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# Peptide TQS169 prevents osteoporosis in rats by enhancing osteogenic differentiation and calcium absorption

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ABSTRACT

The preventive effects of TQS169 peptide on osteoporosis were investigated and the mechanisms of promoting osteogenic differentiation and calcium absorption were also studied *in vitro* and *vivo* in this research. Wistar rats were given a low-Calcium diet treated with different doses of peptide TQS169 and extra $CaCl_2$ for 4 weeks. The content of Calcium, OPG in serum and the Ca content of the femur were increased in high-dose-treated rats. The osteoporosis model induced by retinoic acid was established to measure the related factors. High dosage of TQS169 treated group showed the protective effects. Osteoblastogenesis differentiation of MC3T3-E1 cells treated with TQS169 were accelerated through improving ALP activity, promoting mineral matrix formation and the expression of typical genes of RUNX2, OCN and COL I. Taking all the experiments together, our study confirms the protective effects of TQS169 on osteoporosis and explains the potential mechanism for the first time.

## 1. Introduction

Osteoporosis is an elderly disease that is characterized by massive decreases of bone mineral density (BMD) and the increases of bone fragility, fractures and ostealgia (Kanis, Melton, Christiansen, Johnston, & Khaltaev, 1994). BMD is involved in the formation of new bone and enough bone volume and quality are extremely important for the maturation and functions (Ammann & Rizzoli, 2003). Many researches have indicated that adequate calcium and phosphorus are required to alter the bone remodeling and the formation of bone mineral matrix (Bisson et al., 2018; Fujita, Goto, Ichikawa, Hamaguchi, & Maki, 2016; Kasten et al., 2003; Libouban & Chappard, 2017). When the balance between bone formation and resorption is destroyed, bone metabolic diseases merges (Riggs & Melton, 1992). The bone metabolism on the differentiation of osteoblasts or osteoclasts is accounting for the balance, and the improvement in the osteoblasts differentiation and the inhibition in that of the osteoclasts are crucial for osteoporosis prevention (Al Mamun, Hosen, Khatun, Alam, & Al-Bari, 2017; Kim et al., 2018; Kono et al., 2011; Ying et al., 2014). Retinoic acid can suppress the proliferation of pre-osteoblasts and inhibit the mineralization, and furtherly induce bone resorption and osteoporosis (Saneshige et al., 1995). It is a classic model for anti-osteoporosis activity evaluations, as well as the low-calcium diet model (Fujita et al., 2016; Libouban & Chappard, 2017; Uenishi, Tokiwa, Kato, & Shiraki, 2018). Furthermore, genes such as alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), were related to early osteogenic differentiation while osteocalcin (OCN) and collagen type I (COL I) are often selected to studied the late differentiation in MC3T3-E1 cells, a kind of pre-osteoblasts (Born, Lischer, & Maniura-Weber, 2012; Pratap et al., 2003; Reinholz et al., 2000), while the activity of artrate resistant acid phosphatase (TRAP) as a main marker for the function of osteoclasts (Ueda, Yamazaki, & Yamazaki, 2002).

Ossotides, complex peptides derived from the bone of different animals, can be orally taken as a functional food for anti-oxidant, anti-hypertensive, anti-bacterial, anti-tumor and immunomodulatory improvement (Zhang, Wang, Liu, Xu, & Zhou, 2012). The ossotide derived from fish (fish peptide) can play a positive role in calcium absorption and has been widely used for in fracture healing and osteoporosis (Sun et al., 2016), but the mechanism of whether ossotide promotes the proliferation and differentiation of osteoblasts remains uncertain.

In this study, a new kind product of collagen peptide called peptide TQS169 obtained from bovine through more ecofriendly processes

*Abbreviations:* ALP, alkaline phosphatase; BMD, bone mineral density; COL I, collagen type I; OCN, osteocalcin; OPG, osteoprotegerin; RUNX2, runt-related transcription factor 2; TGF-β, transforming growth factor-β; TRAP, artrate resistant acid phosphatase

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without residue of extracting agents with different amino acid composition was used to study the calcium absorption promotion activities in a low-calcium diet, as well as the anti-osteoporotic effects on osteoporosis rats induced by retinoic acid. The purity of TQS169 reached 95% while peptide molecular weight distribution below 5000D accounts for more than 90%. BMD, weight of femur, calcium content, and some osteogenic differentiation associated factors of ALP, osteoprotegerin (OPG), transforming growth factor-β (TGF-β) and TRAP in the serum were investigated. Finally, we also analyzed the differentiation factors in MC3T3-E1cells such as COL I, OCN and RUNX2 at the RNA level by RT-PCR analysis and at the protein level by western blot analysis after treatment with TQS169. These findings provide a molecular mechanism for the potential treatment of TQS169 on osteoporosis as a food therapy.

It's our work that first demonstrates TQS169 derived from bovine bones has positive and protective effects on bone formation against osteoporosis. And we highlight TQS169 could improve the life quality of patients with osteoporosis.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

Peptide TQS169 was provided by TianQi Pharmacy Co. Ltd., (Chifeng, Neimenggu, China). Retinoic acid was purchased from Dalian Meilun Biotechnology Co. Ltd., (Dalian, China). Alpha modification of Eagle's minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from (Hyclone, Logan, USA). β-glycerophosphate, L-ascorbic acid and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against OCN, Collagen I, and β-actin were purchased from Cloud-Clone Corp. (Katy, TX, USA) and anti-Runx2 antibody from Cell Signaling Technology (Danvers, MA, USA). A secondary antibody linked with horseradish peroxidase was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu Province, China).

### 2.2. The prevention of osteoporosis in vivo

Animals were all offered by the Center of Experimental Animals, Fudan University (Shanghai, China). The animal care and animal experimentations were performed according to the Guide for the Care and Use of Laboratory Animals. The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Fudan University with permission number 2015000521984.

#### 2.2.1. Low-Calcium diet model

Male Wistar rats (200 ± 20 g) were divided into normal group (6 rats), control group (6 rats) and three experimental groups (11 rats each) ad libitum. The control and experimental groups were fed with a low-Calcium diet (0.05% Ca, Slac Laboratory Animal co., Ltd, Shanghai), while the normal group was fed with a normal diet (0.5%Ca). TQS169 suspended in deionized water was administrated orally every day at a dose of 0.5, 1 and 2 g/kg, as well as an extra $CaCl_2$ solution at a dose of 10 mg/kg. Same volume of deionized water and $CaCl_2$ solution were orally administrated to control group every day. Same total volume of deionized water was administrated to normal group. The dosing was adjusted according to the daily weight conditions. After 4 weeks the rats were sacrificed, and the serums and femurs were collected.

#### 2.2.2. Retinoic Acid-induced osteoporosis model

Female Sprague-Dawley rats (200 ± 20 g) were orally administrated retinoic acid (70 mg/kg) once daily for two weeks to induce osteoporosis. 24 rats were randomly divided into control group and three experimental groups (6 rats each group), with daily oral administration of CMC-Na solution, 3 g/kg, 6 g/kg, and 12 g/kg of TQS169 for another 14 days, respectively. In addition, another 6 healthy rats were defined as the normal group and administrated orally with CMC-Na solution once daily.

#### 2.2.3. ALP, OPG, and TGF-β analysis

The contents of ALP, OPG, TGF-β in the serum were measured with corresponding kits (Jiancheng Bioengineering Institute, Nanjing, China). Alkaline phosphatase decomposes disodium phosphate and produces free phenol and phosphoric acid. Phenol reacts with 4-aminoantipyrine in alkaline solution. Potassium ferricyanide oxidizes to red oxime derivative. The enzyme activity can be measured according to the depth of red color. OPG, TGF-β content were measured with Elisa kits.

