Research Square

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# WITHDRAWN: Apigenin neutralizes the inhibitory effect of inflammation on the osteogenic differentiation of human mesenchymal stem cells

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## EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

# Abstract

**Background:** The stimulating effects of apigenin on mesenchymal stem cells (MSCs) osteogenesis, as well as the anti-inflammatory effect of this flavonoid, have been identified. In this study, osteogenic differentiation was investigated under inflammatory conditions and treatment with apigenin.

**Methods and Results:** Along with osteogenic differentiation of MSCs, they became inflamed with LPS/PA, and treated simultaneously with apigenin. The degree of differentiation was assessed by alizarin red staining and alkaline phosphatase (ALP) activity. Also, gene expression of NLRP3 and RUNX2 was performed along with protein expression of IL-1β. Significant increase in NLRP3 and IL-1β were observed in MSCs when exposed to LPS/PA ($p<0.01$). Also, the osteogenesis was significantly decreased ($p<0.01$). Apigenin treatment induced significantly higher gene expression of RUNX2, the activity of ALP, and cell staining ($p<0.01$) which were also associated with reduced inflammation in these cells.

**Conclusions:** The effectiveness of apigenin on osteogenesis under inflammatory conditions was cautiously observed.


# Introduction

Improve general health and reduced fertility, have increased life expectancy. Therefore, age-associated diseases such as cardiovascular and neurodegenerative diseases, cancer, osteoporosis, and other bone-related diseases are more threatening today [1]. Osteoporosis, as a diverse genetic and metabolic bone disease, is one of the most important public health issues characterized by bone loss, bone density, and bone mass [2]. This disorder, commonly referred to as postmenopausal osteoporosis (PMOP) in women over fifty years, is associated with fracture and fat infiltration [3]. The real cause of it is due to an imbalance between the activity of osteoblasts (bone-forming cells) [4] and osteoclasts (bone cells) and a decrease in the differentiation potential of bone marrow mesenchymal stem cells [5]. In fact, in this case, this differentiation potential goes toward adipogenesis, which is associated with a significant change of cells from osteocytes to adipocytes [6].

Age, gender, inactivity, smoking, and alcohol are factors affecting osteoporosis [3]. The process of this disorder is associated with increased inflammation, especially the activation of nlrp3 Inflammasome [7]. If aging is accompanied by an increase in the basal level of inflammation, potentially impairs bone formation [8]. Thus, activation of NLRP3 inflammatory as an intracellular protein complex involved in the innate immune response increases adipogenesis and suppresses osteogenesis [7]. As a result, by controlling the inflammatory mechanism, including Nlrp3 Inflammasome, it seems that the behavior of stem cells can be regulated.

Anti-inflammatory effects of polyphenolic compounds have been reported with their effects in the treatment of diseases such as osteoporosis [9]. Among them, apigenin (4', 5, 7-Terry Hydroxy flavone) is a member of the flavonoid family that has potential anti-cancer, anti-inflammatory, antimicrobial and antioxidant properties [10].

Previous studies have shown that apigenin significantly downregulates TNF-α, IL-6, and IL-1β's mRNA levels in LPS activated mouse macrophages [11]. It also inhibits LPS-induced IL-1β by interfering with NLRP3 inflammasome and also decreases IL-6 and IL-1β production through ERK1 /2 [12].

Anti-osteoclastogenic effects of this compound [13] have also been reported to be associated with increased expression of osteoblast differentiation genes such as alkaline phosphatase, collagen, osteopontin, and BMP in osteoblasts [14]. Apigenin was also able to induce osteogenesis of hMSCs by increasing the expression of Runt-related transcription factor 2 (RUNX2) and osterix (Osx) [15,16]. These results suggest that apigenin enhances osteogenesis of hMSCs from osteoblast precursors to terminal differentiation states.

According to the above, the anti-inflammatory effect of apigenin along with its effect on stimulating osteogenesis has been identified. Due to this, and the role of inflammation in inhibiting osteogenesis, the protective effect of this compound against LPS/PA-induced osteoporosis, as well as its osteogenic stimulatory effect, was investigated.

