. However, the effects of taraxasterol on osteoblastic cells remain unclear. The purpose of this study was to investigate the protective effects and mechanisms of taraxasterol on osteoblastic cells and bone regeneration.

We investigated the osteogenic effects of taraxasterol on osteoblastic cells by detecting cell proliferation, alkaline phosphatase (ALP) activity, and expression of related osteogenic genes *in vitro*. Our results demonstrate that taraxasterol has a strong stimulatory effect on expression of osteoblast marker genes, bone morphogenetic protein-2 (BMP-2), runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), bone sialoprotein (BSP), type I collagen (COL-I), and osteocalcin (OCN), especially at a concentration of 12.5 μM. This study may provide reference for its application to the prevention and therapy of osteoporosis.

Materials and methods

Taraxasterol preparation

Taraxasterol (purity ≥ 98%) was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China) and stored at 4°C. Prior to the experiments, taraxasterol was dissolved in dimethyl sulfoxide (DMSO; Solarbio, China). The stock solutions were stored at 4°C. The taraxasterol stock solution was diluted with the culture medium immediately before treatment. Cells were treated by LBP at various concentrations (0 μM as control, 6.25 μM, 12.5 μM, and 25 μM), and filtered using 0.22 μM filters for sterilization. The concentration of DMSO was less than 0.1% in all experiments.

Cell cultures

Osteogenic cells were harvested from the calvaria bones of newborn (2-day-old) Wistar rats. The calvaria bones were dissociated enzymatically with 0.25% trypsin (Solarbio, Shanghai, China) for 30 min, and then with 2 mg/mL collagenase type I in alpha-modified Eagle's medium (α-MEM, Gibco, Carlsbad, CA) for 3 h. After centrifugation (1,000 r, 5 min), isolated osteogenic cells were re-suspended in alpha-modified Eagle's medium (α-MEM; Gibco, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, USA), and 5% (v/v) antibiotics (penicillin 100 U/l, streptomycin 100 U/l; Solarbio, China). After centrifugation, the cells were suspended in the culture medium, and then maintained under a humidified atmosphere with 5% CO_2 at 37 °C. When reaching 80-90% confluence at 7d, cells were passaged. The logarithmic growth phase of confluence osteogenic cells was used for further study.

Cell cytotoxicity assay

Cell viability was estimated using a colorimetric assay, based on the conversion of MTT into a blue formazan product. The cells were then plated, at 800 cells/well, in a 96-well cell culture cluster pretreated with various concentrations of LBP (0-80 μM) for 3 days in a 5% CO_2 humidified incubator at 37°C. MTT (5 mg/mL)

Table 1. Primer sequences used in qRT-PCR experiments

Gene	Primer sequence (5' to 3')	Length (bp)	Amplicon size (bp)
BMP-2	F: TCCATGTGGACGCTCTTTCA	20	113
	R: AGCAGCAACGCTAGAAGACA	20	
COL1	F: GCCACCTCAAGAGAAGGCTG	20	139
	R: CTCGGGGCTCTTGATGTTCT	20	
BSP	F: CAATCTGTGCCACTCACTGC	20	257
	R: TGCCCTGAACTGGAAATCGTT	21	
ALP	F: CCAGGGCTGTAAGGACATCG	20	181
	R: GCTCTTCCAGGTGTCAACGA	20	
Runx2	F: TGTCATGGCGGGTAACGATG	20	286
	R: CCCTAAATCACTGAGGCGGT	20	
OCN	F: ACACTCCTCGCCCTATTGGC	20	166
	R: CCATTGATACAGGTAGCGCCT	21	
GAPDH	F: GTCATCATCTCAGCCCCCTC	20	99
	R: GGATGCGTTGCTGACAATCT	20	

PCR: polymerase chain reaction; GDNF: glial cell-derived neurotrophic factor; BMP-2: bone morphogenetic protein-2; COL1: type I collagen; BSP: bone sialoprotein; ALP: alkaline phosphatase; Runx2: runt-related transcription factor 2; OCN: osteocalcin; F: forward primer; R: reverse primer.

was added to the cultures in each well and they were incubated in the dark at 37°C for 4 h. Subsequently, the culture medium was removed and the cells were treated with 150 µl DMSO, to dissolve the formazan product. The cells were incubated in DMSO, with agitation, for 10 minutes. The optical density of each sample was measured using a Multiskan GO Microplate Spectrophotometer, at 570 nm. Five individual cultures were used for each test. The experiments were carried out in quintuplicate.

Cell proliferation analysis

Based on the result of the cytotoxicity assay, we chose three doses (6.25 µM, 12.5 µM, and 25 µM taraxasterol) with more obvious positive effects, coupled with the control group (0 ng/mL taraxasterol), for cell proliferation analysis. After trypsinization and determination of the cell concentration, 150 µl of cell suspension, containing 5×10^3 cells/well, was transferred onto 24-well plates. Cells were allowed to attach and to propagate at 37°C and 5% CO_2. At each time point (3, 5, 7, and 14 days after cell seeding), 1 mL of 5 mg/mL MTT solution (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to each scaffold for 3 hours at 37°C and 5% CO_2 in the dark. After the formation of formazan during reduction process, DMSO was added for 10 min at 37°C in the dark, with moderate agitation to disrupt the cells and solubilize the formazan. The samples' optical densities (OD) were measured by a Multiskan GO Microplate Spectrophotometer, at 570 nm. Five individual cultures were used for each test. The experiments were carried out in quintuplicate.

Morphological examination

Cells were cultured for 3, 5, and 7 days, and were fixed in 4% paraformaldehyde for 40 min at room temperature, for subsequent hematoxylin-eosin staining. Cells were incubated with a nuclear dye for 3 minutes, followed by a 10 second incubation with a cytoplasmic dye. Subsequently, the cells were rinsed with PBS, naturally dried, and sealed with neutral gum. Cells were then examined, and images were captured under an upright microscope.

