BIO-BYWORD SCIENTIFIC PUBLISHING PTY LTD

Journal of Clinical and Nursing Research

Collagen Hydrolysate Gly-Pro-Hyp on Osteoblastic Proliferation and Differentiation of MC3T3-E1 Cells

Geng Min[1], Miao He[1], Liu Kaili[1], Han Yantao[2], Chen Xuehong[2], Wang Chunbo[2]

1. Department of Pharmacology, School of Pharmacy, Qingdao University, Qingdao, 266021, China

2. Department of Pharmacology, Medical College, Qingdao University, Qingdao, 266021, China

ARTICLE INFO

Article history:

Published online: 30th Nov, 2017

Corresponding author: Wang Chunbo, Department of Pharmacology, Medical College, E-mail: cbwang666@126.com

Foundation: The project supported by National Natural Science Foundation of China (81473384)

Key words:

Gly-Pro-Hyp

MC3T3-E1 cell

Differentiation

Alkaline phosphatase

ABSTRACT

Objectives: Bone formation and bone resorption continuously occur in bone tissue to prevent the accumulation of old bone, this being called bone remodeling. Osteoblasts especially play a crucial role in bone formation through the differentiation and proliferation. Therefore, in this study, we investigated the effects of collagen hydrolysate Glycine-Prolyl-Hydroxyproline (Gly-Pro-Hyp) on osteoblastic proliferation and differentiation in MC3T3-E1 cells. **Methods:** Four groups including control, Gly-Pro-Hyp 20 μM, 100 μM, 500 μM groups were set up in this study. Cells were cultured with blank control medium or Gly-Pro-Hyp of the different dosages for 24 h. CCK 8 assay was analyzed cell proliferation. Assay of Alkaline phosphatase (ALP)activity was analyzed osteoblast differentiation. The expression levels of ALP, Col 1, Runx 2 and Osterix in MC3T3-E1 cells were measured by Western blot. **Results:** The results indicated the treatment of Gly-Pro-Hyp promoted the proliferation of MC3T3-E1 cells and improved ALP activity. In addition, cells treated with Gly-Pro-Hyp significantly upregulated protein expression of ALP, type 1 collagen, runt-related transcription factor 2 and osterix. **Conclusion:** The results demonstrate that Gly-Pro-Hyp promote differentiation inducement and proliferation of MC3T3-E1 cells, therefore may help to elucidate the transcriptional mechanism of bone formation and possibly lead to the development of bone-forming drugs.

Introduction

Our skeleton is constantly updated about 10 % per year [1]. Osteoporosis is a disease resulting from decreased renewal of bone, which leads to less dense, lose strength and breakmore easily of skeleton [2]. With increasing age, there has been a significant reduction in bone mass due to tipping of this delicate balance towards increased resorption combined with decreased formation. Also, this can lead to net bone loss in the aging peoples that ultimately induces as osteoporosis [3]. The balance between bone formation and bone resorption is essential for maintaining bone homeostasis [4]. Osteoblast