#### 2.2.4. TRAP, OCN, and estradiol expression assay by ELISA

The content of TRAP, estradiol and OCN in the serum were determined by ELISA kits (Jianglai Biological Technology Co. Ltd., Shanghai, China) according to the protocols. The principle of the enzyme-linked immunosorbent assay (sandwich technique) is to bind a specific antibody to a solid-phase support to form a solid-phase antibody, which is then combined with the corresponding antigen in the test serum to form an immune complex.

#### 2.2.5. Femur condition detection

Bilateral femur bones were collected, and the length and wet weight were recorded. Then the left femur was burned in a muffle roaster at 800 °C for 8 h after air dry. The remaining weight was recognized as dry weight. BMD of the right mid-diaphysis femur was tested by a dual energy X-ray bone densitometer (GE Healthcare, Pittsburgh, USA).

#### 2.2.6. Bone mineralization assay

The ashes (0.1 g) of left femur were dissolved in 1 ml HCl (6 mol/L). 10ul solution was used to determine the contents of calcium and 200ul solution to determine the contents of phosphorus in the femurs using inorganic calcium and phosphorus assay kits (Jiancheng Bioengineering Institute, Nanjing, China). The calcium ions in the sample are combined with methylthymol blue (MTB) in an alkaline solution to generate a blue complex; the calcium content of the sample can be calculated by comparing the colorimetric with the same calcium standard at 660 nm.

#### 2.2.7. Histological analysis

Right femoral bones were collected to detect the bone histopathology by hematoxylin–eosin (HE) staining. Briefly, the bone samples were fixed in 4% paraformaldehyde for 24 h and then put into 10% ethylene diamine tetraacetic acid (EDTA) solution at 4 °C for 4 weeks for decalcification. After trimmed and embedded in paraffin, the femur samples were then sliced at 4 um of each section, and stained with hematoxylin and eosin, and examined by a light microscope (Teelen, Shanghai, China).

### 2.3. The osteogenic differentiation in MC3T3-E1 cells

#### 2.3.1. Cell Culture

MC3T3-E1 subclone in 14 cells (Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in α-MEM supplemented with antibiotics (100 units/mL penicillin A and 100 μg/mL streptomycin) and 10% FBS in an atmosphere of 5% $CO_2$ at 37 °C. Cells were induced to differentiate in maintenance medium with 10 mM β-glycerophosphate and 50 μM L-ascorbic acid. The medium was changed every three days. Cells with a final concentration of $1 \times 10^4$ cells/well in 96-well plates was plated

**Table 1**
Primer sequences for RT-qPCR analysis of the target genes.

| Gene | Forward (5′-3′) | Reverse (5′-3′) |
|---|---|---|
| COL I | ATGCCGCGACCTCAAGATG | TGAGGCACAGACGGCTGAGTA |
| OCN | AGCAGCTTGGCCCAGACCTA | TCGTCGAACCGGGTCTGGAT |
| Runx2 | CACTGGCGGTGCAACAAGA | TTTCATAACAGCGGAGGCATTTC |
| β-actin | AGGAGCAATGATCTTGATCTT | TCCTCGTTACTAGAACTAGAA |

for the cell proliferation assay, $2 \times 10^5$ cells/well in 6-well plates for PCR analysis and ALP activity assay, and $3 \times 10^6$ cells/well in 6-well plates for alizarin red staining.

#### 2.3.2. Cell viability and proliferation assay

The effects of TQS169 on the viability and proliferation of MC3T3-E1 cells were investigated by the MTT assay. After incubation with various concentrations ($1{,}10^{-1}$-$10^{-4}$mg/ml) of TQS169 for 1,7 days, and 10 uL MTT solution (0.5 mg/mL) was added for 4 h at 37 °C. Then the supernatant was removed and 150 μL of dimethyl sulfoxide (DMSO) was added into each well. The optical density was recorded at the wavelength of 570 nm by microplate reader (Thermo Fisher Scientific Oy Ratastie 2, FI-01620 Vantaa, Finland).

#### 2.3.3. Alizarin red staining

Extracellular matrix mineralization deposits are crucial for bone nodule formation, the mineralization possibility of MC3T3-E1 cells induced by TQS169 was observed with alizarin red staining. Cells were cultured in osteogenic medium (10% FBS, 50 mM L-ascorbic acid, and 10 mM β-glycerophosphate) containing different concentration of TQS169 for 14 days. The cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 min at room temperature, and rinsed with deionized water. Mineralized nodules were detected by alizarin red staining kit (Yuanye biological technology, Shanghai, China). Culture plates were photographed by a phase contrast microscope with a digital camera (IM50, Leica, Jena, Germany).

#### 2.3.4. RT-qPCR analysis of typical Bone-related gene expressions

On the 4th day, the total RNA was extracted, and reversely transcribed into cDNA using EZ-press cell to Ct Kit (EZ Bioscience, Roseville, USA) following the manufacturer's instructions. RT-qPCR was performed with SYBR premix (PrimeScriptTM RT Master Mix, Takara, China). The specific primers (Sangon Biotech Co. Ltd., Shanghai, China) used for determining mRNA transcripts of Col, OCN, Runx2, and β-actin gene are shown in Table 1. Transcripts were normalized by the β-actin transcript levels and the relative quantity of each gene over β-actin was calculated according to the delt-delt-Ct method.

#### 2.3.5. Western blot analysis

To assess the terminal differentiation of pre-osteoblasts, the protein levels of COL I, OCN, and Runx2 in MC3T3-E1 cells were also determined. Cells were rinsed with PBS after incubation with different concentrations of TQS169 ($1{,}10^{-1}$-$10^{-4}$mg/ml) for 7 days and for 4,7,14 days with 1 mg/ml TQS169. Cells were lysed by RIPA buffer (Beyotime, shanghai, China) on ice for 30 min. The lysates were centrifuged at 4829*g* at 4 °C for 10 min, and the supernatant was collected. The protein concentration was then quantized with bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, China). 20 μg total protein of each sample was subjected to western blot analysis of OCN, Collagen I, and Runx2, with β-actin as an internal control. The membranes were detected with enhanced chemiluminescence reagents (Beyotime Institute of Biotechnology, China) and the pictures were captured using Bio-rad ChemiDoc™ XRS$^+$ (Bio-rad, Hercules, USA).

### 2.4. Statistical analysis

Results are expressed as means ± S.D. of the indicated number of independent experiments. Statistical analyses were performed using SPSS-10.0 software (SPSS Inc., Chicago, IL, USA) by analysis of variance (ANOVA) and Student's *t*-test. The level of significance was set at $p < 0.05$.

## 3. Results

### 3.1. TQS169 can promote the absorption of Ca in rats with a low-calcium diet

At every beginning of a week, the body weights were recorded (Fig. 1A). Compared with the normal group, the body weights of the groups with a low-calcium diet increased more slowly. Only rats treated with high dose (2 g/kg) of TQS169 had higher weights than control group when observed at 4 weeks, though low dose and middle dose of TQS169 brought about 12% and 16% gains of weights compared to control group after 2 weeks. The results indicated that TQS169 could improve the body weight under the low-calcium diet and might be a nutrition source.

The calcium contents of the serums (Fig. 1B) and the bones (Fig. 1C) were also analyzed, the control group with much lower calcium content than those of the normal groups indicated a successful modelling. In treated groups, the bone calcium contents increased in dose dependence and the high dose group obviously increased by 30% compared to that of control group ($p < 0.05$). At the same time, TQS169 also improved the calcium contents in serum significantly, but in a reverse increasing trend, that the low group had a highest concentration which was 3 times higher than the control group. It indicated that TQS169 promoted the calcium absorption from foods and the calcium deposition from serum to bone. But TQS169 had no significant effects on phosphorus absorption (Supplementary Materials Fig. S1) and femoral indexes (Supplementary Materials Fig. S2). Moreover, the osteogenic differentiation associated proteins of OPG (Fig. 1D) and *TGF-β* (Fig. 1E) in serum were also analyzed. Both of these two factors were increased in dose dependence and there were significant differences between the control and the high dose groups, even with 20% higher in TGF-β contents.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2018.08.021.