# Methods

### Cell culture

Mesenchymal stem cells were enzymatically isolated from abdominal adipose tissue isolated from women who underwent liposuction procedure. Due to the use of disposable abdominal fat, this was confirmed by the ethics committee of Isfahan University of Medical Sciences. The MSC isolation steps included washing the adipose tissue with PBS (0.1 M, pH=7.4) – penicillin-streptomycin (10000 U-10000 mg/ml) three times and digesting the tissue with collagenase type I (1 mg/ml in PBS) (Thermo Fisher Scientific, USA) followed by lysis of erythrocytes by RBC lysis buffer (155 mM $NH_4Cl$, 12 mM $NaHCO_3$, and 0.1 mM EDTA) and centrifugation (800 × g for five minutes). The isolated cells were maintained at 37˚C and 5% $CO_2$ in self-renewal medium (DMEM-F12 + 10% FBS + 1% P/S (Thermo Fisher Scientific, USA). The medium was changed every three days and the cells were passaged after each filling of the flask. After four passages, the cells were differentiated under the following conditions. For osteogenesis, the self-renewal medium was changed by an osteogenic medium contained 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate. For chondrogenesis, the chondrogenic medium was used which contained the same complete medium and dexamethasone (100 nM), L-proline (40 µg/ml; Merck Millipore Corporation, Istanbul, Turkey), L-ascorbic acid 2-phosphate (50 µg/ml; Sigma-Aldrich, Germany), and TGF-β3 (10 ng/ml; BioLegend$^{Ò}$, Istanbul-Turkey). For adipogenesis, the adipogenic medium contained dexamethasone (1 µM), indomethacin (100 µM), insulin (10 µg/mL), and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM) (All from Sigma-Aldrich Co. Ltd., Dorset, UK) [17,18].

### hMSCs immunophenotyping and multipotency assays

The cell identification was done by three lineage differentiation and FACS Immunophenotyping with positive (CD73, CD90, CD105) and negative (CD34 and CD45) CD markers include. For isotype controls,

rabbit polyclonal IgG and rat IgG2b were used (all BD Biosciences, San Jose, USA) [17]. In summary, the cells from passage four were trypsinized and transferred to a 15 ml tube and fixed with 4% paraformaldehyde. Finally, surface CD markers were characterized using a flow cytometry device (Partec, Germany) [18].

### MTS assay

The toxicity effects of apigenin in the whole period of treatment was done by cell viability assay using an MTS assay kit (Abcam, Cambridge, UK). Accordingly, after the growth of cells in a 96-well plate at the density of 5 × 10 3, they were treated with apigenin (1–100 μM) for 1, 2, 3, 7, and 14 days. Then, using 20 μL/well MTS reagent for four hours at 37°C the absorbance was read at 490 nm in a microplate reader (BioTek Instruments, USA) [17].

### Osteogenesis induction and treatment

To evaluate the effect of apigenin on the expression of NLRP3 and interleukin induced by LPS and PA, MSCs were exposed to 0.1 $mg.ml^{-1}$ LPS/0.25 mM PA and 1 $mg.ml^{-1}$ LPS/0.25 mM PA for 72 hours. Then, different concentrations of apigenin (25 and 50 μM) were added to the medium for 14 days. To compare the effect of apigenin on osteogenesis in inflammatory conditions, cells were first exposed to LPS/PA for 72 hours and then induced osteogenesis for 21 days while being treated simultaneously with apigenin for 14 days. The protocol for inducing osteogenesis, inflammation, and treatment has been based on the assessment of cell viability and previous studies [7,19,20]

### Alizarin red staining

Briefly, cells cultured on a 24 well plate were washed with PBS, fixed with 4% formaldehyde, and then stained with 0.5% Alizarin Red in deionized water (pH = 4.1) for 30 minutes at room temperature [15].

### Evaluation of ALP activity

The cells cultured on the plate were washed with PBS and lysed using lysis buffer (20 mM Tris – HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100). ALP activity was measured by laboratory kits and protein content was measured by the bicinchoninic acid (BCA) method [21].

### Measurement of interleukin-1β by ELISA

The amount of interleukin-1β released in the culture medium was measured using a human IL-1β ELISA kit in cells cultured on a 24 well plate (Carmania Pars Gene, Iran).