differentiation, an importanprocess for its function, confers marked rigidity and strength to the bone while still maintaining some degree of elasticity. It is regulated by the action of key transcription factors, including runt-related transcription factor 2 (Runx 2) and osterix (Osx), accompanied by the increased expression of bone matrix proteins such as alkaline phosphatase (ALP) and type 1 collagen (Col 1). These osteoblast differentiation factors stimulate differentiation and lead to bone formation [5]. Recently, there has been an increasing interest in the utilization of natural extract in the treatment of diseases since they are basically safe and inexpensive. Moreover, numerous edible natural extracts are showing tremendous potential for the treatment of bone-related diseases. Collagen is the main extracellular matrix protein present mainly in skin and bone [6]. It is present in fibrous tissues, such as tendons and ligaments, as well as in cartilage, bones and blood vessels. Collagen has a unique triple-helix structure. Gelatin is a denatured form of collagen and collagen-derived peptides is formed by protease hydrolysis of this gelatin. Various di- or tripeptides are included in collagen-derived peptides. Furthermore, it is thought that collagen in living tissues is degraded into collagen peptides by various enzymes secreted by osteoclasts or osteoblasts during the process of bone metabolism [7-8].
Collagen-derived peptides can be derived not only from food but also from collagen inconnective tissues such as skin and bone. Several food-derived collagen oligopeptides wereidentified in human blood after oral ingestion of collagen-derived peptides [9-10]. Glycine-prolyl-hydroxyproline (Gly-Pro-Hyp) is the major constituent of collagen-derived peptides which remains within human blood after ingestion of collagen-derived peptides [11–12]. Gly-Pro-Hyp or hydroxyproline-containing peptides are hard to be hydrolyzed in vivo and mayplay important functions in target tissues [13]. It has been reported that Gly-Pro-Hyp affects the growth of fibroblasts and regulates the differentiation of chondrocytes [14-15]. A previous study has shown that oral administration of collagen-derived peptides increases bonemineral density in calcium-deficient rats [16]. Furthermore, some studies have indicated that crude collagen-derived peptide has a beneficial effect on the proliferation or differentiation of osteoblastic cells [17–19]. Therefore, collagen-derived peptides may also play an important role in bone remodeling. However, the role of specific sequences peptide including in collagen-derived peptides on bone metabolism is currently unclear. In this study, we investigated the effect of Gly-Pro-Hyp on the proliferation and differentiation of MC3T3-E1 osteoblastic cells.

1 Material and methods

1.1 Reagents and antibodies

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Hyclone; Fetal bovine serum (FBS) was purchased from Gibco; Cell counting kit (CCK 8) was obtained from Beyotime Biotechnology; Antibody against ALP, Col 1, Runx 2 and Osterix were all purchased from Elabscience; AKP kit assay measured the activity of ALP that was obtained from Nanjingjiancheng; BCA protein assay kit was obtained from Beyotime Biotechnology.

1.2 Cell cultures

MC3T3-E1 cell obtained from China Center for Type Culture Collection was used in vitro study. The mouse osteoblastic cell line MC3T3-E1 was cultured in Dulbecco's Modified Eagle Medium(DMEM) medium supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotics (100 U/mL penicillin and 100 U/mL streptomycin, pH adjusted to 7.6). Cell cultures were maintained at 37 ℃ in a humidified atmosphere of 5 % CO_2.

1.3 Assay of cell differentiation

MC3T3-E1 cells viability were determined using CCK 8 assay. Cells were seeded on a 96-well plate at an initial concentration of 5000 cells/well over night, then were treated with or without Gly-Pro-Hyp at the different concentration (20, 100 and 500 μM) for 24 h.Cultures were washed with PBS. CCK 8 testing solution (0.1 mg/mL) were then added to each well, and the mixture was incubated for 3h at 37 °C. The absorbance of each well was read using a microplate plates reader at a wavelength of 490 nm.

1.4 Assay of alkaline phosphatase (ALP) activity

Cells cultured on 96-well culture plates (1 x 10^4 cells/well) for 24 h, were treated with different concentrations (20, 100, 500 μM) of Gly-Pro-Hyp, including controls, for 24 h. Cells were cultured for an additional 48h. After incubation, cells were fixed with 20 % formalin for 20 min and incubated in 0.05 mol/L 2-amino-2-methyl-1-propanol (AMP) buffer (pH 9.8), containing 10 mM naphtol AS-BI phosphate and 1 mM fastred violet LB salt for 30 min at 37 ℃. The staining solution was aspirated and the cells were washed with deionized water [20]. We normalized the absorbance values to the total protein levels which were quantified by using a BCA kit (Pierce). And detected the absorbance at a test wavelength of 410 nm for the measure of ALP activity, analyzed qualitatively using Image J software.