Taken together, TQS169 can promote the absorption of calcium in rats with low-calcium diet. It illustrated that TQS169 influences the growth of bone and improves the osteogenesis *in vivo*.

### 3.2. TQS169 prevents osteoporosis induced by retinoic acid in vivo

The further functions of TQS169 in preventing osteoporosis were studied in retinoic acid induced rats. As is showed in Fig. 2A, After the 4-week treatment, the body weights of the middle and high dose groups were significant higher than that of the control group, even slightly higher than the normal group. The calcium contents of the femurs in high dose group were significantly increased by 30% compared with control group ($p < 0.05$) (Fig. 2B) and the levels of phosphorus (Fig. 2C) have no significant differences across the board. These results are in consistent with the previous results from the low-calcium diet model.

As for BMD, recognized as the key standard of osteoporosis, was greatly reduced in the control group than that in the normal group,

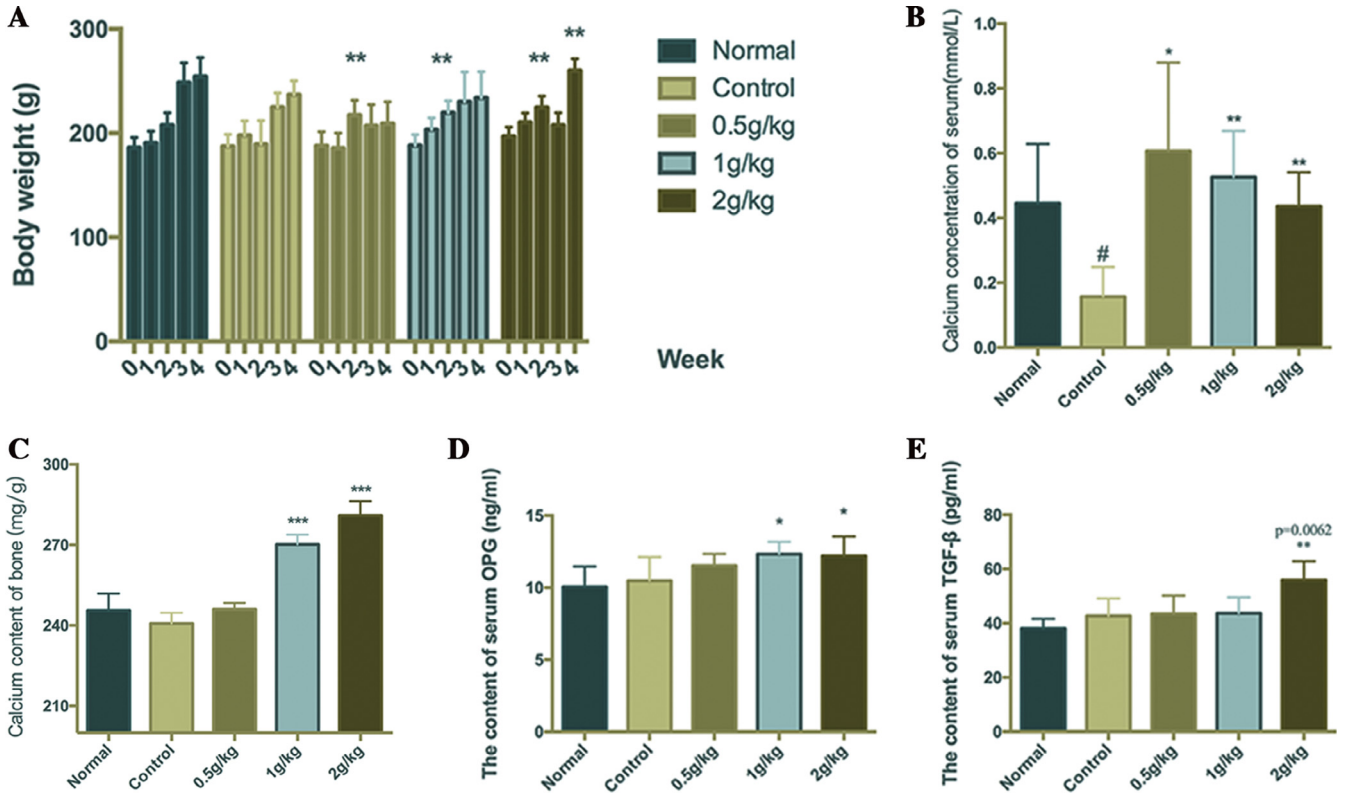


**Fig. 1.** Ossotide can promote the absorption of calcium in rats with a low-calcium diet. (A) The body weights of TQS169-treated rats were measured every week. (B) Rats were euthanized after being administrated different concentration of TQS169 for 4 weeks and the serum was collected to measure the content of calcium. (C) The calcium content of bone was analyzed by inorganic calcium assay kit as described in Materials and Methods. (D) The serum from rats was collected to measure the content of OPG. High dose of TQS169 can increase level of expressed OPG significantly. ($P = 0.046$). (E) The TGF-β content of serum in rats from all group were measured with inferred ELISA Kit. ($P = 0.0062$). Results were represented as mean ± SD ($^{*}p < 0.05$ compared with control group, # $p < 0.05$ compared with normal group, $^{**}P < 0.01$, $^{***}P < 0.001$).

which means the osteoporosis model was established successfully by retinoic acid (Fig. 2D). The rats treated with high dose TQS169 had a much higher BMD compared with rats in the control group, which explains the protective effect of TQS169 on osteoporosis progression. The dry weight analysis of the femurs also showed that both of the middle and the high dose could convert the bone weight decrease induced by retinoic acid (Fig. 2E). Furthermore, HE staining showed that the control group had fewer scattered bone trabeculae, while obviously wider and more trabeculae were found in the TQS169 treated groups with a dose dependence (Fig. 3).

Decreased ALP (Fig. 4A) and increased TRAP (Fig. 4B) activities in the serum showed that the bone absorption had been activated while the bone formation had been inhibited, which was induced by retinoic acid. In contrast, ALP ascending and TRAP falling were definitely founded in the TQS169 treated groups with a dose-dependent manner, which indicated a modification of the disrupted balance of bone formation and absorption. In addition, the content of estradiol was also heavily reduced in the control group, which is another key factor influencing the bone metabolism (Chiba et al., 2003). The estradiol content can be restored completely in rats treated with high-dose TQS169 (Fig. 4C). The contents of osteocalcin showed that OCN in the control group was evidently less than that of the normal group ($p < 0.05$), and the TQS169-treated groups showed an increasing dose-dependent effects, but with no statistical differences (Fig. 4D).

All these dates illustrate that TQS169 prevents bone loss on osteoporosis induced by retinoic acid *in vivo* through elevating the activity of ALP, the content of estradiol and the content of osteocalcin.

### 3.3. The mechanism of TQS169 for improving osteoporosis in vitro

The effects and mechanism of TQS169 in improving osteoblast differentiation were further studied in MC3T3-E1 cells. In the cell viability analysis, high concentration of TQS169 (1 mg/ml) can definitely improve the cell proliferation to approximate 125% of the control on the first day, of the concentrations (Fig. 5A). The activity and protein content of ALP were measured after the treatment with TQS169 on the 1st and 4th day (Fig. 5B). Cells treated with 1 mg/ml TQS169 presented a 50% higher activity compared with the control group on the 1st day ($P < 0.05$), but no significant difference was observed on the 4th day. The protein content could be increased by 60% on the 4th day, while had no effect on the 1st day. The data above indicated that TQS169 can shortly play a positive role in ALP activity and can gradually affect protein level during the treatment.

