Original Paper

Use of an Osteoblast Overload Damage Model to Probe the Effect of Icariin on the Proliferation, Differentiation and Mineralization of MC3T3-E1 Cells through the Wnt/β-Catenin Signalling Pathway

Yingjie Liu\textsuperscript{a, b} Lulu Huang\textsuperscript{c} Baohui Hao\textsuperscript{a, d} Hao Li\textsuperscript{b} Shuanglong Zhu\textsuperscript{a} Qiangsong Wang\textsuperscript{b} Ruixin Li\textsuperscript{b} Yunqiang Xu\textsuperscript{a} Xizheng Zhang\textsuperscript{b}

\textsuperscript{a}Tianjin Medical University General Hospital, Tianjin; \textsuperscript{b}Institute of Medical Equipment, Academy of Military Medical Science, Tianjin; \textsuperscript{c}Xiangya School of Medicine, Central South University, Changsha; \textsuperscript{d}First Hospital of Jilin University, Changchun, China

Key Words
MC3T3-E1 • Icariin • Overload • Wnt/β-catenin

Abstract

\textbf{Background/Aims:} Mechanical loading plays an important role in the regulation of bone mass. However, bone cells are not always under physiological stress. In some cases, bone tissue is subjected to an overloaded mechanical environment. For example, a person who is weight training and a stevedore often experience bone pain, inflammation and other bone fatigue damage symptoms. Icariin is the major ingredient of Herba epimedii, which has been widely used for the treatment of bone injury in traditional Chinese medicine, but its mechanism remains unknown. The aim of this study was to probe the effect of icariin on the proliferation and differentiation of osteoblasts exposed to overload and to determine whether the Wnt/β-catenin signalling pathway is involved in the drug response in osteoblasts.

\textbf{Methods:} Mouse MC3T3-E1 cells were exposed to mechanical tensile strain using a four-point bending device to create an overload damage model. An MTT assay was performed to determine the effects of icariin on MC3T3-E1 cell proliferation. The mRNA and protein levels of ALP, COL-I, OCN, RUNX2 and β-catenin were assessed using RT-PCR and immunoblotting. The protein levels of β-catenin in the MC3T3-E1 cells were also determined using fluorescence microscopy. The mineralization of osteoblasts was assessed using Alizarin Red S staining.

\textbf{Results:} We found that icariin enhanced the proliferation of osteoblasts exposed to overload and promoted MC3T3-E1 cell differentiation and mineralization. Furthermore, the gene and protein expression levels of β-catenin and RUNX2 all increased with icariin treatment compared with those in the damage group.

\textbf{Conclusion:} Our study suggested that icariin promotes proliferation and differentiation in osteoblasts exposed to overload. The effect of icariin on osteoblastic differentiation acted by activating the RUNX2 promoter and the Wnt/β-catenin pathway.
Introduction

Human bone tissue cells are mainly osteoblasts, osteocytes and osteoclasts. Bone tissue maintains its homeostasis through a delicate dynamic balance between bone-forming osteoblasts (bone formation) and bone-eroding osteoclasts (bone resorption) [1]. Such a delicate balance is often regulated by mechanical loads, hormones, and nutrients [2,3]. Additionally, mechanical loading plays an important role in bone homeostasis. Frost’s mechanist theory proposes that the normal physiological level of bone strain is 200-2500 microstrain (με), but bone can withstand 10000 to 30000 με; additionally, 5000 με is the dividing line between bone physiological strain and pathological strain, and their respective strain response mechanisms may be different [4]. An overloaded mechanical environment indicates that the strain level is greater than 5000 με or the physiological overload strain level is under long-term cyclic loading, such as the fatigue load. In many cases, bone tissue is often subjected to overload conditions, such as heavy-load training soldiers, high strength-training athletes, handling workers, etc. [5, 6]. These individuals often have bone pain, inflammation and other fatigue bone injury symptoms. Thus, overload-induced fatigue bone injury has become a serious problem in military training and in heavy manual workers.