1.5 Western blotting

MC3T3-E1 cells were plated on 60 mm dish, then washed with PBS after treatment Gly-Pro-Hyp (20, 100, and 500 μM) for 24 h, the concentration from each sample was determined using a BCA protein assay kit. After centrifuging for 20 min at 13500 rpm, equal amounts (30 μg- 50 μg) of proteins were separated by electrophoresis on 10 % sodium dodecyl sulphate polyacrylamide gels, and these separated protein bands were transferred to 0.45 μM PVDF membranes. The membranes were blocked with 5 % (w/v) skimmed milk powder in Tris-buffered saline containing 0.1 % (v/v) Tween-20 (PBST) for 2 h at room temperature. Then all the antibodies were probed with dilution at 1:1000 at 4 °C overnight. Primary antibodies including ALP, Col 1, Runx 2, Osterix and GAPDH . After washing with PBST, the membranes were incubated with the secondary anti-rabbit antibodies or anti-mouse IgG HRP-linked antibody at the concentration of 1:3000 for 1h at 37 ℃. After washing with PBST again, each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents. Band densities were determined by an image J analyzer and normalized to GAPDH for total protein and nuclear protein.

1.6 Statistical analysis

All data were represented as means ± standard deviation (SD). Statistical analysis was performed using the Student t-test for two-group comparison and the one-way ANOVA analysis for multiple-group comparison. Statistical tests were performed using the software SPSS 22.0. P-values of less than 0.05 were considered as significant.

2 Results

2.1 Gly-Pro-Hyp promotes osteoblast proliferation dose-dependently

We examined the effect of Gly-Pro-Hyp on the cell proliferation by the assay of CCK 8. Compared to control, the Gly-Pro-Hyp promoted osteoblast proliferation dose-dependently, with the greatest role at the dose of 500 μM after 24 h treatment ($P < 0.05$, Figure 1). Thus, these results indicated that Gly-Pro-Hyp promoted osteoblast proliferation.

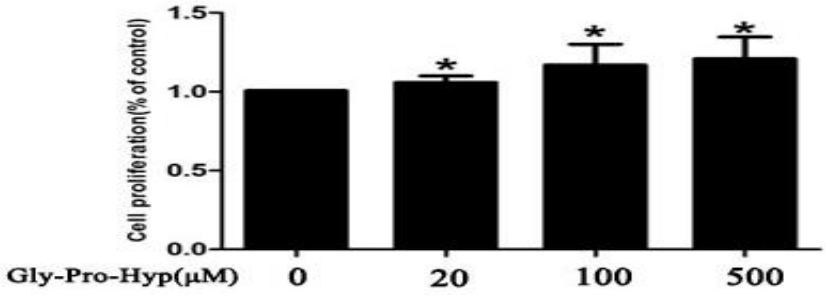

Figure 1 Effect of Gly-Pro-Hyp on the proliferation in MC3T3-E1 cells. CCK 8 assay showed that Gly-Pro-Hyp treated with 20, 100 and 500 μM for 24 h significantly promoted the proliferation. *P < 0.05, compared with control. Each value is expressed as mean ± SD (n = 5).

2.2 Gly-Pro-Hyp Enhances Osteoblast Differentiation Dose-dependently

We examined the effect of Gly-Pro-Hyp on ALP activity as a well-recognized marker of osteoblast differentiation. ALP activity was shown significantly increased after the treatment of Gly-Pro-Hyp in a dose-dependent manner, with a maximal role at a dose of 500 μM ($P < 0.05$) in MC3T3-E1 cells (Figure 2). These findings suggest that Gly-Pro-Hyp stimulates osteoblast differentiation.