To further examine the effects of TQS169 on bone formation, qualitative assays of mineralization (bone nodule formation) were carried out using alizarin red *S* staining. The presence of calcium deposits showed that TQS169 significantly increased the mineralization in MC3T3-E1 cells at 14 days compared with the control cells (Fig. 6). The results dominantly support the idea that TQS169 can improve the mineralization possibility *in vitro*.

To gain further understanding of the molecular mechanisms of the TQS169 in improving osteoblast differentiation, the expressions of typical related genes were examined by RT-PCR and western blot. The mRNA expression level of ALP was markedly upregulated by 300 and 100 folds with 1 and $10^{-1}$ mg/ml TQS169 treatment, respectively

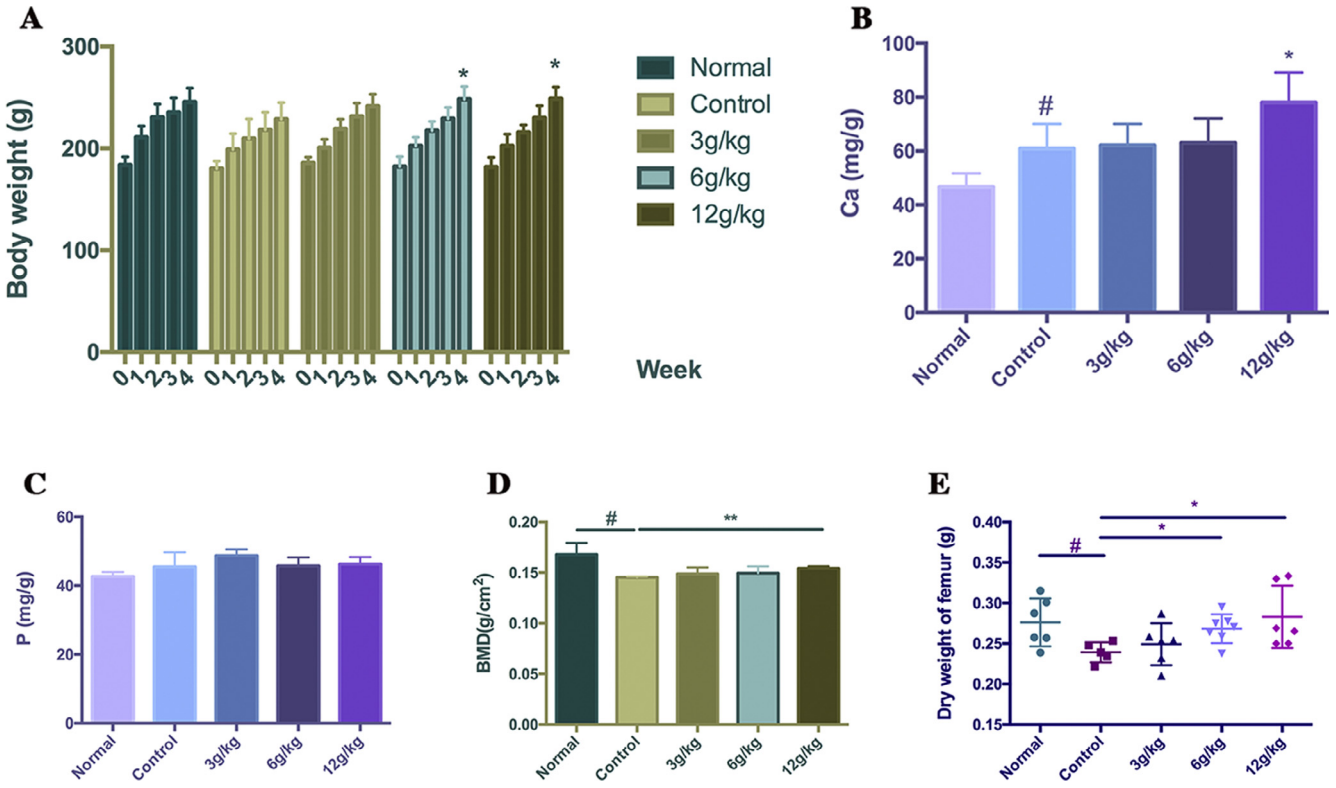


**Fig. 2.** TQS169 prevents bone loss in rats on osteoporosis induced by retinoic acid. (A) The body weights of rats in each group were measured every week during the treatment of the TQS169. (B) The content of bone calcium (Ca) in rats from each group were measured once the rats were killed. (C) The content of bone phosphorus (P) in rats from all groups were analyzed according to the Method and Materials. (D) The BMD of femur bones was measured once the rats were killed. (E) The dry weights of femur bones were measured. Results were represented as mean ± SD ($^{*}p < 0.05$ compared with control group, $^{\#}p < 0.05$ compared with normal group).

($P < 0.05$) (Fig. 7A). The mRNA expression level of RUNX2 was found significantly increased by 12 folds in comparison with the treatment of no TQS169 ($P < 0.05$) (Fig. 7B). OCN and COL-I are recognized as two late osteoblastic markers and they were also significantly promoted by TQS169, with 4 and 2.5 times enhancement respectively when 1 mg/ml TQS169 was added ($P < 0.05$) (Fig. 7C and D). According these RT-PCR analysis results, 1 mg/ml was chosen to perform the western blot assay. Results indicated that the protein levels of RUNX2, COL-I and

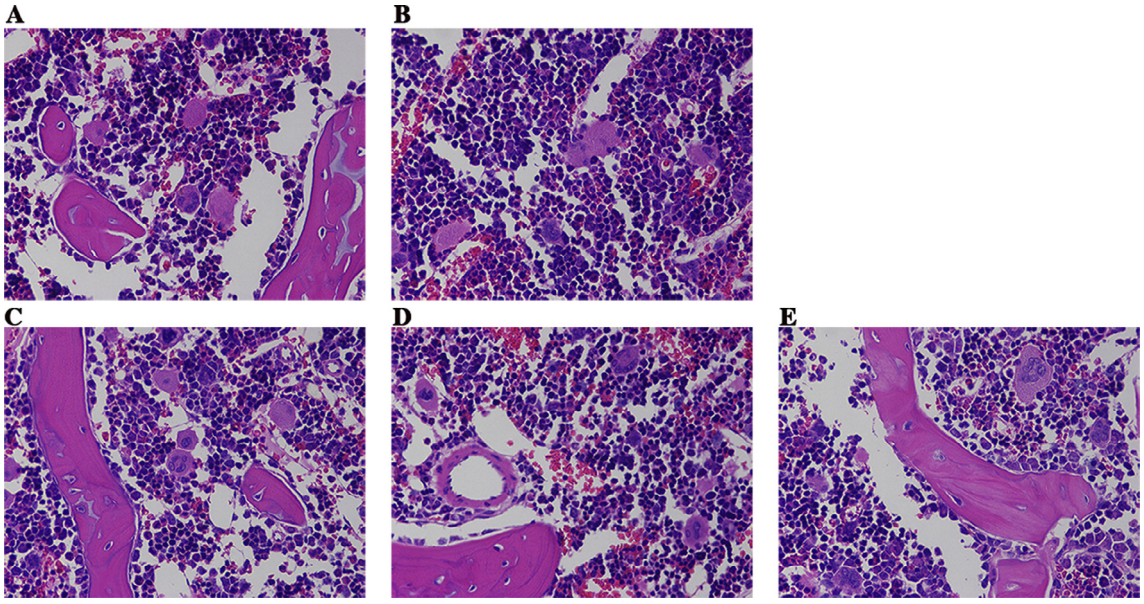


**Fig. 3.** Pathological features of femurs in rats (HE, x400). (A) Normal group, (B) Control group, (C) low-dose-treated group (3 g/kg), (D) middle-dose-treated group (6 g/kg), (E) high-dose-treated group (12 g/kg).