Icariin (C_{33}H_{40}O_{15}; molecular weight: 676.67), the main active flavonoid isolated from *Epimedium brevicornum* Maxim, has been used in traditional Chinese medicine for over 2000 years. Modern pharmacology has found that icariin has many pharmacological and biological activities, including preventing osteoporosis, protecting neurons, facilitating penile erection, and possessing anti-cancer and anti-depression properties [7-9]. Domestic and international studies have shown that icariin can promote oestrogen biosynthesis, increase alkaline phosphatase activity and promote the transition of mesenchymal stem cells into osteoblasts [10, 11]. It is also able to protect mouse chondrocytes from the inflammatory reaction induced by lipopolysaccharide *in vitro* and the acute inflammatory response induced by lipopolysaccharide *in vivo* [12, 13]. Thus, icariin can promote osteogenic differentiation and bone formation while inhibiting free radical inflammation. However, there is almost no literature focusing on the effect of icariin on the damage caused by overload, as it may be an effective method to protect against the damage caused by overload. Additionally, there is limited knowledge about the potential mechanisms that icariin uses to promote the proliferation and differentiation of osteoblasts.

Canonical Wnt/β-catenin signalling plays a key role in regulating osteoblast osteogenesis and bone formation. In recent years, several studies have confirmed that oestrogen is involved in adjusting the biological activity of osteoblasts through Wnt signalling pathways [14]. Because of the important role of the Wnt/β-catenin signalling pathway in osteoblast proliferation and differentiation, we hypothesized that the Wnt/β-catenin signalling pathway is involved in the effect of icariin on osteoblasts.

The purpose of this work was to study (1) the effect of icariin on the proliferation, differentiation and mineralization of osteoblast cells that were damaged by overload and (2) whether icariin could affect Wnt/β-catenin signalling pathways.

Materials and Methods

**Materials**

TRIzol was purchased from Invitrogen (Invitrogen, USA). COL I, OCN, RUNX-2 and β-catenin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The GAPDH antibody was purchased from Tianjin Sungene Biotech Co., Ltd. (Tianjin, China). H$_2$O$_2$, Triton X-100 and DMSO were purchased from Sigma (St. Louis, USA).

**Cell culture**

The preosteoblast MC3T3-E1 Subclone 4 cell line was purchased from the Cell Resource Center IBMS CAMS/PUMS in Beijing, China. The MC3T3-E1 cell line is also a good model for understanding extracellular...
matrix signalling and tissue remodelling. The cells were maintained in alpha-minimum essential medium (α-MEM; Invitrogen, San Diego, CA, USA) supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were cultured at 37 °C and 5% CO₂. After reaching 90% confluency, the cells were detached by treatment with 10% trypsin-EDTA (Sigma) and were seeded at a density of 2×10⁴/cm² in cell culture dishes.

**Application of mechanical strain to cultured cells**

The stretching device was a specially designed four-point bending device described previously [15]. The cells were seeded at a density of 2×10⁴/cm² in cell culture dishes and cultivated until they reached 70% confluency. The medium was then changed to FBS-free medium. The cell cultures were subjected to a mechanical strain of 3000 με at 1.5 Hz for 6 h and 5000 με at 0.5 Hz for 30 min with this method for three consecutive days. The average loading frequency was based on the data of heavy manual-carrying workers and the weight training of new recruits. Unstratned (control) cell cultures were incubated in parallel with loading cell cultures using the same procedures as loaded cells, except for the loading. Each experiment was repeated at least three times to confirm the results.

**Icariin and grouping**

Icariin (Fig. 1) was purchased from Nanjing Chongyuan Biotechnology Co. (Nanjing, China). In a previous work, it was found that a concentration of 10⁻⁷ mol/L icariin was the optimal concentration for the proliferation and differentiation of osteoblasts [16]. The experiment was divided into eight groups: Control (no mechanical strain and no icariin), Icariin (no mechanical strain and the cells were treated with 10⁻⁷ mol/L icariin), Damage (no icariin and cells were subjected to a mechanical strain of 3000 με at 0.5 Hz for 30 min or 5000 με at 1.5 Hz for 6 h with this method of loading for three consecutive days), Damage prevention (during the 24 h before mechanical stimulation, the cells were treated with 10⁻⁷ mol/L icariin), and Damage treatment (during the 24 h after mechanical stimulation, the cells were treated with 10⁻⁷ mol/L icariin).