### Gene expression evaluation of NLRP3 and RUNX2

The flask containing co-culture of apigenin, LPS/PA of all groups were lysed using Kiazol reagent (Kiazist, Iran) for RNA isolation. The concentration and integrity of isolated RNA were then analyzed using a microplate reader (BioTek Instruments, USA). They were reverted to complementary DNA using a cDNA

synthesis kit (gene all, Seoul Korea). NLRP3 and RUNX2 gene expression was amplified by quantitative real-time PCR (qPCR) through an ABI 7500 sequence detection system (Applied Biosystems). The forward and reverse primers include; RUNX2; F, 5´- ACGAGGCAAGAGTTTCACCT-3´, R, 5´- AGCTTCTGTCTGTGCCTTCT-3´, NLRP3; F, 5´- GATCTTCGCTGCGATCAACAG-3´, R, 5´- CGTGCATTATCTGAACCCCAC-3´. Expression of these genes was performed based on RPII reference gene with primer sequence RPII; F, 5´- GCACCATCAAGAGAGTCCAGT-3´, R, 5- ATTTGATGCCACCCTCCGTCA-3 based on cycle threshold (Ct) $2-(\Delta\Delta Ct)$ using SYBR green qPCR master mix (Odense M, Denmark) [22].

### Statistical analyses

Data analyses were performed using SPSS software (SPSS version 16.0) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). Statistical significance was performed using one-way analysis of variance (ANOVA) followed by *post hoc* Tukey and $p<0.05$ was considered as statistically significant.

## Results

### Mesenchymal stem cell confirmation

Mesenchymal stem cell confirmation was performed by examining surface CD markers as well as the ability to differentiate cells into three categories: adipocytes, osteocytes, and chondrocytes (Fig. 1). According to Fig. 1, the results showed high expression of positive markers (CD73; 86.9%, CD90; 88.7% and CD105; 88.3%), and low expression of negative markers (CD34; 2.41% and CD45; 2.06%), which along with their high ability to differentiate into these three categories by specific staining confirmed cell type.

### Cell viability assay

Initially, a cell viability test was performed by multiple doses of apigenin (1 to 100 μM) by the MTS method (Fig.2). The results showed that in a 14-day period of cell treatment, different doses had no toxic effect.

### LPS/PA caused inflammation in the MSCs.

To evaluate the response of MSCs to inflammatory agents, they were inflamed by LPS/PA at different doses and the gene expression of NLRP3 along with the protein level of IL-1β was examined (Figure 3). LPS / PA-induced inflammation was observed as a significant increase in NLRP3 gene expression and IL-1β levels compared with controls ($p<0.001$). The anti-inflammatory effect of apigenin was also investigated by examining NLRP3 gene expression and IL-1β levels under the influence of this compound. Statistically apigenin in both concentrations significantly decrease protein level of IL-1β and down-regulated gene expression of NLRP3 compared to cells that didn't receive the compound ($p<0.001$).

### LPS/PA-induced inflammasome suppresses osteogenesis in MSCs.

The effect of apigenin and LPS/PA on osteogenesis were subsequently examined (Figure 3). For this purpose, ALP activity, RUNX2 and NLPRP3 gene expression, and IL-1β protein were determined. As shown in Figure 3A, the stimulatory effect of apigenin on osteogenesis in non-inflammatory conditions was observed with increasing ALP activity ($p<0.01$), and increased RUNX2 expression ($p<0.001$), which was statistically significant. Inflammation, induced by LPS/PA was associated with a significant increase in the expression of the NLRP3 gene and IL-1β protein ($p<0.001$; group OSX+LPS/PA *v.s* OSX). These inflammatory conditions were also associated with a significant reduction in osteogenesis, which was observed by measuring RUNX2 expression and ALP activity in comparison with the control group ($p<0.001$). Interestingly, also in inflammatory conditions, apigenin was able to improve ossification at both concentrations, citing a significant increase in RUNX2 expression and ALP activity, compared with the untreated group ($p<0.01$ *v.s* OSX+LPS/PA). To confirm their effects on the degree of osteogenesis, the differentiation of mesenchymal stem cells into osteocytes was evaluated by evaluating alizarin red staining. In staining, increased bone calcium staining was observed in treatment with 50μM apigenin compared to the untreated group in both inflammatory and non-inflammatory conditions.

# Discussion

The hypothesis of this study was based on the relationship between inflammation and osteoporosis. Osteoporosis is a chronic disease that occurs in old age, especially in postmenopausal women, due to decreased estrogen secretion, which is associated with loss of bone mass and increased fat penetration into the bone marrow [23]. Also, the role of inflammation on bone turnover and osteoporosis has been identified [24], which is also strongly associated with menopause [25]. NLRP3-based inflammation has also been shown to play an important role in this process [7].

If so, targeted interventions to modulate inflammation are one of the potential strategies to optimize bone repair in the elderly [8]. Mesenchymal stem cells play an important role in bone modeling and remodeling by forming basic osteoblasts while maintaining a balance between bone formation and resorption [26].