Cell viability assay

Cell viability was determined using the LIVE/DEAD Viability Assay Kit. Cells on coverslips were rinsed quickly with PBS (0.01 mol/L, pH 7.4), to remove the medium. Subsequently, 1 µM calcein-acetoxymethyl (calcein-AM) and 1 µM propidium iodide (PI) were added to the cell cultures and were incubated in the dark for 5 min at 37°C. Images were captured using a laser scanning confocal microscope.

ALP activity assay

To examine the effect of taraxasterol on osteoblastic cells, cells were seeded onto coverslips and were cultured in media containing various concentrations of taraxasterol. After 3, 5, 7, and 14 days of culture, cells were washed with phosphate-buffered saline (PBS), and lysed with 200 µl RIPA Lysis Buffer (Beyotime Institute of Biotechnology, China), which was added together with phenylmethanesulfonyl fluoride (PMSF) to give a final concentration of 1 mM, before the analysis of ALP activity. Total protein concentration (mg/mL) and ALP activity (units/mL) were measured, following the manufacturer's instructions, with an enhanced BCA protein assay kit (Beyotime Institute of Biotechnology, China) and an ALP reagent kit (Nanjing Jiancheng Bioengineering Research Institute,

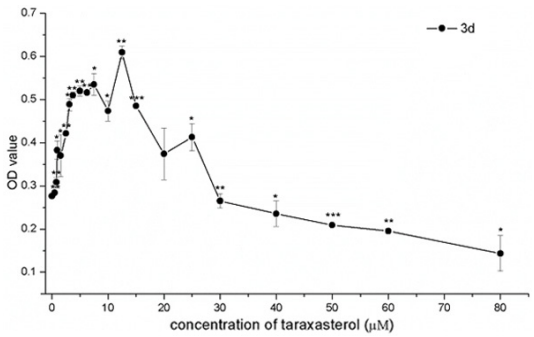

Figure 2. Cytotoxicity of taraxasterol on osteoblastic cells after 3d (mean ± SD, n = 3). *p < 0.05, **p < 0.01.

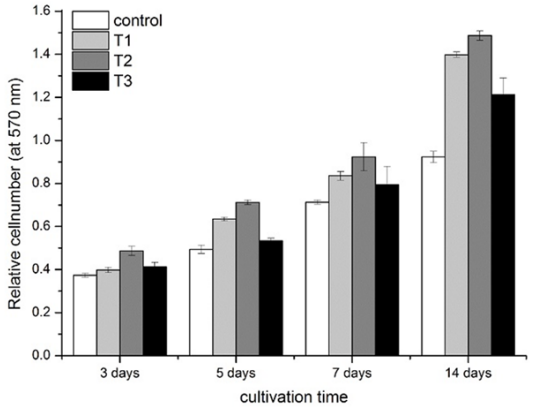

Figure 3. Quantification of cell proliferation by the MTT assay. The proliferation of osteoblastic cells cultured *in vitro* with 0 μM (Control), 6.25 μM (T1), 12.5 μM (T2) and 25 μM (T3) taraxasterol for 3, 5 and 7 days. The data represent the mean ± SD of five independent culture experiments. Bars with different letters are significantly different from each other at P < 0.05.

China), respectively. ALP levels were normalized to the total protein content. All samples were examined in triplicate.

Alizarin red staining

Extracellular matrix (ECM) mineralization of the taraxasterol-treated osteoblasts was tested by using Alizarin red staining. After 5, 7, and 14 days, cells were washed with distilled water and fixed in ice-cold 70% (v/v) ethanol for 1 h. Cells on coverslips were then rinsed twice with deionized water (Tiangen Biotech [Beijing] China), and stained with Alizarin red S (Sigma) solution (40 mM, pH 4.2) for 10 minutes, at room temperature. The dye was prepared in the TRIS-HCl (Sigma) buffer solution and adjusted to the target pH. After the staining step, excess dye was gently washed away with running water. Calcification deposits were, typically, stained red and were examined under a microscope (Nikon, Japan).

Immunohistochemical assay

After 5, 7, and 14 days of culture, immunohistochemical staining of BMP-2 and COL-I was performed, following the manufacturer's instructions. Briefly, cells on coverslips were washed with PBS, rinsed with 0.01% Triton X-100 (Beyotime Institute of Biotechnology), washed thoroughly in PBS, treated with 3% hydrogen peroxide (BOSTER, Wuhan, China), washed again in PBS, and then blocked with 3% bovine serum albumin. After incubation with the primary antibody (BMP-2 and COL-I; BOSTER, Wuhan, China), at a dilution of 1:200, a second antibody and biotin-labeled horseradish peroxidase were added to the cells. Following incubation with DAB and counterstaining with hematoxylin, the cells were air-dried and then sealed with the neutral resin.

RT-qPCR analysis

Real-time polymerase chain reaction assays were performed for the detection of the osteogenic gene expression of cells cultured in 6-well plates, with various concentrations of taraxasterol (0 μM, 6.25 μM, 12.5 μM, 25 μM). Total RNA was harvested by using a Reverse Transcription System (Promega, USA) on days 3, 5, 7, and 14. Next, 1 mg of total RNA was reverse-transcribed to cDNA. Finally, an ABI 7300 Sequence Detection System (Applied Biosystems, USA) was used to conduct quantitative real-time PCR with TaqMan Universal PCR Master Mix and gene-specific TaqMan PCR primers (Applied Biosystems, USA), including bone morphogenetic protein-2 (BMP-2), type I collagen (COL-I), bone sialoprotein (BSP), osteocalcin (OCN), ALP, runt-related transcription factor 2 (RUNX2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene expression was normalized to GAPDH by using the comparative $2^{-\Delta\Delta Ct}$ method. The primers used in this experiment are shown in **Table 1**. All PCRs were carried out in triplicate.