2.3 Protein expression of ALP, Col 1 in MC3T3-E1 cells

The osteoblast-associated molecules ALP, Col 1 are considered osteoblast differentiation markers, as their expression levels increased during osteoblast differentiation. To investigate the effect of Gly-Pro-Hyp on osteoblast differentiation in MC3T3-E1 cells, protein levels of ALP, Col 1 were examined by western blot. The protein expression levels of ALP, Col 1 were significantly increased by Gly-Pro-Hyp treatment in a dose dependent manner (Figure 3). These results suggest that Gly-Pro-Hyp promotes osteogenesis by increasing markers of osteoblast differentiation.

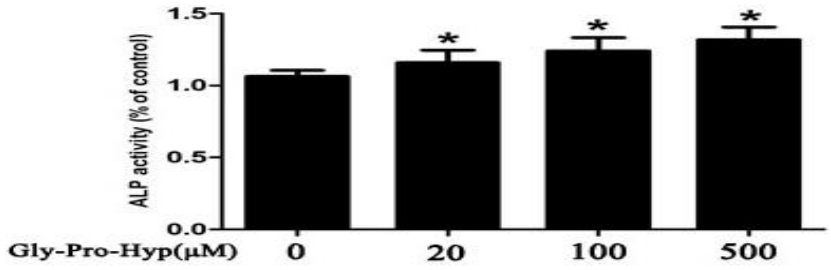

Figure 2 Effect of Gly-Pro-Hyp on alkaline phosphatase (ALP) activity in MC3T3-E1 cells. Cells were treated with Gly-Pro-Hyp at 20, 100 and 500 μM for 24 h significantly stimulated osteoblast differentiation dose-dependently. *P < 0.05, compared with control. Each value is expressed as mean ± SD (n = 5).

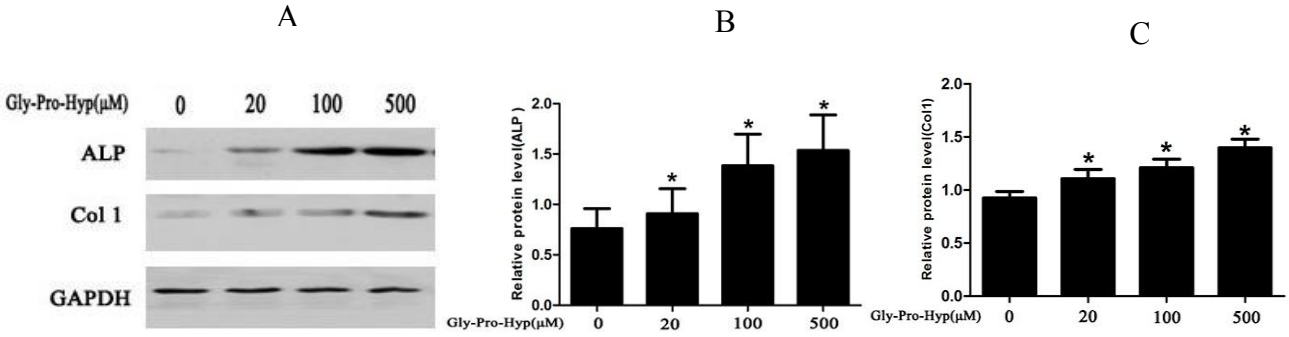

Figure 3 Effects of Gly-Pro-Hyp on osteoblast differentiation in MC3T3-E1 cells. Cells were treated with Gly-Pro-Hyp at 20, 100 and 500 μM for 24 h. The protein expression of osteoblast differentiation factors, such as ALP, Col 1 were detected by western blot. Each value is expressed as mean± SD (n = 3). GAPDH was used as loading control. *P < 0.05, compared with control.

2.4 Protein expression of Runx2, Osterix in MC3T3-E1 cells

To examine the effect of Gly-Pro-Hyp on osteoblastic gene expression in MC3T3-E1 cells, MC3T3-E1 cells were cultured with in the presence of 0, 20, 100 and 500 μM Gly-Pro-Hyp for 24 h and osteoblastic gene expression was determined by western blot. Runx 2 and Osterix protein expression levels were increased in a dose-dependent manner in the presence of Gly-Pro-Hyp ($p < 0.05$, Figure 4).