**MTT assay**

An MTT assay was performed to determine the effects of icariin on MC3T3-E1 cell viability. MC3T3-E1 cells were continually cultured for 24 h after mechanical stimulation, and then, proliferation was detected. After 24 h of culture, the cells were washed with PBS, and then, 1 ml of an MTT (Promega) solution (5 mg/ml, diluted by PBS) was added to each well. The cells were incubated for 4 h at 37 °C. After 4 h, the supernatant was removed. Then, 2 ml of dimethyl sulfoxide (DMSO) was added to all the wells and mixed thoroughly to dissolve the dark blue crystals. After 10 min at room temperature, to ensure that all crystals were totally dissolved, the plates were read on an ELISA reader at wavelength of 490 nm.

**Alkaline phosphatase (ALP) activity analysis**

After 24 h, the cells were harvested and lysed in 2000 µl/dish of lysis buffer (10 mmol/L HEPES, 250 mmol/L sucrose, 5 mmol/L Tris-HCl, and 0.1% Triton X-100, pH 7.5), sonicated, and centrifuged to remove the cell debris. The ALP activity in the cellular fraction was measured using a fluorometric detection kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China). The ALP activity of each sample was normalized to the protein concentration.

**RNA isolation and real-time RT-PCR**

To investigate the influence of mechanical strain on gene expression, reverse transcription (RT)-polymerase chain reaction (PCR) was performed as previously described. The cells were collected, and the total RNAs were prepared as described previously using TRIzol (Invitrogen, San Diego, CA, USA). The integrity of the extracted RNA was verified by denaturing agarose gel electrophoresis. Following RNA elution, 10 µl of RNA were reverse transcribed using the Superscript III Reverse Transcriptase Kit (Invitrogen, San Diego, CA, USA) transcriptase and primed with random hexamers. Quantitative RT-PCR was performed to determine the mRNA levels of the genes ALP, OCN, Runx2 and β-catenin using a pair of primers specific for each of these genes (Tab. 1) using an iQ5 Bio-Rad real-time PCR System (ABI, USA) with
the Brilliant SYBR Green Master Mix. The amplification reaction included 3 steps: (1) incubation at 95°C for 3 min, (2) incubation at 95 °C for 15 s, and (3) annealing and extension at each annealing temperature for 60 s. Steps (2) and (3) were repeated for 40 cycles. All reactions were run in triplicate and analysed using the 2^ΔΔCT method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control gene. Three independent experiments were carried out to determine the relative mRNA levels.

Fluorescence microscopy

The cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Subsequently, the cells were penetrated using 0.5% Triton X-100 for 5 min. Then, the cells were blocked using 1% BSA (bovine serum albumin) for 30 min at room temperature. Afterwards, the cells were incubated with a rabbit anti-beta catenin antibody (1:200 in PBS) overnight at 4°C. Then, labelled goat anti-rabbit IgG antibodies (1:100 in PBS) were applied for 40 min at room temperature. Finally, DAPI was added to the cells for 2 min. After washing three times with PBS, the cells were visualized using fluorescence microscopy.

Western blot analysis

Each culture was rinsed twice with PBS and solubilized with radio immunoprecipitation assay (RIPA) buffer. The protein concentration was determined with the Micro BCA Protein Assay Reagent Kit (Kangchen Bio-tech Company, Shanghai, China). Approximately 35 μg of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the levels of COL I, RUNX2, OCN and β-catenin. Subsequently, the proteins were transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The blots were blocked by incubation in 5% milk with TBST for 1 h and probed overnight at 4°C with the rabbit anti-COL I, rabbit anti-RUNX2, rabbit anti-OCN and rabbit anti-β-catenin antibodies, respectively. After washing, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (1:5000 in 5% milk) for 60 min at room temperature. The immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (TIANGEN BIOTECH CO., LTD., Beijing, China). The optical density of the protein bands was determined using a Gel Doc 2000 (Bio-Rad, CA, USA). The expression of GAPDH was used as a loading control, and the data were normalized against the corresponding GAPDH band. The results were expressed relative to the control.

Mineralization assay

The cultures were stained as described by Majors et al. [17]. MC3T3-E1 cells were continually cultured for 14 days after mechanical stimulation, and then, proliferation was detected. After 24 h of culture, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, ddH2O or PBS was used to wash the plate three times. After washing, the cells were incubated with Alizarin Red S (Keygen Bio-tech Company, Nanjing, China) at room temperature with mild shaking for 30 min to stain the calcium nodes. The unincorporated dye was aspirated. The plate was washed with PBS and agitation for 5 min for a total of three times, and then, the cells were visualized using microscopy.