Accordingly, in the present study, mesenchymal stem cells were inflamed with LPS/PA in the osteogenic differentiation and their behavior under the influence of inflammation was examined. As expected, inflammation disrupted the process of this differentiation. This was consistent with previous information about the association of aging with inflammation and subsequent osteoporosis [24]. Also, the effect of lipopolysaccharide-induced inflammation on inhibition of osteogenesis has been accurately expressed in other studies [27,7].

These changes in osteogenesis were characterized by changes in RUNX2 gene expression, ALP activity, and calcium deposition stained by Alizarin Red. RUNX2 transcription, as a major osteogenic factor, is a nuclear transcription factor and regulator of bone differentiation [28]. The essential role of the RUNX2 transcription factor in this process has been proven in various molecular and genetic studies [29]. ALP is an early marker of osteoporosis that plays an important role in bone formation and its peak activity

indicates differentiation [30], Therefore, changes in these cases indicate an inhibitory effect of inflammation on the osteogenesis.

In the present study, the anti-inflammatory effects of apigenin were evaluated. Accordingly, while the stem cells were inflamed with LPS/PA, the anti-inflammatory effects of this flavonoid compound were observed via significant down-regulation of NLRP3 and IL-1β when compared with the non-treatment group.

The anti-inflammatory role of flavonoid derivatives by inhibiting NLRP3 inflammasome has been well demonstrated [31]. Apigenin in particular has an anti-inflammatory effect by inhibiting oligomerization of inflammasome components by blocking Syk and Pyk2 signaling pathways and other pathways [31]. NLRP3 as the most important member of this complex, and sensors of pathogen-associated molecular patterns (PAMPs), facilitates interaction with caspase-1 and is necessary for IL-1β processing [32]. Therefore, down-regulation of its expression as well as IL-1β secretion showed its anti-inflammatory activity.

Apigenin, as a plant flavone, has also shown its osteogenic effects by inhibiting osteoclasts, prevent bone loss, and stimulation of osteogenic differentiation of hMSCs in various studies [33,34,15]. On the other hand, as mentioned, the anti-inflammatory effect of this flavonoid has been identified in various studies [35,31], which is done by inhibiting the NLRP3 inflammasome [35,36,31]. In the present study, the effect of inflammation on bone differentiation and the effect of apigenin on ossification stimulation were investigated, most likely through inhibition of inflammation. These effects were based on altering the expression of the NLRP3 inflammasome gene and the amount of IL-1β along with the stimulation of ossification based on the study of RUNX2 gene expression, ALP activity, and Alizarin Red staining. Therefore, the stimulatory effect of apigenin on osteogenesis can be exerted in two ways: reducing NLRP expression and inflammation, and the intrinsic effect of apigenin on differentiation.

According to previous reports, in 2005, more than 2 million people suffered from osteoporotic fractures, which will bring a lot of treatment costs by 2025 due to the aging of the population [37]. The drugs developed have two functions: inhibiting bone resorption or stimulating bone formation, which lead to many problems, from inhibiting bone formation in long-term use, gastrointestinal and hormonal problems to muscle pain, and finally osteosarcoma [38-40]. Therefore, the importance of developing a safer compound based on natural origin is important. Due to the limitations of the present study concerning the evaluation of adipogenic pathway, as well as using pathway inhibitors, more detailed assessments have been considered to obtain more complete results in subsequent studies.

In conclusion, our study showed the stimulatory effect of apigenin on osteogenesis in inflammatory conditions. This could be due to the anti-inflammatory effect of this compound, as well as the inherent stimulatory effect of apigenin on osteogenesis. It also confirms the role of inflammation in osteogenesis and the importance of inhibiting inflammation in this process. The importance of using flavonoids with a focus on the anti-inflammatory role of these compounds was also identified in this study.