3 Discussion

Osteoporosis is a systemic skeletal disease characterized by decreased bone mass and loss of bone tissue that may lead to weak and fragile bones [21]. Because new bone formation is primarily mediated by osteoblast, agents that act to either increasing osteoblast proliferation or inducing osteoblast differentiation enhance bone formation [22]. A wide variety of natural materials with therapeutic effects on bone formation and skeleton construction have been reported and several enhance osteogenic differentiation [23]. A decrease in osteoblastic proliferation is an important factor in the pathogenesis of osteoporosis [24]. But the

effects of Collagen Hydrolysate Gly-Pro-Hyp on proliferation and differentiation of osteoblastic cells have not yet to be determined. Therefore, in the present study, we investigated the effect of Gly-Pro-Hyp on the proliferation and differentiation of MC3T3-E1 cells.The effect of Gly-Pro-Hyp on cell proliferation was assayed by using CCK 8 assay. As shown in figure 20, 100, 500 μM of the Gly-Pro-Hyp increased in MC3T3-E1 cell proliferation dose dependently. Thus, these results indicated that Gly-Pro-Hyp stimulated cell proliferation and had no cytotoxicity toward the cells.

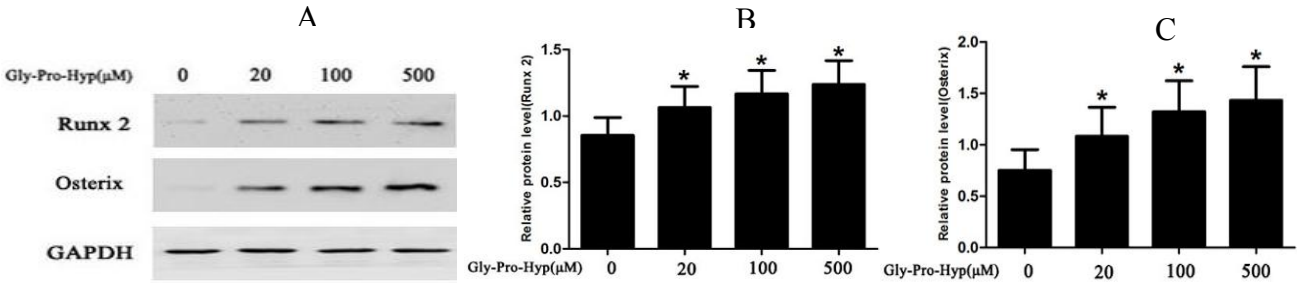

Figure 4 Effects of Gly-Pro-Hyp on Runx 2 and Osterix in MC3T3-E1 cells. Cells were treated with indicated concentrations (0, 25, 50 and 100 μM) of Gly-Pro-Hyp for 24 h. The protein expression levels of Runx 2 and Osterix were determined by western blot analysis. GAPDH was used as loading control. *p < 0.05, indicates the significant difference compared with control group.

Therefore, further studies are necessary to investigate the effects of other specific sequence peptides included in collagen-derived peptides on the proliferation of osteoblasts. During the osteoblast differentiation phase, osteoblasts synthesize type 1 collagen, ALP, and various non-collagen proteins that are associated with matrix mineralization. ALP is a marker enzyme of early-stage osteoblast differentiation [25–26]. We revealed that Gly-Pro-Hyp could significantly increase ALP activity in MC3T3-E1 cells compared with the control. This result suggests that Gly-Pro-Hyp enhanced osteoblast proliferation and differentiation.