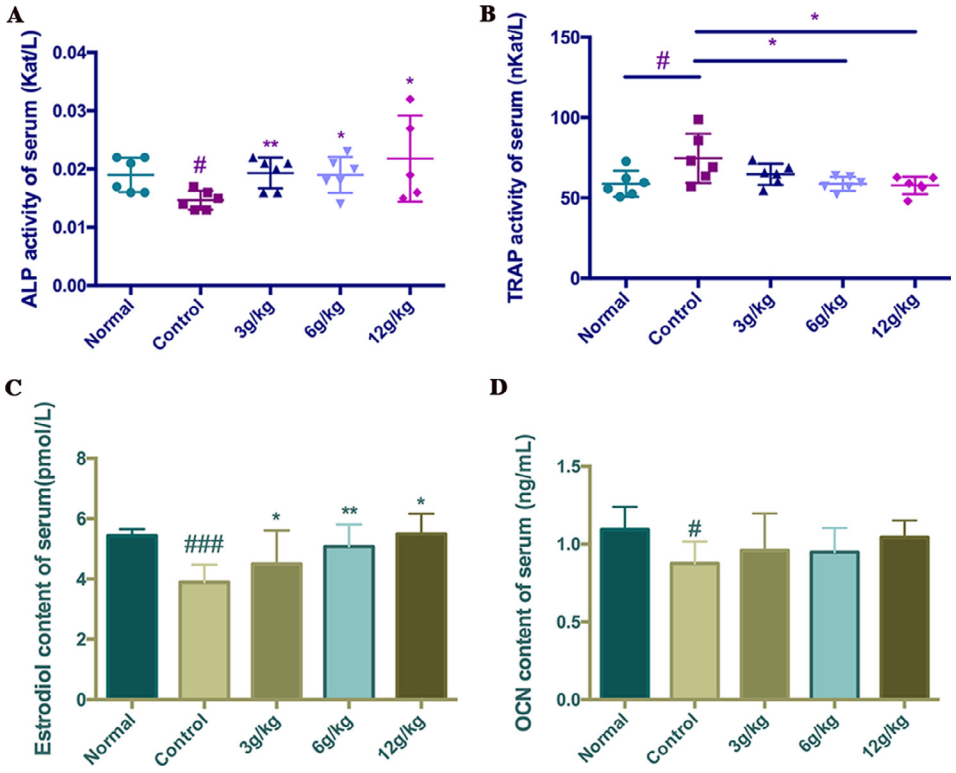


**Fig. 4.** The content of serum ALP, TRAP, estradiol and osteocalcin in rats from different groups. (A) The activities of ALP in serum of rats from each group. (B) The activities of TRAP in serum of rats from each group. (C) Effect of TQS169 on serum estradiol (E2) was detected by the Elisa kit. (D) Effect of TQS169 on serum osteocalcin (OCN) was detected by the Elisa kit. Results were represented as mean ± SD ($^{\#}p < 0.05$ compared with normal group; $^{*}p < 0.05$ compared with control group).

OCN were time-dependently enhanced in cells when incubated with TQS169 for 0,4,7,14 days (Fig. 7E).

These data indicated that TQS169 can improve osteoblast differentiation by upregulating ALP and RUNX2 at the transcription and expression levels, further increasing COL-I and OCN expression at the late stage of differentiation and promoting mineralization in MC3T3-E1 cells.

## 4. Discussion

Osteoporosis is an outcome of an imbalance between bone resorption and formation. The disorder of the two processes acts as a basic regulator which leads to bone construction and reconstruction (Bharadwaj, Naidu, Betageri, Prasadarao, & Naidu, 2009). In this study, we first found that TQS169 markedly prevents osteoporosis in a dose dependent manner *in vivo*. According to our results, it improved the bone formation positively. The content of calcium in femurs had an obvious increase during the TQS169 treatment in both low-calcium diet and retinoic acid models, even no changes were found in phosphorus. HE staining also showed that more trabeculaes were found in the TQS169 treated rats. BMD was considered to diagnose osteoporosis and evaluate the bone mass and quality (Kanis et al., 2002). Our results indicated TQS169 can increase the BMD and the dry wet of femur as well as the body weight of rats.

From the results about the absorption of calcium, TQS169 can alter the bone metabolism, and how it promotes the osteogenic differentiation and reduces bone loss need to be clarified. OPG was one of new biochemical markers for bone metabolism (Price, Parthemore, & Deftos, 1980), which can contribute to bone formation in a long steady period. TGF-β was recognized for dual effects on osteogenic differentiation (Suzuki et al., 2014), especially influences the early differentiation of osteoblasts like MC3T3-E1. Lycopene in tomato can decrease the formation of TRAP which led to the inhibitory effects on osteoclastic mineral resorption (Rao, Krishnadev, Banasikowska, & Rao, 2003). In this study, the contents of OPG, TGF-β in serum were increased significantly, while TRAP activity was inhibited when TQS169 was fed. Interestingly, besides above osteogenic differentiation related factors, the estradiol in serum was also found to be improved significantly by TQS169. Estradiol is a necessary estrogenic hormone (Rao et al., 2003). Previous study showed the absence of estradiol is perhaps the main cause of osteoporosis among premature and menopause women (Štěpán, Pospíchal, Presl, & Pacovský, 1987) and parathyroid hormone had a dose-dependent effect on bone formation (Milstrey et al., 2017). Our results first found that estradiol in serum was significantly inhibited by retinoic acid, as compared with the normal, and highly increased in TQS169 treated groups. We predicated one of the main possible mechanism of TQS169 against osteoporosis may be that TQS169 improves the level of estradiol.

To our knowledge, no study has yet evaluated the effects of TQS169 on osteoblastic differentiation. Osteoblast originates from bone marrow mesenchymal stem cells and differentiates to osteocyte with extra cellular associated genes secretion including ALP, RUNX2, COL I and OCN. These genes are major phenotypic markers for pre-osteoblast differentiation during bone formation (Nagasawa et al., 2005). Then osteocyte secrets cytokines which activates osteoblasts again. In present study, MC3T3-E1 cells were used to investigate the influence of TQS169 on the regulation of these genes for the first time. ALP is an enzyme related to the cell membrane, appeared in early stage during the period of osteoblast differentiation. It was the most associated marker of osteoblastic differentiation (Serigano et al., 2010). COL I, followed by the production of ALP and other osteoblastic differentiation markers, ultimately leading to the induction of extracellular matrix calcification (König, Oesser, Scharla, Zdzieblik, & Gollhofer, 2018). Runx2 regulates osteoblast proliferation and accelerates the transition from the proliferative to post-proliferative stage during the osteoblast differentiation (Galindo et al., 2005). OCN was reported to be involved in regulating