Statistical analysis

Data are presented as the mean ± standard error of the mean. The expression activity dif-

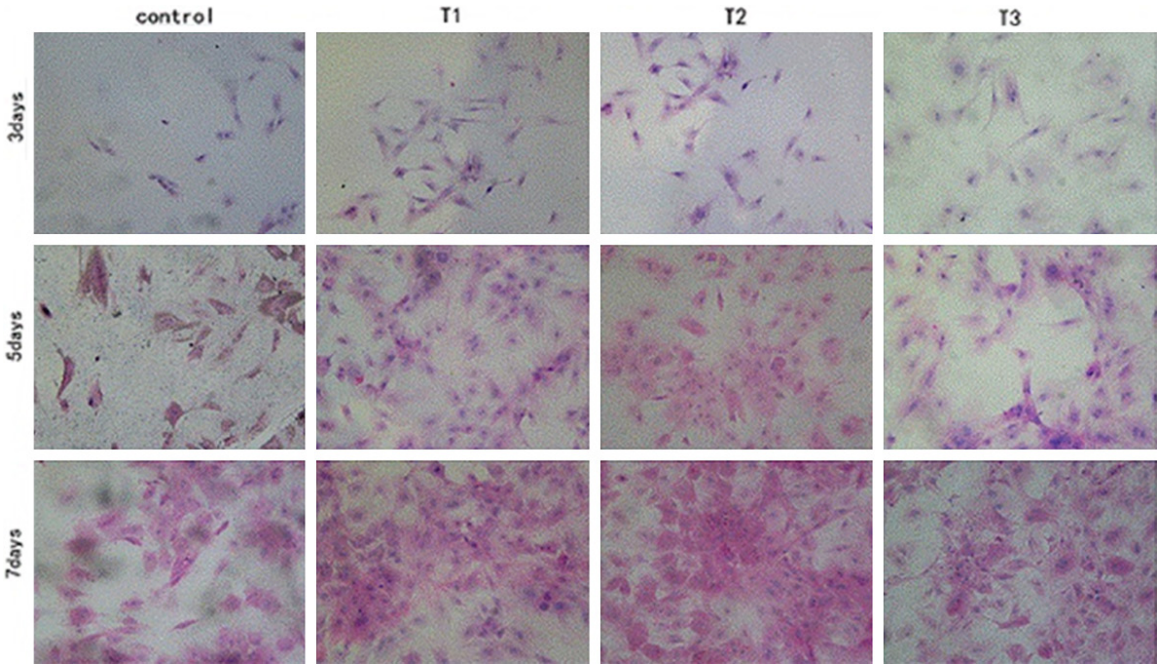

Figure 4. Hematoxylin-eosin staining images showing the morphology of taraxasterol cells cultured *in vitro* with 0 μM (Control), 6.25 μM (T1), 12.5 μM (T2) and 25 μM (T3) taraxasterol for 3, 5 and 7 days. Cell seeding density: 4 × 10^3/mL (original magnification × 100).

ferences (for ALP, RUNX2, and BMP-2), among different taraxasterol dose groups (control group and doses of 6.25 μM, 12.5 μM, and 25 μM), were analyzed by two-tailed one-way analysis of variance (ANOVA) following by Dunnett's post-hoc test or by Wilcoxon signed-rank test at different time points, respectively. Statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell cytotoxicity assay

The present study examined the cytotoxicity of various concentrations of taraxasterol on osteoblastic cells, using the MTT assay. Cells were treated with increasing concentrations of taraxasterol (0-80 μM). As shown in **Figure 2**, compared with the control group (0 μM), treatment with taraxasterol between 0.49 and 30 μM exhibited low cytotoxicity. In addition, 0.49-30 μM taraxasterol significantly accelerated cell growth ($P < 0.05$), with the most obvious effect observed when used at 12.5 μM ($P < 0.05$). However, taraxasterol exhibited a suppressive effect on osteoblastic cells *in vitro*, when used between 30-80 μM, as compared with the control group.

Cell proliferation

As presented in **Figure 3**, osteoblastic cells treated with 6.25 μM, 12.5 μM, and 25 μM taraxasterol, exhibited increased proliferation compared with the control group (0 μM taraxasterol). Proliferation of osteoblastic cells cultured with various concentrations of taraxasterol was detected by the MTT assay, which was markedly higher in the taraxasterol groups, compared with the control group after the same culture period. Among the three concentrations, 12.5 μM taraxasterol exhibited the strongest effect on cell growth at all time points.

Cell morphology

H&E staining was conducted using an upright microscope to assess the morphology of osteoblastic cells. The images indicated that the taraxasterol groups grew better, compared with the control group at the same time point (**Figure 4**). Compared with the control group, osteoblastic cells in the presence of taraxasterol grew better and had a distinct proliferative tendency, that gradually increased with time. In addition,