In addition, we examined the effects of Gly-Pro-Hyp on the regulation of osteoblast associated genes. Runx 2 is a transcription factor that regulates the differentiation of osteoblasts and the expression of Col 1 and other extracellular matrix protein genes during bone formation [27]. Osterix is also an essential transcription factor for osteoblast differentiation [28]. We found Gly-Pro-Hyp significantly up-regulated Runx 2, Osterix and Col 1 protein levels in MC3T3- E1 cells compared with the control. Furthermore, Runx 2 and Osterix protein levels were significantly increased with addition of lower concentrations of Gly-Pro-Hyp. These results indicate that Gly-Pro-Hyp can promote the differentiation of osteoblasts by upregulating gene expression of Runx 2 and Osterix. Previous reports have shown that treatment with crude Collagen-derived peptide increases the expression of the Runx 2 and the Col 1 genes in osteoblastic cells [29-30]. The regulation by Gly-Pro-Hyp in MC3T3-E1 cells would explain a mechanism of the protective effect of collagen-derived peptides in bone metabolism.

In conclusion, this study demonstrates for the first time that Gly-Pro-Hyp, food-derived collagen hydrolysate has a positive effect on osteoblast differentiation. These results indicate that bone formation is modulated by collagen-derived peptides from oral ingestion (exogenous) or bone metabolites (endogenous). These findings may indicate the potential utility of Gly-Pro-Hyp as a food supplement or biomaterial in bone health. In this experiment Gly-Pro-

Hyp also could improve the proliferation and differentiation of MC3T3-E1 cell systems, and could be developed as a promising drug with little toxicity to regulate bone metabolism. But the current research is still in the initial stage which could to be further explored.

References

[1] Michaelsson K, Melhus H, Ferm H, et al. Genetic liability to fractures in the elderl [J]. Arch Intern Med, 2005, 165: 1825-30.

[2] Ivanova S, Vasileva L. Current and emerging strategies in osteoporosis management [J]. Curr Pharm Des, 2017, 14: 2174.

[3] Riggs BL, Khosla S, Melton LJ. Sex steroids and the construction and conservation of the adult skeleton [J]. Endocr Rev, 2002, 23: 279-302.

[4] Isomura H, Fujie K, Shibata K, et al. Bone metabolism and oxidative stress in postmenopausal rats with iron overload [J]. Toxicology, 2004, 197: 93-100.

[5] Yoon HJ, Seo CR, Kim M, et al. Dichloromethane extracts of Sophora japonica L. stimulate osteoblast differentiation in mesenchymal stem cells [J]. Nutr Res, 2013, 33: 1053-62.

[6] Kamranpour NO, Miri AK, James-Bhasin M, et al. A gel aspiration-ejection system for the controlled production and delivery of injectable dense collagen scaffolds [J]. Biofabrication, 2016, 22, 8(1): 015018.

[7] Deng Y, Wu A, Li P, et al. Yap1 Regulates Multiple Steps of Chondrocyte Differentiation during Skeletal Development and Bone Repair [J]. Cell Rep, 2016, 14(9): 2224-37.

[8] Ortega N, Behonick DJ, Werb Z. Matrix remodeling during endochondral ossificatio [J].Trends Cell Biol, 2004, 2: 86–93.

[9] Ichikawa S, Morifuji M, Ohara H, et al. Hydroxyproline-containing dipeptides and tripeptides quantified at high concentration in human blood after oral administration of gelatin hydrolysate [J]. Food Sci. Nutr, 2010, 61: 52–60.

[10] Sugihara F, Inoue N, Kuwamori M, et al. Quantification of hydroxyprolylglycine (Hyp-Gly) in human blood after ingestion of collagen hydrolysate [J]. Biosci Bioeng, 2012, 113: 202–203.

[11] Iwai K, Hasegawa T, Taguchi Y, et al. Identification of food-derived peptides in human blood after oral ingestion of gelatin hydrolysates [J]. Agric. Food Chem, 2005, 53: 6531–6536.

[12] Ohara H, Matsumoto H, Ito K, et al. Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources [J]. Agric. Food Chem, 2007, 55: 1532–1535.

[13] Liu C, Sugita K, Nihei K, et al. Absorption of hydroxyproline containing peptides invascularly perfused rat small intestine in situ [J]. Biosci Biotechnol, 2009, 73: 1741–1747.

