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Original Article

Evaluation of the osteogenic effect of apigenin on human mesenchymal stem cells by inhibiting inflammation through modulation of NF-κB/ IκBα

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Abstract

Background and purpose: Apigenin has stimulatory effects on osteogenic differentiation of human mesenchymal stem cells (hMSCs) as well as anti-inflammatory properties. This study investigated the osteogenic differentiation of hMSCs in inflammatory conditions treated with apigenin focusing on nuclear factor kappa-light-chain-enhancer of activated B (NF-κB), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) and nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3 (NLRP3) inflammatory pathways.
Experimental approach: Along with osteogenic differentiation of the hMSCs, they became inflamed with lipopolysaccharide (LPS)/palmitic acid (PA) and treated with apigenin. Alizarin red staining, alkaline phosphatase (ALP) activity, and Runt-related transcription factor 2 (RUNX2) gene expression were used to determine the degree of differentiation. Also, gene expression of NLRP3 was performed along with protein expression of interleukin 1-beta (IL-1β), NF-κB, and IκBα.
Findings / Results: Apigenin was shown to be effective in neutralizing the inhibitory impact of LPS/PA on osteogenesis. Apigenin increased MSC osteogenic capacity by inhibiting NLRP3 expression and the activity of caspase-1. It was also associated with a considerable decrease in the protein expression of NF-κB and IκBα, as well as IL-1β, in these cells.
Conclusion and implications: The effects of apigenin on osteogenesis under inflammatory conditions were cautiously observed.

Keywords: Apigenin; Inflammation; Mesenchymal stem cells; NF-κB; NLRP3; RUNX2.

INTRODUCTION

Public health and the prevention of infectious diseases have improved longevity and life expectancy. As a result, age-related diseases such as cardiovascular and neurological diseases, cancer, and osteoporosis have become more common (1). Osteoporosis is a serious public health problem defined by bone loss, low bone density, and decreased bone mass (2). It is also associated with fractures and fat infiltration (3). It is common around the world, but it is most prominent in Africa and Europe, which has detrimental effects on society, economy, and health (4). The main mechanisms are a loss of differentiation ability of bone marrow mesenchymal stem cells and an imbalance in the activity of osteoblasts (bone-forming cells) and osteoclasts (bone cells) (5). This transition in differentiation from osteogenesis to adipogenesis is accompanied by a considerable shift in the cell population from osteocytes to adipocytes (6).

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The progression of this disorder is associated with increased inflammation, particularly the activation of the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome (7), which stimulates the production of inflammatory cytokines *via* activating the nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) pathway (8). Thus, activation of NLRP3 and the NF-κB pathway promotes adipogenesis while suppressing osteogenesis (7). For this reason, as people get older, basal inflammation increases, which potentially impairs bone formation (9). As a consequence, by modulating the inflammatory process, it seems that the behavior of stem cells can be regulated.

The medications have two functions: blocking bone resorption or stimulating bone formation, which can cause various issues in long-term usage, including inhibiting bone growth, gastrointestinal and hormonal issues, muscular soreness, and, in the worst-case scenario, osteosarcoma (10,11). Therefore, developing a safer compound with natural origins is critical.

Polyphenolic compounds have considerable anti-inflammatory effects (12). Among them, apigenin (4′,5,7-trihydroxyflavone) is a member of the flavonoid family with anticancer, anti-inflammatory, antimicrobial, and antioxidant potential (13). Various studies have revealed that this compound has anti-inflammatory characteristics by reducing tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 production, inhibiting NLRP3 inflammasomes *via* extracellular signal-regulated protein kinase (ERK1/2) and preventing NF-κB and IκBα phosphorylation (14).

The anti-osteoclastogenic effects of this compound (15) have also been connected to increased expression of osteoblast differentiation genes such as alkaline phosphatase, collagen, osteopontin, and bone morphogenetic protein in osteoblasts (16).

It also stimulates osteogenesis in human mesenchymal stem cells (hMSCs), as evidenced by the expression of Runt-related transcription factor 2 (RUNX2) and osterix (Osx) (17,18). These findings imply that apigenin promotes the osteogenesis of hMSCs at all stages of the differentiation, from osteoblast precursors to final states.