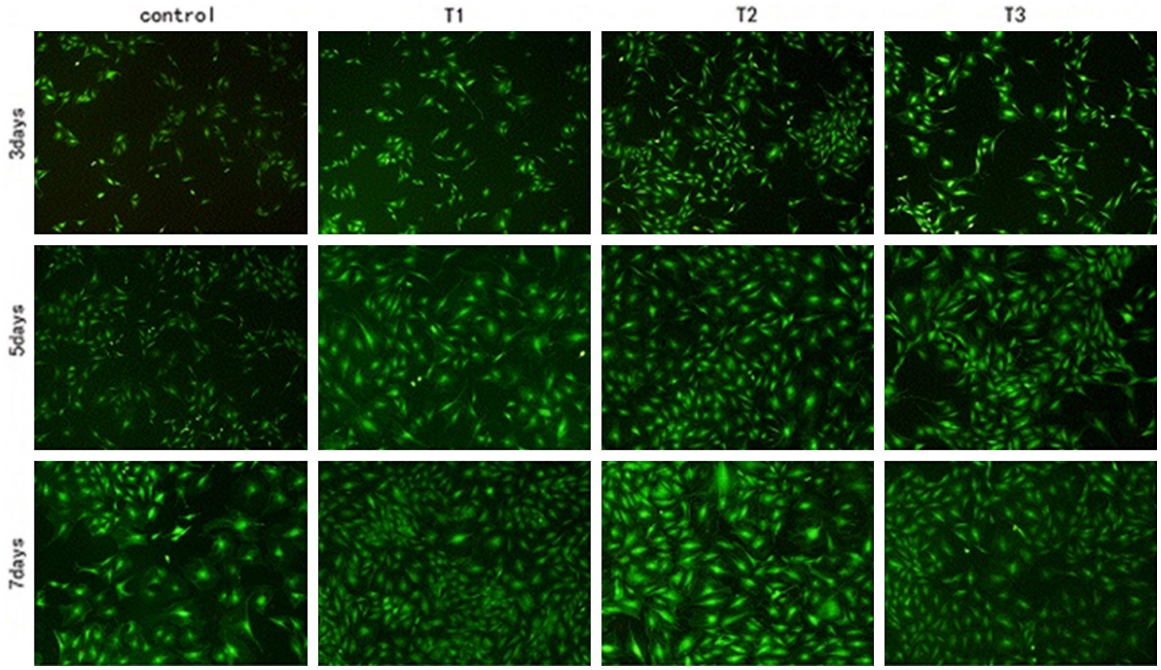

Figure 5. Confocal laser scanning microscopy images showing the viability of osteoblastic cells, cultured *in vitro* with 0 μM (Control), 6.25 μM (T1), 12.5 μM (T2) and 25 μM (T3) taraxasterol for 3, 5 and 7 days. Cell seeding density: 4 × 10^3/mL (original magnification × 100).

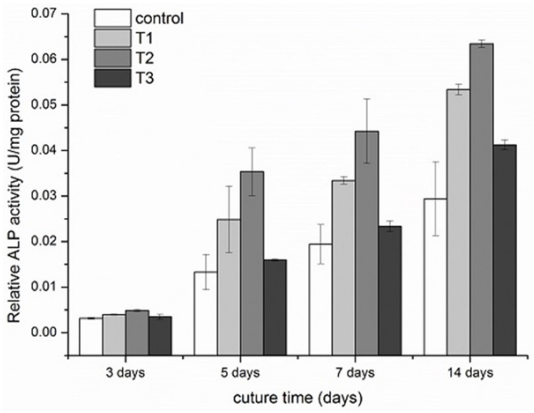

Figure 6. Time-course of alkaline phosphatase (ALP) activity and ALP staining of osteoblastic cells at various concentrations (0 μM (Control), 6.25 μM (T1), 12.5 μM (T2) and 25 μM (T3)) of taraxasterol. Relative ALP activity (units/mg protein) expressed as mean ± 2SD. ALP activity in 12.5 μM taraxasterol was significantly higher than that in other groups.

when used at 12.5 μM, taraxasterol was able to enhance the proliferation of osteoblastic cells, compared with the other two concentrations *in vitro*.

Cell viability assay

As presented in **Figure 5**, viable cells and dead cells were stained with calcein-AM/PI. The results demonstrated that taraxasterol exerted positive effects on survival. Images of calcein-AM/PI staining demonstrated that the survival of cells, in the taraxasterol groups, was increased compared with the control group. Consistent with the results of a cell proliferation assay (**Figure 3**), more viable cells than dead cells were detected in the taraxasterol groups, thus implying that taraxasterol was able to better support cell growth, compared with the control group. Among the taraxasterol groups, treatment with 12.5 μM exhibited the best effects, as evidenced by an increase in the number of viable cells.

ALP activity assay

ALP is produced by osteoblasts and is likely to be involved in the degradation of inorganic pyrophosphate to provide sufficient local phosphate or inorganic pyrophosphate, for mineralization to occur. Commonly used as a marker of osteogenesis, ALP activity is assumed to reflect the degree of osteogenic differentiation.

As evidenced by ALP activity assay (**Figure 6**), taraxasterol groups showed much higher ALP activity, compared with the control, after 7 days, although no significant difference was observed at day 3. In particular, ALP levels