# Declarations

**Conflicts of interest** None.

### Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [**Azita Asadi**], [**Farjam Goudarzi**], [Mustafa Ghanadian] and [**Adel Mohammadalipour**]. The first draft of the manuscript was written by [**Azita Asadi**] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

### Consent to participate

All authors whose names appear on the submission

1) made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work;

2) drafted the work or revised it critically for important intellectual content;

3) approved the version to be published; and

4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Consent for publication

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities tacitly or explicitly at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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# Figures

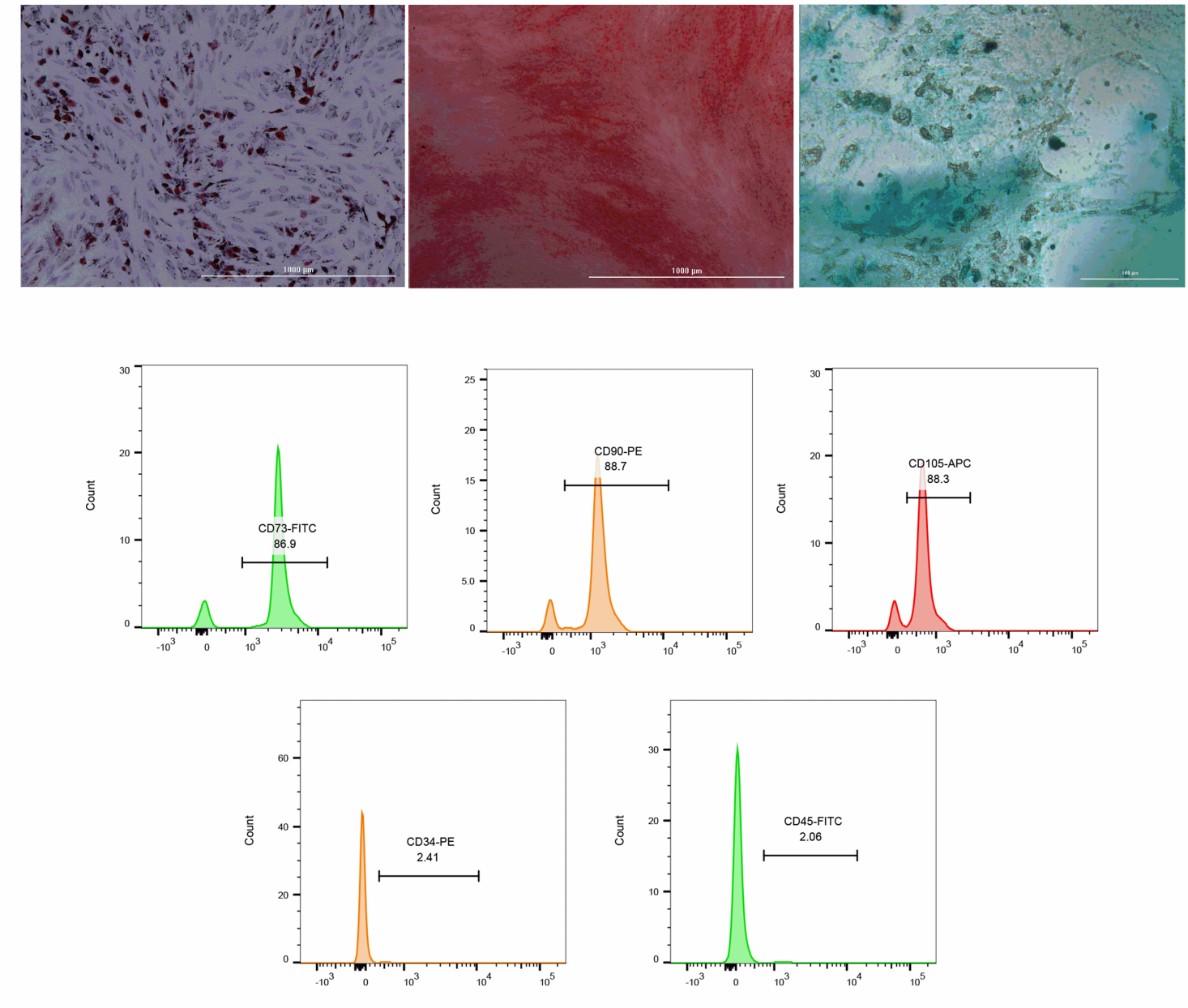



**Figure 1**

Confirmation of mesenchymal stem cells by determining surface markers and multi-potency properties of stem cells in differentiating into three cell lines: osteocytes, adipocytes, and chondrocytes. A; Flow

cytometric analysis of the CD markers of human-derived MSCs. Accordingly, While a high percentage of cells was observed with CD73, CD90 and CD105, only 2.41% of cells were positive in CD34 and 2.06% were positive in CD45. B: Differentiation of these cells into adipocytes by Oil Red O (red spots) staining and into chondrocytes by glycosaminoglycan (GAG) staining using safranin and differentiation into osteocytes by alizarin red staining were observed.