Therefore, the role of apigenin in inhibiting inflammation and stimulating osteogenesis has been determined. However, due to the role of inflammation in this process, no study has been conducted to measure the effect of this compound on hMSCs osteogenesis in inflammatory conditions. Hence, in this study, the osteogenesis effect of this compound was investigated in inflammatory conditions.

MATERIALS AND METHODS

Chemicals and reagents

Sodium dodecyl sulfate-polyacrylamide gels, Dulbecco's modified eagle's medium F12 (DMEM-F12) cell culture media, fetal bovine serum (FBS), and penicillin-streptomycin (P/S) were purchased from Gibco-BRL (Invitrogen Corporation; Carlsbad, CA, USA). The antibodies used included NF-κB (RRID:AB_443394; Abcam), p-NFκB (RRID: AB_1524028; Abcam), IκBα (RRID: AB_733068; Abcam), p-IκBα antibody (RRID:AB_2801653), β-actin (RRID: AB_2305186), CD105 (RRID: AB_303134), CD45 (RRID: AB_442810; Abcam), CD34 (RRID: AB_1640331; Abcam), CD73 (RRID: AB_2747828; Abcam), and CD90 (RRID: AB_297794; Abcam). Rabbit polyclonal IgG and rat IgG2b were purchased from BD Biosciences (BD Biosciences, San Jose, USA). Dexamethasone, β-glycerophosphate, and L-proline were purchased from Merck Millipore (Merck Millipore Corporation, Istanbul, Turkey). Apigenin was purchased from Sigma-Aldrich (St Louis, MO, USA). Lipopolysaccharide (LPS), palmitic acid (PA), L-ascorbic acid 2-phosphate, insulin, indomethacin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St Louis, MO, USA). Transforming growth factor-beta 3 (TGF-β3) was purchased from BioLegend (BioLegend®, USA). Kiazol reagent (Kiazist, Iran), cDNA synthesis kit (GeneAll, Seoul, Korea), and SYBR green master mix high ROX (Ampliqon, Denmark) were used for gene expression analysis. Caspase-1 assay kit (Biovision, California, USA), and alkaline phosphatase (ALP) activity kit (Darman Faraz Kave) were used. Alizarin red S dye, oil red O dye, and alcian blue dye were purchased from Sigma-Aldrich (St Louis, MO, USA). IL-1β ELISA kit

was purchased from Carmania Pars Gene (Tehran, Iran).

Cell culture

According to our earlier study, hMSCs were enzymatically extracted from abdominal adipose tissue obtained from women who had liposuction (19). The ethics committee confirmed this due to the usage of disposable abdominal fat. The isolated cells were maintained at 37 °C and 5% CO_2 in self-renewal medium (DMEM-F12 + 10% FBS + 1% P/S). Every three days, the medium was replaced, and the cells were passaged after each filling of the flask. The cultured cells were passaged four times and then differentiated. Osteogenesis was created by changing the medium with a medium containing dexamethasone (100 nM), β-glycerophosphate (10 mM), and L-ascorbic acid-2-phosphate (0.05 mM). Chondrogenesis was created using a complete medium enriched by L-proline (40 µg/mL), dexamethasone (100 nM), L-ascorbic acid 2-phosphate (50 µg/mL), and TGF-β3 (10 ng/mL). Adipogenesis was created by the complete medium supplemented with insulin (10 µg/mL), dexamethasone (1 µM), indomethacin (100 µM), and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM) (19).

hMSCs immunophenotyping and multipotency assays

Three lineage differentiation and fluorescence-activated cell sorting (FACS) immunophenotyping using positive (CD73, CD90, CD105) and negative (CD34 and CD45) CD markers were used to identify the cells. Rabbit polyclonal IgG and rat IgG2b were utilized as isotype controls (19). Their multipotency assay was evaluated by the differentiated ability of osteocytes, adipocytes, and chondrocytes. For this purpose, staining by alizarin red, O red oil, and alcian blue was performed according to our previous article (19).