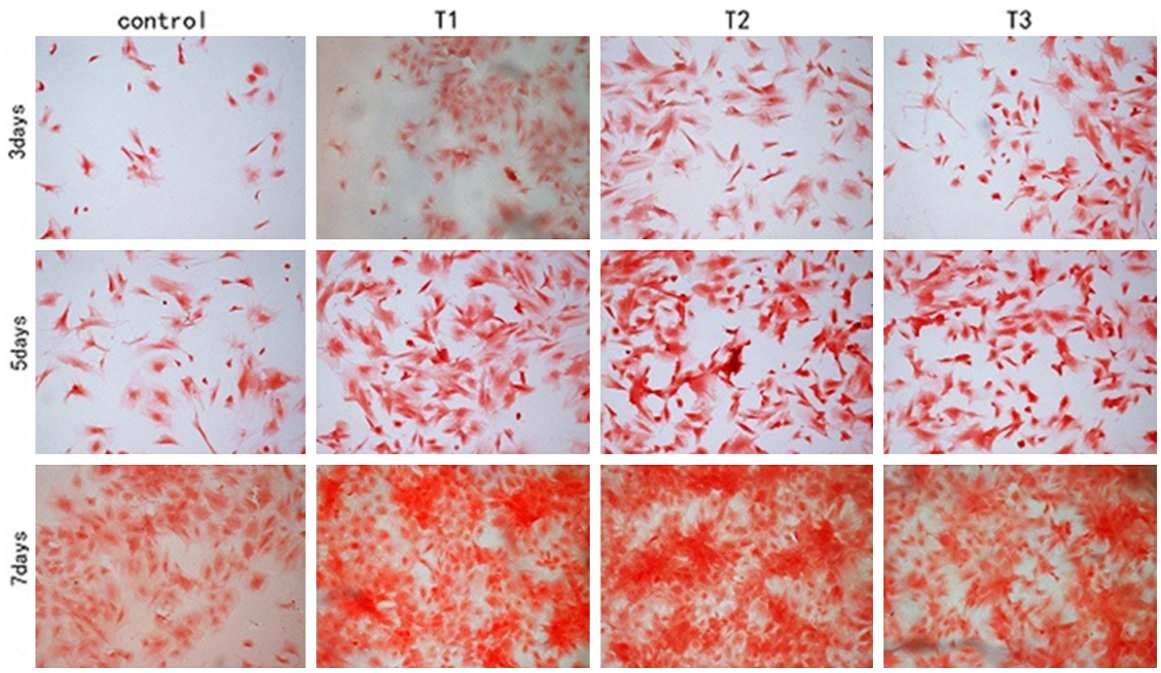

Figure 7. Alizarin red staining of osteoblastic cells cultured with various concentrations (0 μM (Control), 6.25 μM (T1), 12.5 μM (T2) and 25 μM (T3) *in vitro*) of taraxasterol for 3, 5 and 7 days. Cell seeding density: 4 × 10^3/mL (original magnification × 100).

were the highest in the 12.5 μM taraxasterol group.

Alizarin red staining

Alizarin red staining was used to determine the calcium content of the constructs. Our investigation revealed that bone-like nodules were formed in a time-dependent manner in all of the groups (**Figure 7**). Taraxasterol was effective in enhancing mineralization, as evidenced by the stronger expression of staining, especially at 12.5 μM.

Immunohistochemical assay

Further exploration of the expression of osteogenesis-related proteins was carried out by immunocytochemistry, with specific antibodies for COL-I (**Figure 8A-D**) and BMP-2 (**Figure 8E-H**). COL-I, a major specific marker of the bone matrix, is highly expressed during the entire process of bone formation [21]. BMP-2 is a key molecule in the early stages of bone healing [22, 23], and it has been widely used for bone tissue regeneration [24, 25]. Consistent with the real-time PCR findings, the result clearly shows that taraxasterol up-regulated the levels of COL-I and BMP-2, with stronger positive staining than the control, especially at a concentration of 12.5 μM. In all the groups, the expression of both COL-I and BMP-2 increased over time.

RT-qPCR analysis

The proliferation-enhancing effect of taraxasterol on osteoblastic cells was further examined with respect to the gene expression of BMP-2, COL-I, BSP, OCN, ALP, and RUNX2 after 3, 5, 7, and 14 days of culture. **Figure 9** shows that genes associated with osteogenic differentiation, including BMP-2, RUNX2, ALP, BSP, OCN, and COL-I, were all significantly up-regulated by taraxasterol treatment ($P < 0.05$), especially at a dose of 12.5 μM. The effect of taraxasterol on osteogenic differentiation was dose-dependent from 0 μM to 12.5 μM. However, at a concentration of 30 μM, slight down-regulation was seen for all of the genes, compared with those in the group of 12.5 μM taraxasterol. Among all of the osteogenesis-related genes, an exception was RUNX2, which peaked at day 14. The other genes showed a continuous increase during the culture period.

Discussion

Osteoporosis, a musculoskeletal disease, is characterized by compromised bone strength, leading to an increased risk of fracture and electrolyte imbalance [26]. It is a severe dis-

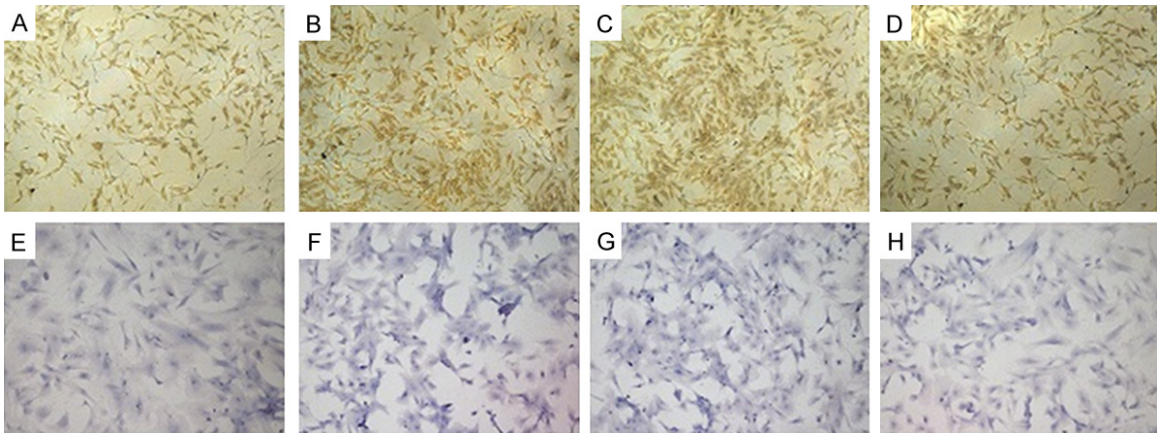

Figure 8. Immunohistochemical staining images revealed the presence of BMP-2 and Col I. Osteoblastic cells cultured *in vitro* with 0 μM (Control), 6.25 μM (T1), 12.5 μM (T2) and 25 μM (T3) taraxasterol for 7 days. Cell seeding density: 4 × 10^3/mL (original magnification × 100).

ease characterized by decreased bone mineral density and degraded bone fiber structure, which reduces the quality of life. Previous studies have shown that taraxasterol had anti-inflammatory, antioxidant, and protective effects [27]. In this study, osteogenic cells from rat calvaria bones were used to examine the effects of taraxasterol on osteogenesis, *in vitro*. Our study indicates that taraxasterol exhibits potent abilities to treat osteoporosis through enhancing osteoblastogenesis, and may serve as an appropriate pro-osteogenic agent.