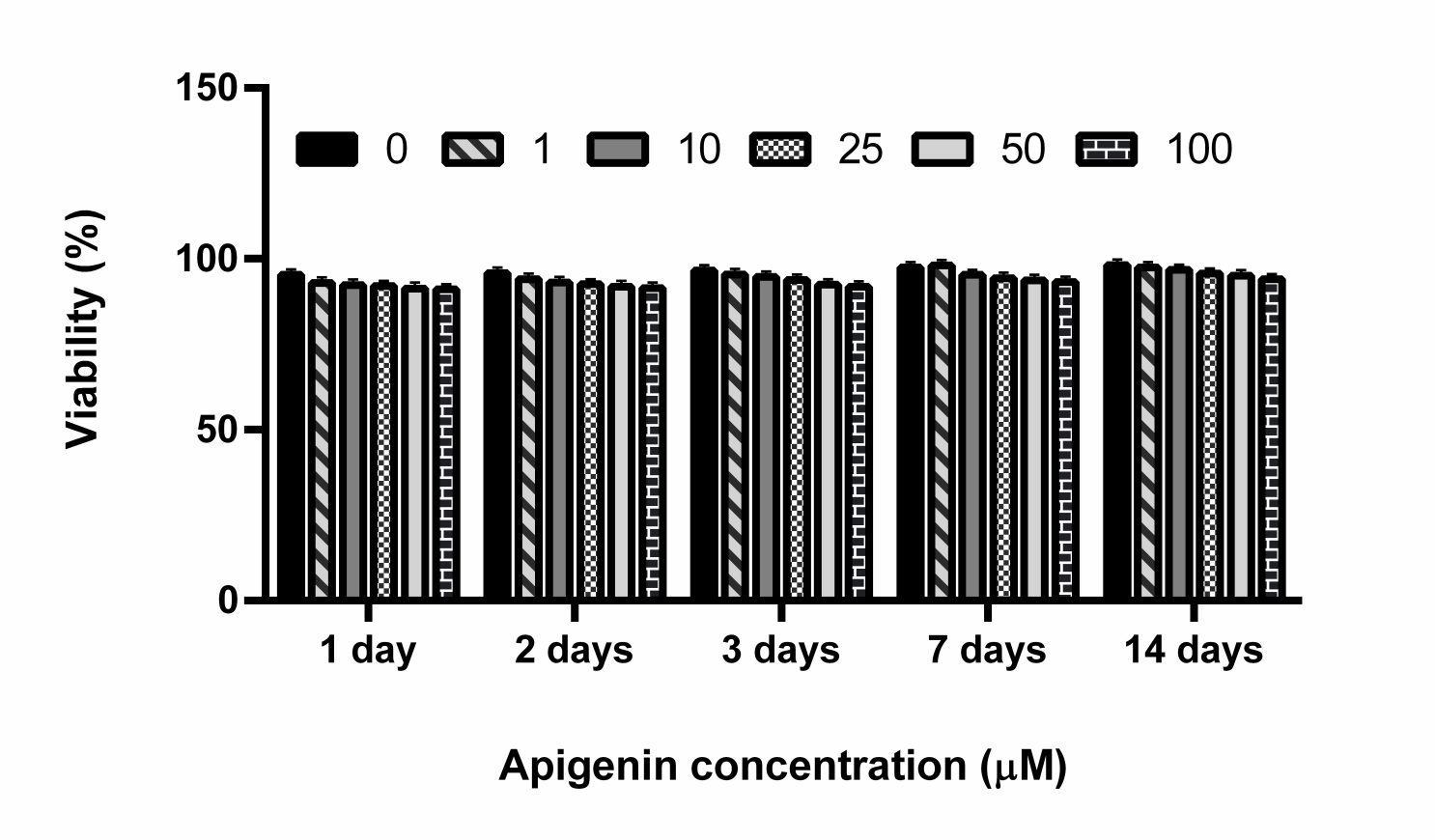