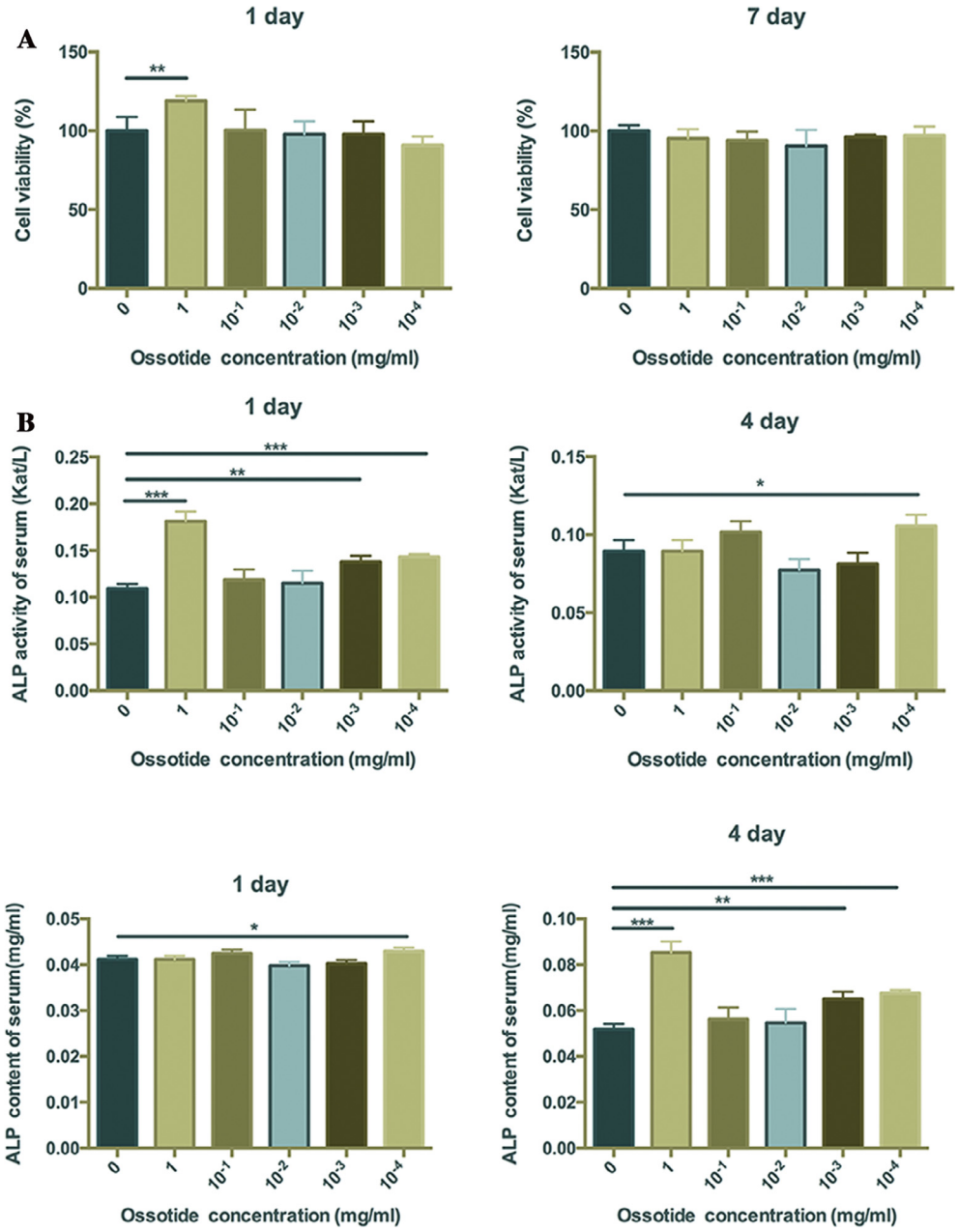


**Fig. 5.** TQS169 can improve the cell viability of MC3T3-E1 and enhance the activity of ALP. (A) MC3T3-E1 cells treated with different concentrations of TQS169 were cultured in α-MEM medium for 1,7 days. Then the 10ul MTT was exposed to determine the viability of the cell at 570 nm by a microplate reader. (B) The activity and quantity of alkaline phosphatase (ALP) were measured on the 1st and 4th day. The data are represented as mean ± standard deviation. $^{*}P < 0.05$ vs. control value.

the formation of mineralization matrix, and was recognized as the marker appeared in the late stage of osteogenic differentiation and as the representative of mature osteoblastic cells (Komori, 2006). In our results, TQS169 didn't influence the viability of cells but improved the activity and quantity of ALP significantly, just as it improved the serum ALP in osteoporosis model rats induced by retinoic acid. The expression of OCN was both upregulated at mRNA and protein level in MC3T3-E1 cells significantly by the TQS169, and the growing trend of OCN content in serum was also observed in a dose dependent manner in the retinoic acid induced rats. Runx2 has been shown to regulate the expressions of COL I and OCN. In this study, we found that both the mRNA and protein levels of Runx2 were increased in the TQS169-treated cells, indicating that the TQS169 might improve the differentiation of osteoblasts by upregulating the level of Runx2 expression and then promoting the following factors.

Osteoporosis is an elderly disease that causes a massive loss of bone matrix and increases bone fragility and susceptibility to fractures. The purpose of osteoporosis treatment is to protect patients against the bone fractures by reducing bone loss, perhaps by improving bone density and consolidating its strength (American Association of Clinical Endocrinologists Medical Guidelines for Clinical Practice for the Prevention and Treatment of Postmenopausal Osteoporosis, 2001 Edition, with selected updates for 2003, 2003; Ammann & Rizzoli, 2003). In addition, although there are some potential novel anabolic

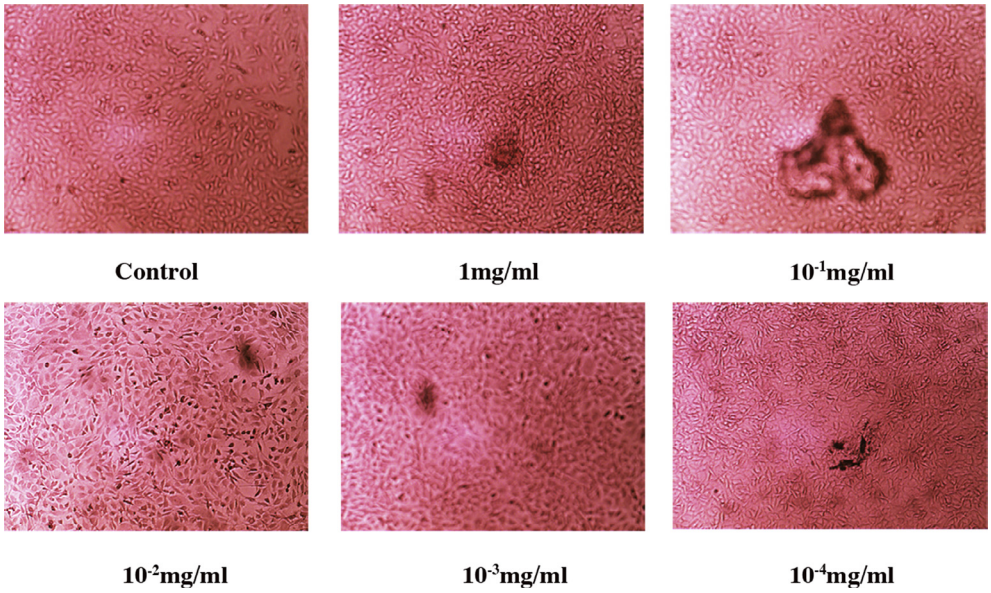


**Fig. 6.** TQS169 can improve the mineralization possibility *in vitro*. The mineralization in cell matrix was found in the TQS169 treated groups on the 14th day, especially in the $10^{-1}$mg/ml TQS169 treated group.

treatments like teriparatide, abaloparatide and romosozumab, no available treatment can completely cure osteoporosis (Canalis, 2017). Earlier detection and treatment of osteoporosis could decrease the risk of bone diseases in the future. So early prevention of osteoporosis is very crucial (McMillan, Zengin, Ebeling, & Scott, 2017). Our study confirmed the preventive effects of TQS169 in osteoporosis animal model and revealed the mechanism in promoting osteogenic differentiation for the first time. And, as a nutritional food, TQS169 is easier to intake and much safer for patients than chemicals. Therefore, our study provides a potential theoretical strategy for the further treatment of osteoporosis.