In our study, we found that taraxasterol markedly up-regulates ALP activity (**Figure 6**) and COL-I (**Figure 8A-D**) during the culture period. The process of osteoblast maturation includes three main developmental stages: proliferation, development of the ECM, and mineralization [28]. ALP and COL-I are the most abundant elements in the osteoblast, which are generally increased during osteoblast differentiation [29]. COL-I comprises almost 90% of organic material during the formation of inorganic bone matrix to support the structure of osteogenesis [30, 31]. Among the groups, taraxasterol (6.25 μM, 12.5 μM, and 25 μM) markedly increased the cell viability, ALP activities, and alizarin red staining area, which indicated that taraxasterol promoted the proliferation, differentiation, and mineralization of osteogenic cells. Our study has provided more evidence that taraxasterol exhibits potent abilities to stimulate osteoblast differentiation.

In addition, we investigated the proliferation of osteoblast cells. The MTT results have shown that taraxasterol accelerates cell growth in a dose-dependent manner. Our data indicated that taraxasterol at a low concentration (< 12.5 μM) dramatically promotes proliferation of osteoblast cells (**Figure 2**). Oxidative damage is an important contributor to the morphological and functional changes in osteoporotic bone. Aging increases the levels of reactive oxygen species (ROS) that cause oxidative stress, and induce osteoblast apoptosis [32]. Being a potent antioxidant [33], taraxasterol is a major mediator for reducing the levels of excessive ROS, which are considered to contribute to several clinical symptoms [7, 8, 34]. Increased ROS with advancing age represents a pathophysiological mechanism underlying age-related (involutional) osteoporosis [8]. Previous studies have shown that treatment of taraxasterol significantly inhibited production of inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and MCP-1 [35, 36], and the protective effects of taraxasterol *in vivo* [37]. These findings are consistent with our results, indicating that taraxasterol, at a proper dosage, holds great promise for promoting the proliferation of osteoblasts in order to provide a promising cell reservoir, for bone regeneration therapy.

Furthermore, we also analyzed the mRNA levels of bone morphogenetic protein-2 (BMP-2), runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), bone sialoprotein (BSP), type I collagen (COL-I), and osteocalcin (OCN). Osteocalcin is a major protein produced by osteoblasts during bone formation. RUNX2, a master regulator essential for osteoblast development and osteoblast maturation, plays a cru-

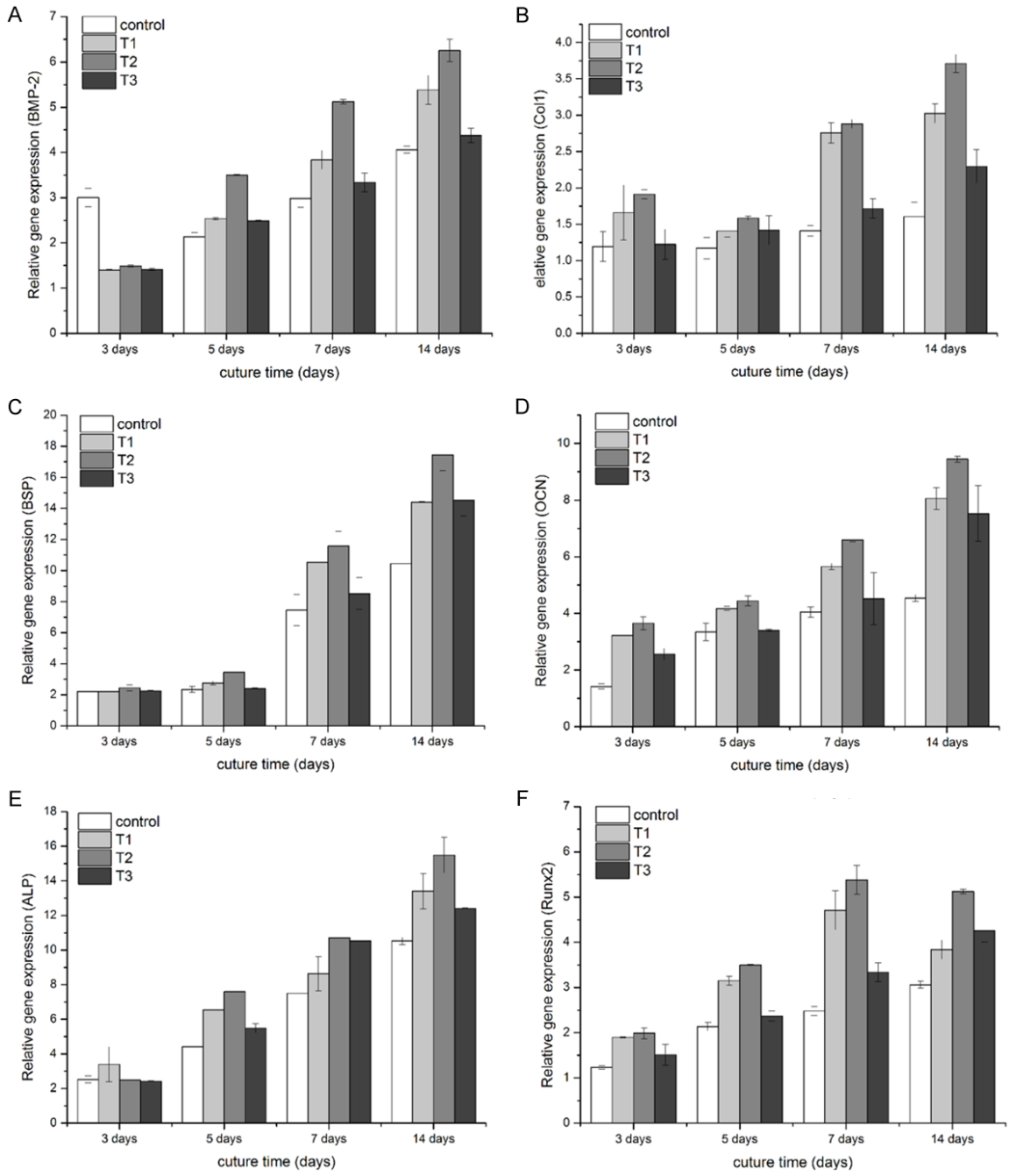

Figure 9. Quantitative comparison of neurotrophic-related gene expression by qRT-PCR. The taraxasterol cells were cultured with 0 μM (Control), 6.25 μM (T1), 12.5 μM (T2) and 25 μM (T3) taraxasterol for 3 days, 5 days, 7 days and 14 days. The gene expression levels in taraxasterol media relative to the control group were analyzed by the 2-ΔΔCt method using GAPDH as the internal control. The data represent the mean ± SD of three independent culture experiments.