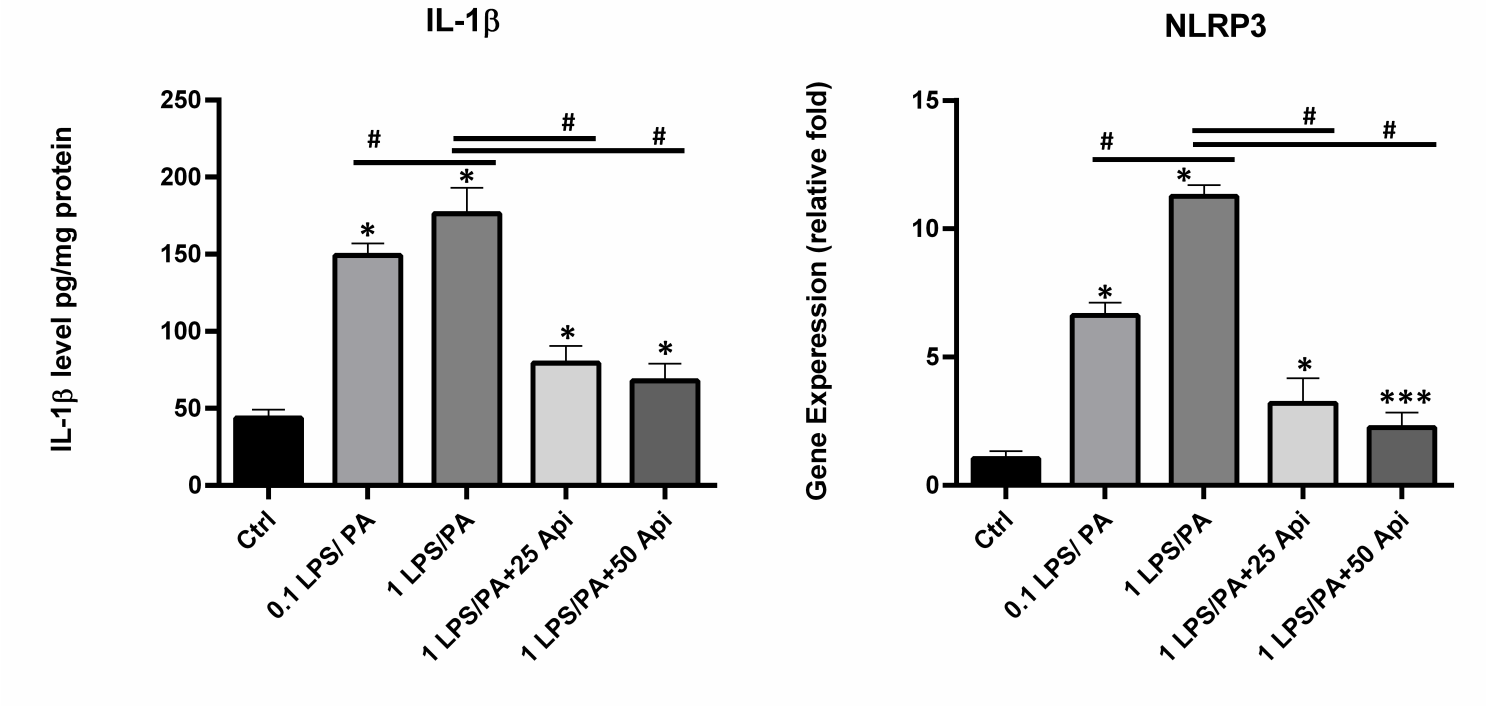