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. The data were presented as the mean±SD. Variance was homogenous for use of the standard ANOVA methodology. After statistical significance was established by an ANOVA, individual comparisons were made using Tukey’s multiple comparison test. Statistical analysis was performed using the SPSS 13.0 software. A value of p<0.05 was considered to be significant.
Results

Icariin promotes the proliferative activity of MC3T3-E1 cells

Osteoblasts undergo three stages of maturation, i.e., proliferation, differentiation, and mineralization. We first estimated the effect of icariin on osteoblast proliferation using an MTT assay. MC3T3-E1 cells were loaded with 3000 με (continuous loading for 3 days, 6 h per day) and 5000 με (continuous loading for 3 days, 0.5 h per day). They were then treated with icariin for 72 h. As shown in Fig. 2, compared with the control group, overload mechanics inhibited cell proliferation. However, icariin treatment significantly increased osteoblast proliferation compared to the untreated damage group. Furthermore, the damage prevention group had more cell proliferation than the damage treatment group (Fig. 2).

Icariin promotes MC3T3-E1 cell differentiation and mineralization

The effect of icariin on MC3T3-E1 cell differentiation in response to overload was tested. The effect of icariin treatment on the gene and protein expression level of common genetic markers of osteoblast differentiation was examined. COL I is an early marker of pre-osteoblast lineage that progressively expresses ALP during maturation stage and OCN during mineralization. Compared with the damage group, icariin treatment dramatically increased the mRNA expression of COL I, ALP and OCN. Compared with the control group, overload mechanics inhibited the gene expression of the common genetic markers of osteoblasts differentiation (Fig. 3A-3C). ALP activity was enhanced by icariin (Fig. 3D), and the protein expression of COL I and OCN in the bone matrix (Fig. 3E-3F) was also enhanced by icariin. These data showed that icariin treatment influenced the early stage and the maturation stage of osteoblast differentiation.

The effect of icariin on the MC3T3-E1 cell mineralization response to overload injury was also tested. MC3T3-E1 cells were exposed to an overload with the mechanical parameters 3000 με and 1.5 Hz for 6 h/day and 5000 με and 0.5 Hz for 0.5 h/day for 3 days, and then, the cells were treated with icariin for 7 days. Osteoblasts were stained with Alizarin Red S at 14 days for the identification of mineralized nodules. As shown in Fig. 3G, icariin dramatically increased the staining density of Alizarin Red S, as shown by observational data and the data quantification done using the ImageJ software compared to untreated damage group.

Fig. 2. The effect of icariin on osteoblast proliferation. The proliferation was evaluated by an MTT assay after different intensities (3000 με or 5000 με) and different frequencies (1.5 Hz or 0.5 Hz) of strain were applied once a day for different strain times (6 h or 0.5 h) over 3 consecutive days after treatment with icariin. All data represent the mean ± SD of at least three biological replicates. *p<0.05, **p<0.01, versus the control group; p<0.05, **p <0.01, versus the damage group.
Fig. 3. The effect of icariin on osteoblast differentiation and mineralization. (A-C) The mRNA expression levels of ALP, COL I and OCN in MC3T3-E1 cells were determined by real-time PCR after overloading with different intensities (3000 με or 5000 με) and different frequencies (1.5 Hz or 0.5 Hz) was applied to cells once a day for different strain times (6 h or 0.5 h) over 3 consecutive days after treatment with icariin. (D) The ALP activity was measured using a commercial ALP assay kit. (E-F) The protein expression levels of COL I and OCN in MC3T3-E1 cells were determined by Western-blot after overloading with different intensities (3000 με or 5000 με) and different frequencies (1.5 Hz or 0.5 Hz) was applied to cells once a day for different strain times (6 h or 0.5 h) over 3 consecutive days after treatment with icariin. GAPDH was used as an internal control. (G) The mineralization of MC3T3-E1 cells was assessed by Alizarin Red S staining. MC3T3-E1 cells were first cultured in osteogenic differentiation medium for 7 days, and then, overloading with different intensities (3000 με or 5000 με) and different frequencies (1.5 Hz or 0.5 Hz) were applied to cells once a day for different strain times (6 h or 0.5 h) over 3 consecutive days after treatment with icariin. MC3T3-E1 cells were stained with Alizarin Red S at 14 days for the identification of mineralized nodule. Scale bar: 40 μm. Each value is the mean ± SD. *p<0.05, **p<0.01, versus the control group; #p<0.05, ##p<0.01, versus the damage group. Each figure is representative of data from three to five independent experiments.
which had almost no mineralized nodules. Furthermore, compared with the control group, the overload mechanics inhibited osteoblast mineralization. Thus, icariin could promote the osteoblast differentiation and mineralization response to overload injury.
Icariin regulates osteoblast differentiation through the Wnt/β-catenin signalling pathway