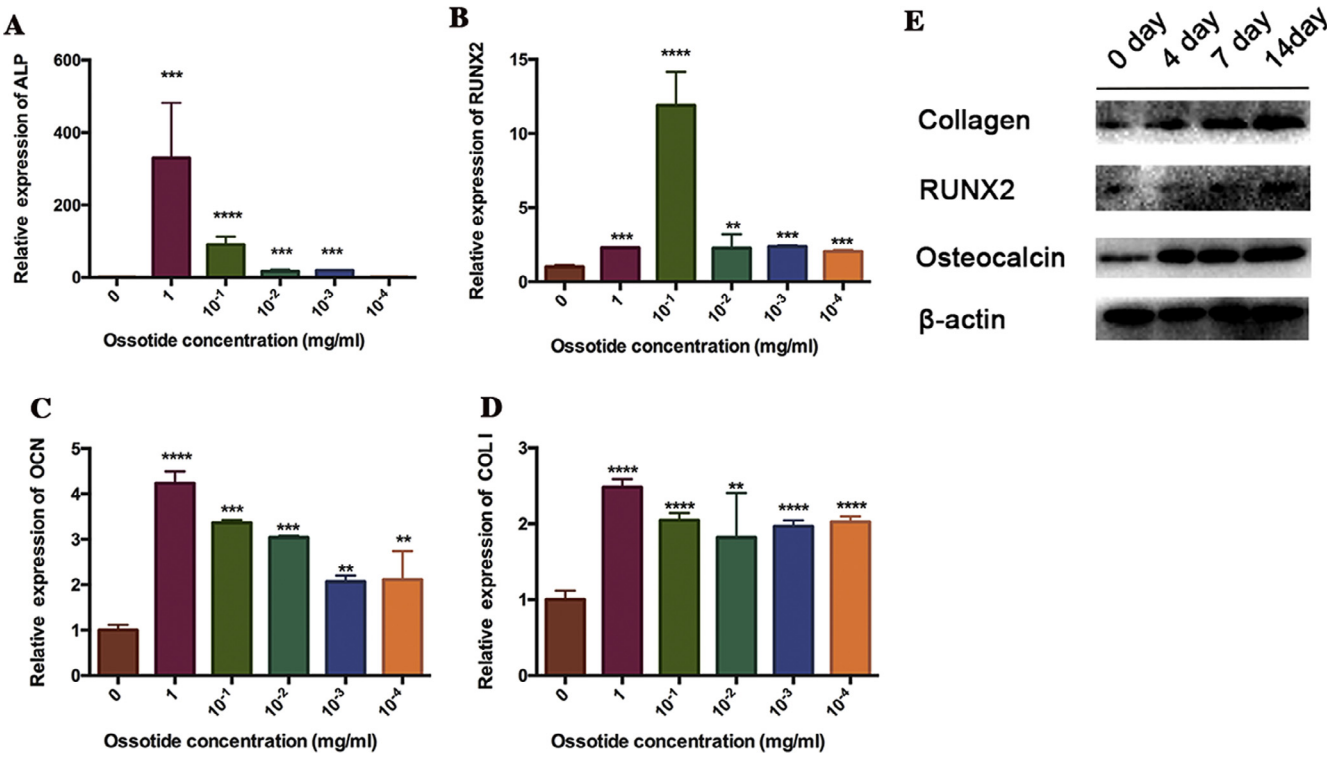


**Fig. 7.** TQS169 can promote the osteogenic differentiation in MC3T3-E1 cells. (A) Real-time PCR analysis of ALP was performed using primers on the 4th day. (B) Real-time PCR analysis was performed using primers for RUNX2 on the 4th day. (C) Real-time PCR analysis was performed using primers for OCN on the 4th day. (D) Real-time PCR analysis was performed using primers for COL I on the 4th day. (E) MC3T3-E1 cells were treated with 1 mg/ml of TQS169 for 0,4,7,14 days. Then the expressions of related protein were assessed by western blot analysis. The experiments were repeated three times independently. Each bar represents the mean ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

## 5. Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

## 6. Ethics statement

The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Fudan University with a permission number 2015000521984.

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## References

Al Mamun, M. A., Hosen, M. J., Khatun, A., Alam, M. M., & Al-Bari, M. A. A. (2017). Tridax procumbens flavonoids: A prospective bioactive compound increased osteoblast differentiation and trabecular bone formation. *Biological Research, 50*(1), 28. https://doi.org/10.1186/s40659-017-0134-7.

American Association of Clinical Endocrinologists Medical Guidelines for Clinical Practice for the Prevention and Treatment of Postmenopausal Osteoporosis: 2001 Edition, With Selected Updates for 2003. (2003). Endocrine Practice, 9(6), 544-564. doi: 10.4158/ep.9.6.544.

Ammann, P., & Rizzoli, R. (2003). Bone strength and its determinants. *Osteoporosis International, 14*(3), 13–18. https://doi.org/10.1007/s00198-002-1345-4.

Bharadwaj, S., Naidu, A. G. T., Betageri, G. V., Prasadarao, N. V., & Naidu, A. S. (2009). Milk ribonuclease-enriched lactoferrin induces positive effects on bone turnover markers in postmenopausal women. *Osteoporosis International, 20*(9), 1603–1611. https://doi.org/10.1007/s00198-009-0839-8.

Bisson, S.-K., Ung, R.-V., Picard, S., Valade, D., Agharazii, M., Larivière, R., & Mac-Way, F. (2018). High calcium, phosphate and calcitriol supplementation leads to an osteocyte-like phenotype in calcified vessels and bone mineralisation defect in uremic rats. *Journal of Bone and Mineral Metabolism*. https://doi.org/10.1007/s00774-018-0919-y.

Born, A. K., Lischer, S., & Maniura-Weber, K. (2012). Watching osteogenesis: Life monitoring of osteogenic differentiation using an osteocalcin reporter. *Journal of Cellular Biochemistry, 113*(1), 313–321. https://doi.org/10.1002/jcb.23357.

Canalis, E. (2017). Management of endocrine disease: Novel anabolic treatments for osteoporosis. *European Journal of Endocrinology*. https://doi.org/10.1530/eje-17-0920.

Chiba, H., Uehara, M., Wu, J., Wang, X., Masuyama, R., Suzuki, K., ... Ishimi, Y. (2003). Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. *The Journal of Nutrition, 133*(6), 1892–1897.

Fujita, Y., Goto, S., Ichikawa, M., Hamaguchi, A., & Maki, K. (2016). Effect of dietary calcium deficiency and altered diet hardness on the jawbone growth: A micro-CT and bone histomorphometric study in rats. *Archives of Oral Biology, 72*, 200–210. https://doi.org/10.1016/j.archoralbio.2016.08.036.

Galindo, M., Pratap, J., Young, D. W., Hovhannisyan, H., Im, H.-J., Choi, J.-Y., ... van Wijnen, A. J. (2005). The bone-specific expression of Runx2 oscillates during the cell cycle to support a G1-related antiproliferative function in osteoblasts. *Journal of Biological Chemistry, 280*(21), 20274–20285. https://doi.org/10.1074/jbc.M413665200.

Kanis, J. A., Black, D., Cooper, C., Dargent, P., Dawson-Hughes, B., De Laet, C., The National Osteoporosis Foundation, U. A. (2002). A new approach to the development of assessment guidelines for osteoporosis. Osteoporosis International 13(7), 527–536. doi: 10.1007/s001980200069.