Osteogenesis induction and treatment

hMSCs were exposed to 0.1 µg/mL LPS and 0.25 mM PA and for 72 h according to Wang *et al*. study (7). Following the evaluation of apigenin cytotoxicity, different concentrations of apigenin (25 and 50 M) were added to the medium for 14 days to assess apigenin's influence on osteogenesis in inflammatory conditions. So, the cells were subsequently submitted to osteogenesis differentiation for 21 days while also receiving apigenin treatment. The procedure for inducing osteogenesis, inflammation, and treatment was developed based on the assessment of cell viability and previous studies (20,21).

Alizarin red staining, ALP, and caspase-1 activity

Briefly, cells cultured on a 24-well plate were washed with PBS, fixed with formaldehyde (4%), and then stained with alizarin red (0.5%), in deionized water (pH = 4.1) for 30 min at room temperature (17). For evaluating ALP activity, the cells were washed with PBS and lysed using lysis buffer (20 mM tris-HCl, pH 7.5; 1% Triton® X-100, and 150 mM NaCl). Then, using laboratory kits, ALP activity was determined, and protein content was determined using the bicinchoninic acid method (22). Caspase-1 activity was determined using a colorimetric assay using the caspase-1 assay kit.

Measurement of IL-1β

IL-1β released in the medium was measured using a human IL-1β ELISA kit in cells cultured on a 24-well plate.

Gene expression evaluation of NLRP3 and RUNX2

The cells were lysed using a Kiazol reagent, and isolated RNA was analyzed using a microplate reader (BioTek Instruments, USA). Then, they were reverted to complementary DNA using a cDNA synthesis kit. NLRP3 and RUNX2 gene expression was amplified by quantitative real-time polymerase chain reaction (qRT-PCR) through an ABI 7500 sequence detection system (Applied Biosystems, Foster City, USA). The forward and reverse primers include RUNX2: 5´-ACGAGGCAAGAGTTTCACCT-3´ (forward), 5´- AGCTTCTGTCTGTGCC TTCT-3´ (reverse); NLRP3: 5´- GATCTTCGCT GCGATCAACAG-3´ (forward), 5´- CGTGCA TTATCTGAACCCCAC-3´ (reverse). Expression of these genes was performed based on RPII reference gene with primer sequence RPII: 5´- GCACCATCAAGAGAGTCCAGT-3´ (forward) , 5- ATTTGATGCCACCCTCCGT CA-3 (reverse) based on cycle threshold (Ct) $2^{-(\Delta\Delta Ct)}$ using SYBR green qRT-PCR master mix (19).

Western blotting

Western blot analysis was performed as in our previous research (23) and the following antibodies were used; anti-β-actin, anti-NF-κB P65, anti-phospho-NF-κB P65, IκBα, and phospho -IκBα (1:1000 diluted for all).

Statistical analyses

In this study, each test was run twice with three replicates in each test. Data analyses were processed using SPSS software (SPSS version 16.0) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). One-way ANOVA was used to determine statistical significance, followed by post hoc Tukey analysis. *P*-values < 0.05 were considered statistically significant.

RESULTS

Mesenchymal stem cell confirmation

Surface CD markers and the capacity to differentiate cells into three categories: adipocytes, osteocytes, and chondrocytes, were used to validate mesenchymal stem cell identity (Fig. 1).