To investigate the effect of icariin on the Wnt/β-catenin pathway, the key components of the Wnt/β-catenin pathway, including β-catenin and RUNX2, were initially examined using real time RT-PCR, Western blotting and fluorescence microscopy. Compared with damage group, the real time RT-PCR analysis showed that the mRNA expression levels of β-catenin and RUNX2 were obviously up-regulated after the treatment of icariin (Fig. 4A-4B). Furthermore, the Western blot results also indicated that icariin significantly up-regulated the total protein expression of β-catenin and RUNX2 (Fig. 4C-4D). Subsequently, we also examined the effect of icariin on the translocation of β-catenin to the nucleus, which is a critical step in controlling the Wnt/β-catenin pathway, using an immunofluorescence assay. As shown in Fig. 4E, icariin increased β-catenin translocation into the nucleus (Fig. 4E). These results suggest that icariin enhanced the stabilization and nuclear translocation of β-catenin in osteoblasts with overload injury. Meanwhile, icariin also up-regulated the gene and protein expression of RUNX2. These results suggest that the effect of icariin on osteoblastic differentiation activated the RUNX2 promoter and the Wnt/β-catenin pathway.

Discussion

Bone has optimized its load-bearing role by adapting its architecture and function to mechanical forces [18, 19]. In vitro experiments have shown that cyclic stretching can cause deformation of the extracellular matrix and make osteoblasts change their biochemical behaviours, such as proliferation and protein production, in response to such mechanical stimulation [20]. In recent years, many researchers have studied the effect of overload mechanical strain on the biochemical behaviour of osteoblasts. Several recent studies have indicated that overloaded strain could lead to pathological bone modelling, remodelling, or microdamage that might result in fractures when they accumulated [21, 22]. Previous studies have shown the mechanical strain of 5000 με inhibited MC3T3-E1 cell proliferation [23]. In this study, the overload osteoblast damage model was constructed by stimulating MC3T3-E1 cells with a four-point bending device. The effects of icariin on the proliferation, differentiation and mineralization of osteoblasts exposed to a mechanical overload were estimated. The results in this study indicated that the proliferation and differentiation ability of the cells was significantly decreased in the damage group compared to the control group.

Epimedium is commonly used in traditional Chinese medicine to “strengthen the kidney” and providing nutrition to the bones. Icariin is a major active ingredient in Epimedium, and previous studies have indicated that it can prevent osteoporosis in late postmenopausal women [9]. This effect of icariin on osteoblasts is likely due to its promotion of osteoblastic proliferation and differentiation. However, the effect of the icariin concentration on osteoblasts with overload injury during proliferation and differentiation has not been investigated. In this study, we examined that the effect of icariin on osteoblast damage caused by overload. Classically, the ability of the bone tissue to respond to the mechanical environment relies on the bone cells [24]. Therefore, we used the non-transformed preosteoblastic cell line MC3T3-E1, a well-accepted model of osteogenesis in vitro, in our study. MC3T3-E1 cells can respond to mechanical strain in vitro. Some studies have shown that icariin can promote the proliferation of osteoblasts [25]. In this study, the MTT results indicated that icariin could improve the proliferation of osteoblasts exposed to overload. It is generally known that specific bone proteins are used to monitor osteoblastic anabolic action during differentiation [26, 27]. Type I collagen (COL I) and alkaline phosphatase (ALP) activity might be early markers of osteoblastic differentiation. Osteocalcin (OCN) and calcium nodules are characteristically expressed in mature osteoblasts. Several other studies have shown that icariin increases the levels of the bone matrix proteins ALP, COL I and OCN, which are required for matrix synthesis [28]. Moreover, numerous studies have shown that icariin promotes the formation of calcium nodules [29]. Studies have shown that icariin may be useful in the prevention of endothelial cell damage induced by reactive
oxygen species [30]. In addition, icariin can promote the biosynthesis of oestrogen and antagonize free radical-induced oxidative damage [31, 32]. Thus, with the ability of icariin to inhibit free radicals and inflammatory reactions, it may be able to reverse cell damage caused by mechanical overloads. The results in this study indicated mechanical-overload stimulation inhibited the expression of the bone matrix proteins ALP, COL I and OCN. On the other hand, we used an osteoblast overload damage model to assess the effect of icariin on the proliferation, differentiation and mineralization of MC3T3-E cells. Thus, in this way, it is possible to verify whether icariin can treat cell damage caused by mechanical overload.