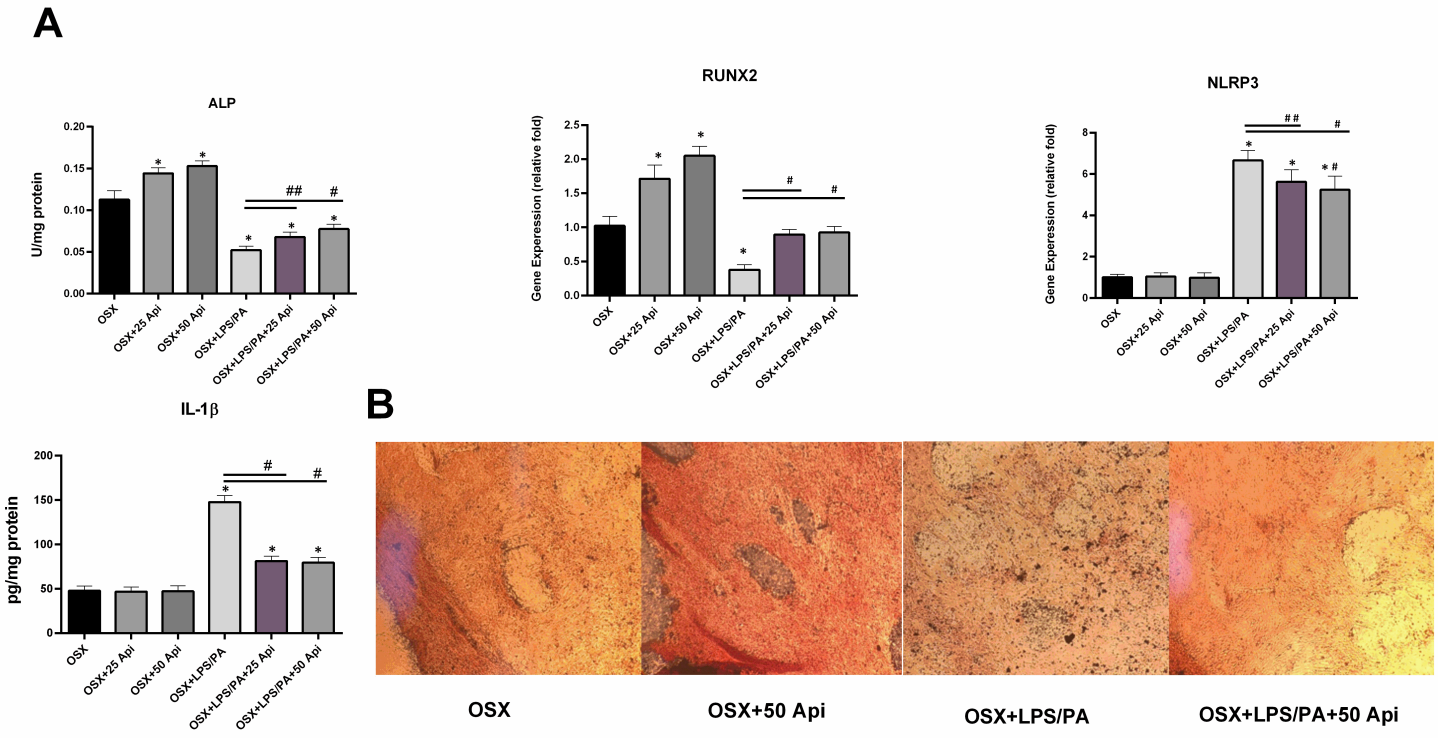

**Figure 2**

Effect of apigenin on the viability of hMSCs. Different concentrations of apigenin (1 μM to 100 μM) were used for 14 days to assess cell viability, which showed no significant change in cell viability.




## Figure 3

Levels of IL-1β (A), and gene expression of NLRP3 (B) in cultured MSCs in the presence and absence of LPS/PA and Apigenin. LPS/PA: MSCs received 0.1 mg.ml-1 LPS/0.25 mM PA, 1 LPS/PA: MSCs received 1 mg.ml-1 LPS/0.25 mM PA, 1 LPS/PA + 25 Api: MSCs received 0.1 mg.ml-1 LPS/0.25 mM PA and 25 µM apigenin, 1LPS/PA + 50 Api: MSCs received 0.1 mg.ml-1 LPS/0.25 mM PA and 50 µM apigenin.* p<0.001 v.s Ctrl. ** p<0.01 v.s Ctrl. ***p<0.05 v.s Ctrl. # p<0.001 v.s 1 LPS/PA. ## p<0.01 v.s 1 LPS/PA.



## Figure 4

A: ALP activity, gene expression of RUNX2, Levels of IL-1β, and gene expression of NLRP3 in cultured MSCs in the osteogenic medium in the presence and absence of LPS/PA and Apigenin. OSX; MSC cultured in osteogenic medium, OSX+25 Api; MSC cultured in osteogenic medium and treated with 25 μM apigenin, OSX+50 Api; MSC cultured in osteogenic medium and treated with 50 μM apigenin, OSX+LPS/PA; MSC cultured in osteogenic medium and treated with 1 mg.ml-1 LPS/0.25 mM PA. OSX+LPS/PA+25 Api; ; MSC cultured in osteogenic medium and treated with 1 mg.ml-1 LPS/0.25 mM PA and 25 μM apigenin; OSX+LPS/PA+50 Api; ; MSC cultured in osteogenic medium and treated with 1 mg.ml-1 LPS/0.25 mM PA and 50 μM apigenin. B: Alizarin red staining indicates the degree of differentiation of cells in different groups for 21 days. * $p<0.001$ v.s Ctrl. ** $p<0.01$ v.s Ctrl. ***$p<0.05$ v.s Ctrl. # $p<0.001$ v.s 1 LPS/PA. ## $p<0.01$ v.s 1 LPS/PA.