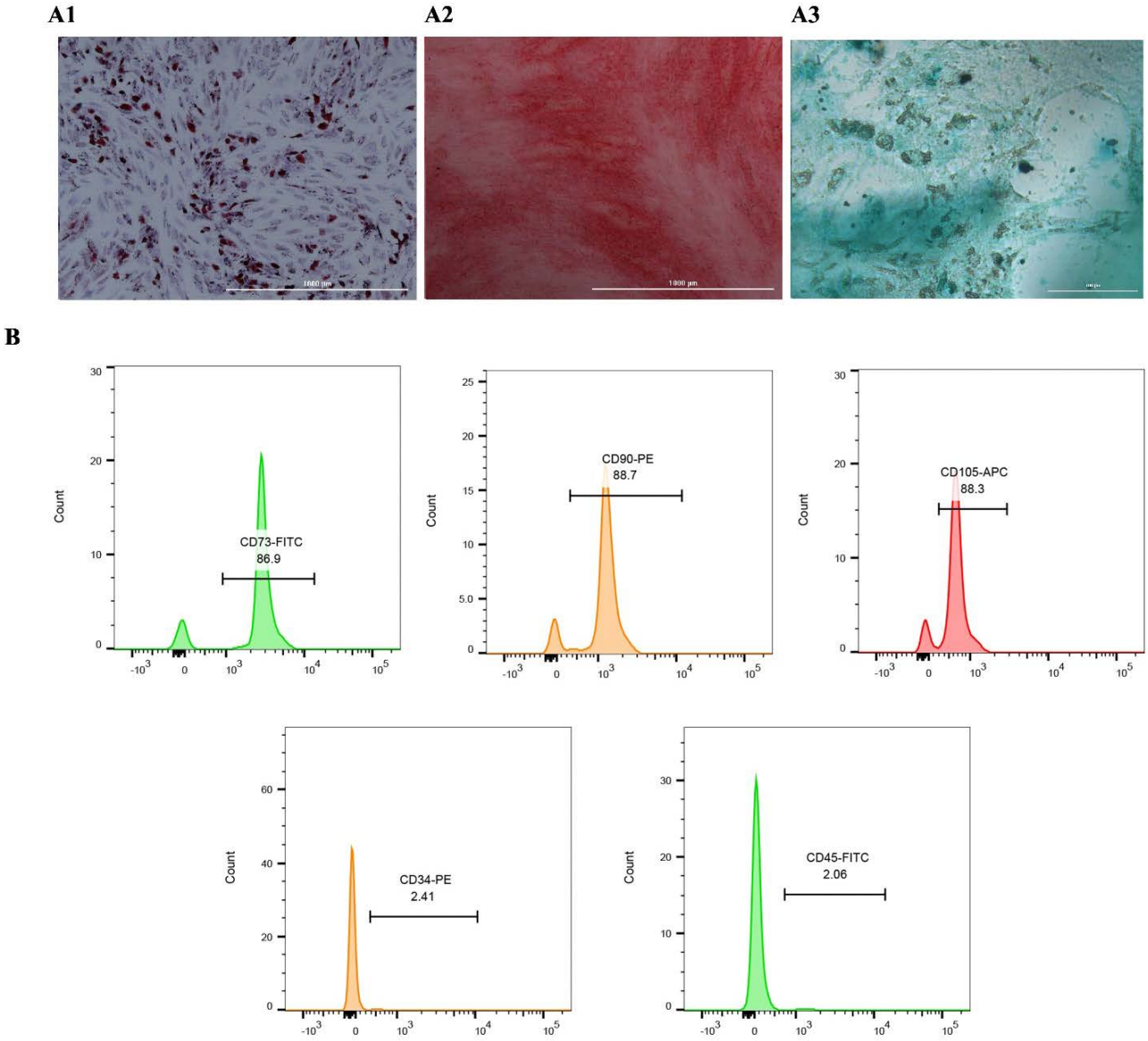

Fig. 1. Confirmation of hMSCs by their ability to three lineage differentiation (A1-A3) and (B) flow cytometric characterization of surface CD markers. (A) The hMSCs differentiation potency into (A1) adipocytes by oil red O staining, (A2) osteocytes by alizarin red, and (A3) chondrocytes by alcian blue staining were observed. The white line below the images indicates a magnification of 1000 µm for (A1 and A2) and 100 µm for (A3). (B) Flow cytometric examination of human-derived mesenchymal stem cells CD markers. As a result, while CD73, CD90, and CD105 were found in a high percentage of cells, only 2.41 percent of cells were positive for CD34, and 2.06 percent for CD45

The results revealed a high level of positive marker expression (CD90: 88.7%, CD73: 86.9%, and CD105: 88.3%) and a low level of negative marker expression (CD45: 2.06% and CD34: 2.41%), which, when paired with their capacity to differentiate into these three lineages using specific staining, validated cell type.