[14] Suqihara F, Inoue N, Kuwamori M, et al. Quantification of hydroxyprolyl-glycine (Hyp-Gly) in human blood after ingestion of collagen hydrolysate [J]. Biosci Bioeng, 2012, 113(2): 202-3.

[15] Misato Y, Yukihiko I, Masayoshi Y, et al. Oral Ingestion of Collagen Hydrolysate Leads to the Transportation of Highly Concentrated Gly-Pro-Hyp and Its Hydrolyzed Form of Pro-Hyp into the Bloodstream and Skin [J]. Agric Food Chem, 2017, 65: 2315−2322.

[16] Wu J, Fujioka M, Sugimoto K, et al. Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats [J]. Bone Miner. Metab, 2004, 22: 547–553.

[17] Yamada S, Nagaoka H, Terajim M, et al. Effects of fish collagen peptides on collagen post-translational modifications and mineralization in an osteoblastic cell culture syste [J]. Dent. Mater, 2013, 32: 88–95.

[18] Liu J, Zhang B, Song S, et al. Bovine collagen peptides compounds promote the proliferation and differentiation of MC3T3-E1 pre-osteoblasts [J]. PLoS

One, 2014, 9: e99920.
[19] Kim HK, Kim MG, Leem KH. Osteogenic activity of collagen peptide via ERK/MAPK pathway mediated boosting of collagen synthesis and its therapeutic efficacy in osteoporotic bone by back-scattered electron imaging and microarchitecture analysis [J]. Molecules, 2013, 18: 15474–15489.
[20] Mano H, Nakatani S, Aoyagi R, et al. IF3, a novel cell-differentiation factor, highlyexpressed in murine liver and ovary [J]. Biochem Biophys, 2002, 297: 323–328.
[21] Gong B, Mandair GS, Wehrli FW, et al. Novel assessment tools for osteoporosis diagnosis and treatment [J]. Curr Osteoporos Rep, 2014, 12(3): 357-65.
[22] Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance [J]. Science, 2000, 289: 1501-4.
[23] Hsieh TP, Sheu SY, Sun JS, et al. Icariin isolated from Epimedium pubescens regulates osteoblasts anabolism through BMP-2, SMAD4, and Cbfa1 expression [J]. Phytomedicine, 2010, 17: 414-23.
[24] Bai XC, Lu D, Bai J, et al. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappaB [J]. Biochem Biophys, 2004, 314:197-207.
[25] Hu B, Yu B, Tang D, et al. Daidzein promotes osteoblast proliferation and differentiation in OCT1 cells through stimulating the activation of BMP-2/Smads pathway[J]. Genetics and Molecular Research, 2016, 15 (2): 1-11.
[26] Don MJ, Lin LC, Chiou WF. Neobavaisoflavone stimulates osteogenesis via p38-mediated up-regulation of transcription factors and osteoid genes expression in MC3T3-E1 cells[J]. Phytomedicine, 2012, 19: 551–561.
[27] Komori T. Regulation of bone development and extracellular matrix protein genes by Runx 2 [J]. Cell Tissue Res, 2010, 339: 189–195.
[28] Lee YJ, Park SY, Lee SJ, et al. Ucma, a direct transcriptional target of Runx2 and Osterix, promotes osteoblastdifferentiation and nodule formation[J]. Osteoarthritis Cartilage, 2015, 23(8):1421-31.
[29] Liu J, Zhang B, Song S, et al. Bovine collagen peptides compounds promote theproliferation and differentiation of MC3T3-E1 pre-osteoblasts[J]. PloS One, 2014, 9(6): e99920.
[30] Shimizu K, Sato M, Zhang Y, et al. Molecular size of collagen peptide reverses the permeability of Caco-2 cells [J]. Biosci Biotechnol Biochem, 2010, 74(5): 1123-5.