Kanis, J. A., Melton, L. J., Christiansen, C., Johnston, C. C., & Khaltaev, N. (1994). The diagnosis of osteoporosis. *Journal of Bone and Mineral Research, 9*(8), 1137–1141. https://doi.org/10.1002/jbmr.5650090802.

Kasten, P., Luginbühl, R., van Griensven, M., Barkhausen, T., Krettek, C., Bohner, M., & Bosch, U. (2003). Comparison of human bone marrow stromal cells seeded on calcium-deficient hydroxyapatite, β-tricalcium phosphate and demineralized bone matrix. *Biomaterials, 24*(15), 2593–2603. https://doi.org/10.1016/S0142-9612(03)00062-0.

Kim, H.-S., Zheng, M., Kim, D.-K., Lee, W.-P., Yu, S.-J., & Kim, B.-O. (2018). Effects of 1,25-dihydroxyvitamin D3 on the differentiation of MC3T3-E1 osteoblast-like cells. *Journal of Periodontal & Implant Science, 48*(1), 34–46.

Komori, T. (2006). Regulation of osteoblast differentiation by transcription factors. *Journal of Cellular Biochemistry, 99*(5), 1233–1239. https://doi.org/10.1002/jcb.20958.

König, D., Oesser, S., Scharla, S., Zdzieblik, D., & Gollhofer, A. (2018). Specific collagen peptides improve bone mineral density and bone markers in postmenopausal women—A randomized controlled study. *Nutrients, 10*(1), 97.

Kono, R., Okuno, Y., Inada, K.-I., Tokuda, A., Hashizume, H., Yoshida, M., ... Utsunomiya, H. (2011). A prunus mume extract stimulated the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Bioscience, Biotechnology, and Biochemistry, 75*(10), 1907–1911. https://doi.org/10.1271/bbb.110264.

Libouban, H., & Chappard, D. (2017). Altered bone microarchitecture and gene expression profile due to calcium deficiency in a mouse model of myeloma. *Micron, 96*, 77–85. https://doi.org/10.1016/j.micron.2017.01.009.

McMillan, L. B., Zengin, A., Ebeling, P. R., Scott, D. (2017). Prescribing physical activity for the prevention and treatment of osteoporosis in older adults. Healthcare (Basel, Switzerland), 5(4). Retrieved from http://europepmc.org/abstract/MED/29113119, http://europepmc.org/articles/PMC5746719?pdf=render, http://europepmc.org/articles/PMC5746719, https://doi.org/10.3390/healthcare5040085. doi: 10.3390/healthcare5040085.

Milstrey, A., Wieskoetter, B., Hinze, D., Grueneweller, N., Stange, R., Pap, T., ... Garcia, P. (2017). Dose-dependent effect of parathyroid hormone on fracture healing and bone formation in mice. *Journal of Surgical Research, 220*, 327–335. https://doi.org/10.1016/j.jss.2017.07.019.

Nagasawa, H., Takahashi, S., Kobayashi, A., Tazawa, H., Tashima, Y., & Sato, K. (2005). Effect of retinoic acid on murine preosteoblastic MC3T3-E1 cells. *Journal of Nutritional Science and Vitaminology, 51*(5), 311–318.

Pratap, J., Galindo, M., Zaidi, S. K., Vradii, D., Bhat, B. M., Robinson, J. A., ... van Wijnen, A. J. (2003). Cell growth regulatory role of Runx2 during proliferative expansion of preosteoblasts. *Cancer Research, 63*(17), 5357–5362.

Price, P. A., Parthemore, J. G., & Deftos, L. J. (1980). New biochemical marker for bone metabolism. Measurement by radioimmunoassay of bone GLA protein in the plasma of normal subjects and patients with bone disease. *Journal of Clinical Investigation, 66*(5), 878–883.

Rao, L. G., Krishnadev, N., Banasikowska, K., & Rao, A. V. (2003). Lycopene I—Effect on osteoclasts: Lycopene inhibits basal and parathyroid hormone-stimulated osteoclast formation and mineral resorption mediated by reactive oxygen species in rat bone marrow cultures. *Journal of Medicinal Food, 6*(2), 69–78. https://doi.org/10.1089/109662003322233459.

Reinholz, G. G., Getz, B., Pederson, L., Sanders, E. S., Subramaniam, M., Ingle, J. N., & Spelsberg, T. C. (2000). Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. *Cancer Research, 60*(21), 6001–6007.

Riggs, B. L., & Melton, L. J. I. (1992). The prevention and treatment of osteoporosis. *New England Journal of Medicine, 327*(9), 620–627. https://doi.org/10.1056/nejm199208273270908.

Saneshige, S., Mano, H., Tezuka, K., Kakudo, S., Mori, Y., Honda, Y., ... Kumegawa, M. (1995). Retinoic acid directly stimulates osteoclastic bone resorption and gene expression of cathepsin K/OC-2. *Biochemical Journal, 309*(3), 721–724. https://doi.org/10.1042/bj3090721.

Serigano, K., Sakai, D., Hiyama, A., Tamura, F., Tanaka, M., & Mochida, J. (2010). Effect of cell number on mesenchymal stem cell transplantation in a canine disc degeneration model. *Journal of Orthopaedic Research, 28*(10), 1267–1275. https://doi.org/10.1002/jor.21147.

Štěpán, J. J., Pospíchal, J., Presl, J., & Pacovský, V. (1987). Bone loss and biochemical indices of bone remodeling in surgically induced postmenopausal women. *Bone, 8*(5), 279–284. https://doi.org/10.1016/8756-3282(87)90002-0.

Sun, K., Wang, J., Liu, F., Ji, Z., Guo, Z., Zhang, C., & Yao, M. (2016). Ossotide promotes cell differentiation of human osteoblasts from osteogenesis imperfecta patients by up-regulating miR-145. *Biomedicine & Pharmacotherapy, 83*, 1105–1110. https://doi.org/10.1016/j.biopha.2016.08.025.

Suzuki, E., Ochiai-Shino, H., Aoki, H., Onodera, S., Saito, A., Saito, A., & Azuma, T. (2014). Akt activation is required for TGF-β1-induced osteoblast differentiation of MC3T3-E1 pre-osteoblasts. *PloS One, 9*(12), e112566. https://doi.org/10.1371/journal.pone.0112566.

Ueda, H., Yamazaki, C., & Yamazaki, M. (2002). Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*. *Biological and Pharmaceutical Bulletin, 25*(9), 1197–1202. https://doi.org/10.1248/bpb.25.1197.

Uenishi, K., Tokiwa, M., Kato, S., & Shiraki, M. (2018). Stimulation of intestinal calcium absorption by orally administrated vitamin D3 compounds: A prospective open-label randomized trial in osteoporosis. *Osteoporosis International, 29*(3), 723–732. https://doi.org/10.1007/s00198-017-4351-2.

Ying, X., Chen, X., Feng, Y., Xu, H. z., Chen, H., Yu, K., ..., Peng, L. (2014). Myricetin enhances osteogenic differentiation through the activation of canonical Wnt/β-catenin signaling in human bone marrow stromal cells. European Journal of Pharmacology 738(Supplement C), 22–30. doi: https://doi.org/10.1016/j.ejphar.2014.04.049.

Zhang, M.-X., Wang, X.-C., Liu, Y., Xu, X.-L., & Zhou, G.-H. (2012). Isolation and identification of flavour peptides from Puffer fish (Takifugu obscurus) muscle using an electronic tongue and MALDI-TOF/TOF MS/MS. *Food Chemistry, 135*(3), 1463–1470. https://doi.org/10.1016/j.foodchem.2012.06.026.