In this study, icariin enhanced the expression of bone matrix proteins in osteoblasts with overload injury. Meanwhile, the density of calcium nodules in the treated group was far more than that in the damage group. These results demonstrate that icariin enhances the osteoblast differentiation process from the early to the terminal stage and up-regulates the maturation of osteoblasts with overload injury. Thus, icariin may be useful in the prevention of osteoblast cell damage induced by mechanical overload. However, the mechanism of its action is still unknown.

The Wnt/β-catenin signalling pathway serves as an important modulator of osteoblast differentiation and bone formation [33, 34]. This pathway is triggered by Wnt glycoprotein family members (such as Wnt-1 and 3) binding to a co-receptor complex, including Frizzled and low density receptor-like proteins 5 and 6. This binding is followed by the phosphorylation of glycogen synthase kinase to stabilize β-catenin. Then, β-catenin translocates into the nucleus, where it forms a complex with a T-cell factor to induce the transcription of osteoblastic genes. Many studies have shown that the Wnt/β-catenin pathway is involved in osteoblastic differentiation [35]. However, it remains unknown whether the Wnt/β-catenin pathway is involved in the effect of icariin on osteoblasts exposed to mechanical overload. The Wnt/β-catenin signalling pathway is complex and contains numerous receptors, activators, modulators and other components. Of them, β-catenin (Ctnnb1) is the critical molecular node of the Wnt/β-catenin pathway [36]. Several reviews have shown that β-catenin expression and translocation are required for osteoblasts to complete the differentiation process and synthesize properly-formed bone [37, 38]. Increasing the expression and translocation of β-catenin leads to the enhanced expression of osteoblastic genes, such as ALP, COL I and OCN, in osteoblasts [39]. In this study, our results indicated that the increased gene expression of ALP, COL I and OCN were because of the increased protein expression and nuclear translocation of β-catenin in icariin-treated osteoblasts exposed to overload. RUNX2, a transcription factor, regulates osteoblast-related genes (COL I, ALP, OCN) in osteoblasts [40, 41]. Several recent findings have indicated that RUNX2 is a direct target of the Wnt/β-catenin pathway [42]. At the same time, RUNX2 plays a key role in osteoblastic differentiation and bone formation. Our results showed that icariin increases the protein and gene expression of RUNX2 in osteoblasts exposed to overload. Finally, it can be concluded that icariin increases the protein and gene expression of RUNX2 through the activation of Wnt/β-catenin signalling. Thus, icariin activates the canonical Wnt/β-catenin pathway in osteoblasts with overload injury through the up-regulation of the gene and protein expression of RUNX2.

In conclusion, this study demonstrated that icariin can promote the proliferation, differentiation and mineralization of osteoblasts exposed to overload. Second, our study demonstrated that icariin stimulated the expression of the components of the canonical Wnt/β-catenin signalling pathway, resulting in the enhanced expression of osteoblast differentiation markers. Furthermore, this study provides novel insight into the molecular mechanism of icariin for further study of its effects on overload bone injury.

**Abbreviations**

ALP (alkaline phosphatase); COL I (Type I collagen); OCN (osteocalcin); με (microstrain); BSA (bovine serum albumin); FBS (Foetal bovine serum); DMSO (dimethyl sulfoxide).
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Disclosure Statement

The authors declare that they have no conflict of interest.

References

Liu et al.: Icariin Affect PDM of Osteoblast Exposed Overload through Wnt/β-Catenin