cial role in the early stage of bone calcification, since it favors bone formation and calcification [38]. OCN expression is regulated by RUNX2 and osterix levels [39, 40]. The present study demonstrates that taraxasterol treatment of osteoblasts, after 14 days, increased RUNX2, BSP, and OCN gene expression to levels that were significantly higher than those of osteoblasts without taraxasterol treatment (**Figure 9**). Therefore, these data further demonstrate that taraxasterol exhibited the probable underlying mechanism for therapy of osteoporosis, through enhancing BMP-2, RUNX2, ALP, BSP, COL-I, and OCN expression.

Importantly, the results of real-time PCR, biochemical, and immunohistochemical assay, showed that expression of BMP-2, one of the major pathways for governing bone regeneration, was consistently increased (**Figures 8** and **9**). The probable underlying mechanism is that taraxasterol affects osteogenetic differentiation through BMP-2 signaling, which regulates RUNX2 expression. BMP-2 signaling has been reported to control the expression and function of RUNX2 through the BMP/Smad signaling pathway. ALP is a primary marker of osteoblast differentiation. In our results, ALP, COL-I, and BMP-2 immunohistology staining directly indicated an increase in bone matrix deposition over time, which was in agreement with RT-PCR data, particularly at 12.5 μM taraxasterol (**Figure 9B**, **9C**, and **9E**). This suggests that taraxasterol adjusts the whole process of osteogenic differentiation by controlling BMP-2 expression. Thus, our findings provide evidence that taraxasterol may have potential therapeutic value for treating bone metabolism disturbances in osteoblasts, such as osteoporosis.

In conclusion, taraxasterol has a regulatory effect on osteogenic function by increased ALP activity, upregulating expression of osteogenic genes, and promoting formation of bone-like nodules. This compound probably affects osteogenetic differentiation through the modulation of ALP, RUNX2, and BMP-2 expression. Moreover, taraxasterol has been found to promote the proliferation of osteoblasts, which may be related to its potential as an antioxidant, producing favorable amounts of ROS in the cellular environment. All the results indicate that taraxasterol is a novel and potent candidate agent for the treatment of osteoporosis. Of course, the mechanisms and clinical applications of taraxasterol need to be further elucidated to make it a practical agent for the therapy of osteoporosis.

Acknowledgements.

This study was supported by the Project of Administration of Traditional Chinese Medicine of Guizhou Province of China (QZYY-2016-074) and the National Natural Science Foundation of China (No. 81560356).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jin-Min Zhao, Guangxi Medical University, 22 Shuangyong Street, Nanning 530021, China, E-mail: myronlee2014@hotmail.com; Xiao-Bin Tian, Department of Orthopedics, Guizhou Provincial People's Hospital, 83 East of Zhongshan Road, Guiyang 550002, China. E-mail: txb6@vip.163.com

References

[1] Karsenty G and Wagner EF. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2002; 2: 389-406.

[2] Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest 2005; 115: 3318-3325.

[3] Jilka RL, Almeida M, Ambrogini E, Han L, Roberson PK, Weinstein RS and Manolagas SC. Decreased oxidative stress and greater bone anabolism in the aged, when compared to the young, murine skeleton with parathyroid hormone administration. Aging Cell 2010; 9: 851-867.

[4] Huang Q, Goh JC, Hutmacher DW and Lee EH. In vivo mesenchymal cell recruitment by a scaffold loaded with transforming growth factor beta1 and the potential for in situ chondrogenesis. Tissue Eng 2002; 8: 469-482.

[5] Jimbo R, Sawase T, Shibata Y, Hirata K, Hishikawa Y, Tanaka Y, Bessho K, Ikeda T and Atsuta M. Enhanced osseointegration by the chemotactic activity of plasma fibronectin for cellular fibronectin positive cells. Biomaterials 2007; 28: 3469-3477.

[6] Lee DH, Park BJ, Lee MS, Lee JW, Kim JK, Yang HC and Park JC. Chemotactic migration of human mesenchymal stem cells and MC3T3-E1 osteoblast-like cells induced by COS-7 cell line expressing rhBMP-7. Tissue Eng 2006; 12: 1577-1586.

[7] Martindale JL and Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 2002; 192: 1-15.

[8] Almeida M and O'Brien CA. Basic biology of skeletal aging: role of stress response pathways. J Gerontol A Biol Sci Med Sci 2013; 68: 1197-1208.

[9] Ben-Mordechai T, Holbova R, Landa-Rouben N, Harel-Adar T, Feinberg MS, Abd Elrahman I, Blum G, Epstein FH, Silman Z, Cohen S and Leor J. Macrophage subpopulations are essential for infarct repair with and without stem cell therapy. J Am Coll Cardiol 2013; 62: 1890-1901.

[10] Crupi A, Costa A, Tarnok A, Melzer S and Teodori L. Inflammation in tissue engineering: the Janus between engraftment and rejection. Eur J Immunol 2015; 45: 3222-3236.

[11] Brown BN, Sicari BM and Badylak SF. Rethinking regenerative medicine: a macrophage-centered approach. Front Immunol 2014; 5: 510.

[12] Mills CD. M1 and M2 Macrophages: oracles of health and disease. Crit Rev Immunol 2012; 32: 463-488.
[13] Sica A and Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 2012; 122: 787-795.
[14] Zhao H, Zhang T, Xia C, Shi L, Wang S, Zheng X, Hu T and Zhang B. Berberine ameliorates cartilage degeneration in interleukin-1beta-stimulated rat chondrocytes and in a rat model of osteoarthritis via Akt signalling. J Cell Mol Med 2014; 18: 283-292.
[15] Sun JL, Jiao K, Niu LN, Jiao Y, Song Q, Shen LJ, Tay FR and Chen JH. Intrafibrillar silicified collagen scaffold modulates monocyte to promote cell homing, angiogenesis and bone regeneration. Biomaterials 2017; 113: 203-216.
[16] Boehler RM, Graham JG and Shea LD. Tissue engineering tools for modulation of the immune response. Biotechniques 2011; 51: 239-240, 242, 244 passim.
[17] Xiong H, Cheng Y and Zhang X. Effects of taraxasterol on iNOS and COX-2 expression in LPS-induced RAW 264.7 macrophages. J Ethnopharmacol 2014; 155: 753-757.
[18] Piao T, Ma Z, Li X and Liu J. Taraxasterol inhibits IL-1beta-induced inflammatory response in human osteoarthritic chondrocytes. Eur J Pharmacol 2015; 756: 38-42.
[19] San Z, Fu Y, Li W, Zhou E, Li Y, Song X, Wang T, Tian Y, Wei Z, Yao M, Cao Y and Zhang N. Protective effect of taraxasterol on acute lung injury induced by lipopolysaccharide in mice. Int Immunopharmacol 2014; 19: 342-350.
[20] Zhang X, Xiong H and Liu L. Effects of taraxasterol on inflammatory responses in lipopolysaccharide-induced RAW 264.7 macrophages. J Ethnopharmacol 2012; 141: 206-211.
[21] Farea M, Husein A, Halim AS, Abdullah NA, Mokhtar KI, Lim CK, Berahim Z and Mokhtar K. Synergistic effects of chitosan scaffold and TGFbeta1 on the proliferation and osteogenic differentiation of dental pulp stem cells derived from human exfoliated deciduous teeth. Arch Oral Biol 2014; 59: 1400-1411.
[22] Nauth A, Ristiniemi J, McKee MD and Schemitsch EH. Bone morphogenetic proteins in open fractures: past, present, and future. Injury 2009; 40 Suppl 3: S27-31.
[23] Axelrad TW and Einhorn TA. Bone morphogenetic proteins in orthopaedic surgery. Cytokine Growth Factor Rev 2009; 20: 481-488.
[24] Bishop GB and Einhorn TA. Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. Int Orthop 2007; 31: 721-727.
[25] Haidar ZS, Hamdy RC and Tabrizian M. Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part B: Delivery systems for BMPs in orthopaedic and craniofacial tissue engineering. Biotechnol Lett 2009; 31: 1825-1835.
[26] Sambrook P and Cooper C. Osteoporosis. Lancet 2006; 367: 2010-2018.
[27] Zhang X, Xiong H, Li H and Cheng Y. Protective effect of taraxasterol against LPS-induced endotoxic shock by modulating inflammatory responses in mice. Immunopharmacol Immunotoxicol 2014; 36: 11-16.
[28] Aubin JE. Advances in the osteoblast lineage. Biochem Cell Biol 1998; 76: 899-910.
[29] Aubin JE. Regulation of osteoblast formation and function. Rev Endocr Metab Disord 2001; 2: 81-94.
[30] Ni P, Fu S, Fan M, Guo G, Shi S, Peng J, Luo F and Qian Z. Preparation of poly (ethylene glycol)/polylactide hybrid fibrous scaffolds for bone tissue engineering. Int J Nanomedicine 2011; 6: 3065-3075.
[31] Niu LN, Sun JQ, Li QH, Jiao K, Shen LJ, Wu D, Tay F and Chen JH. Intrafibrillar-silicified collagen scaffolds enhance the osteogenic capacity of human dental pulp stem cells. J Dent 2014; 42: 839-849.
[32] Lee HY, Chae HJ, Park SY and Kim JH. Porcine placenta hydrolysates enhance osteoblast differentiation through their antioxidant activity and effects on ER stress. BMC Complement Altern Med 2016; 16: 291.
[33] Sharma K and Zafar R. Optimization of methyl jasmonate and beta-cyclodextrin for enhanced production of taraxerol and taraxasterol in (Taraxacum officinale Weber) cultures. Plant Physiol Biochem 2016; 103: 24-30.
[34] Haigis MC and Yankner BA. The aging stress response. Mol Cell 2010; 40: 333-344.
[35] Kolaczkowska E and Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 2013; 13: 159-175.
[36] Likhitpanichkul M, Torre OM, Gruen J, Walter BA, Hecht AC and Iatridis JC. Do mechanical strain and TNF-alpha interact to amplify pro-inflammatory cytokine production in human annulus fibrosus cells? J Biomech 2016; 49: 1214-1220.
[37] Xueshibojie L, Duo Y, Tiejun W. Taraxasterol inhibits cigarette smoke-induced lung inflammation by inhibiting reactive oxygen species-induced TLR4 trafficking to lipid rafts. Eur J Pharmacol 2016; 789: 301-307.
[38] Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem 2006; 99: 1233-1239.
[39] Gersbach CA, Byers BA, Pavlath GK and Garcia AJ. Runx2/Cbfa1 stimulates transdifferentiation of primary skeletal myoblasts into a mineralizing osteoblastic phenotype. Exp Cell Res 2004; 300: 406-417.
[40] Hassan MQ, Javed A, Morasso MI, Karlin J, Montecino M, van Wijnen AJ, Stein GS, Stein JL and Lian JB. Dlx3 transcriptional regulation of osteoblast differentiation: temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to chromatin of the osteocalcin gene. Mol Cell Biol 2004; 24: 9248-9261.