Apigenin inhibited LPS/PA-induced inflammatory changes

LPS/PA was introduced to the medium at various concentrations to assess MSC response to inflammatory agents, and the gene expression of NLRP3, as well as the protein level of IL-1, were measured (Fig. 2). In comparison to the control group, LPS/PA dramatically enhanced NLRP3 gene expression and IL-1 protein levels. Apigenin's anti-inflammatory impact on hMSCs was also studied. This treatment significantly decreased NLRP3 gene expression and IL-1 protein levels as compared to the group that only received LPS/PA. The effect of LPS/PA on caspase-1 activity was also investigated. The results demonstrated a substantial increase in caspase-1 protein activity with an increasing number of inflammatory compounds as compared to the control group. Both concentrations of apigenin considerably reduced the activity of caspase-1 activity compared to the 1 LPS/PA group. As a result, these findings suggested that LPS/PA causes inflammation, while apigenin has an anti-inflammatory impact *via* blocking the NLRP3/IκBα and caspase-1 pathway.

Effect of apigenin on osteogenic differentiability

Following that, the effects of apigenin and LPS/PA on osteogenesis were investigated (Fig. 3). Changes in osteogenesis in the control and inflammatory groups, as well as the apigenin therapy groups, were tracked for 21 days. Then, using alizarin red staining, which shows calcium as a bright red stain, more precise evaluations were made. In both inflammatory and non-inflammatory conditions, treatment with apigenin (50 M), increased calcium amount relative to the untreated group. ALP activity and RUNX gene expression were also used to identify the osteogenic alterations. As a result, adding two concentrations of apigenin to the non-inflammatory medium induced osteogenesis, which was accompanied by a considerable increase in ALP activity and RUNX2 gene expression.

When LPS/PA was added to the medium, inflammation caused alterations in osteogenesis, as evidenced by a substantial drop in ALP activity and RUNX gene expression when compared to the non-inflammatory group. In inflammatory settings, apigenin at two distinct concentrations dramatically compensated for this process. As such, RUNX2 expression and ALP activity were significantly higher in the apigenin-treated groups than OSX+LPS/PA group ($P < 0.01$).

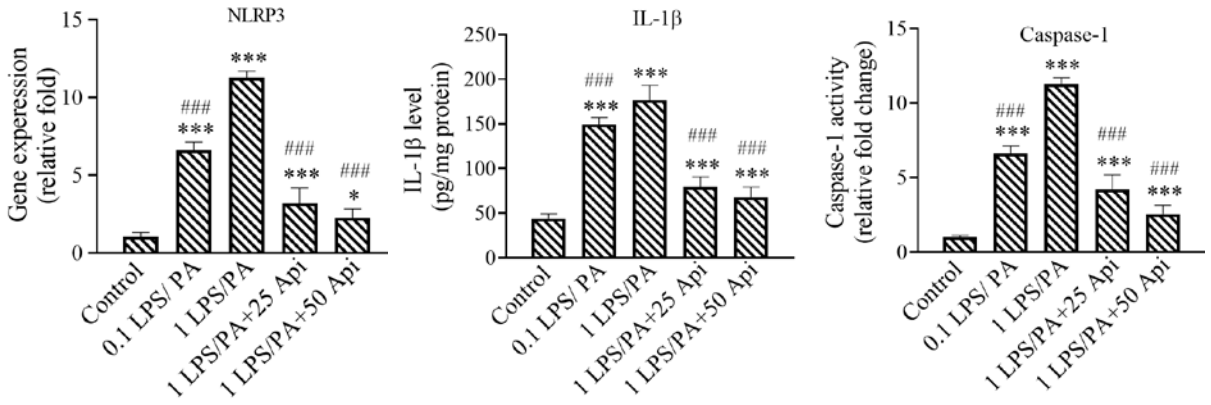

Fig. 2. Apigenin treatment prevented the inflammatory changes of human mesenchymal stem cells caused by LPS/PA. Cells from three donors (n = 3) were used in duplicate in the experiment. NLRP3 gene expression and interleukin-1β protein levels, as well as caspase-1 activity, have been demonstrated in mesenchymal stem cells cultured in the presence of LPS/PA and treated with apigenin. Data represent the means ± SD.*$P < 0.05$ and ***$P < 0.001$ indicate significant differences compared to the control group; $^{###}P < 0.001$ versus 1 LPS/PA group. LPS, Lipopolysaccharide; PA, palmitic acid; Api, apigenin; NLRP3, leucine-rich repeat and pyrin domain containing 3.

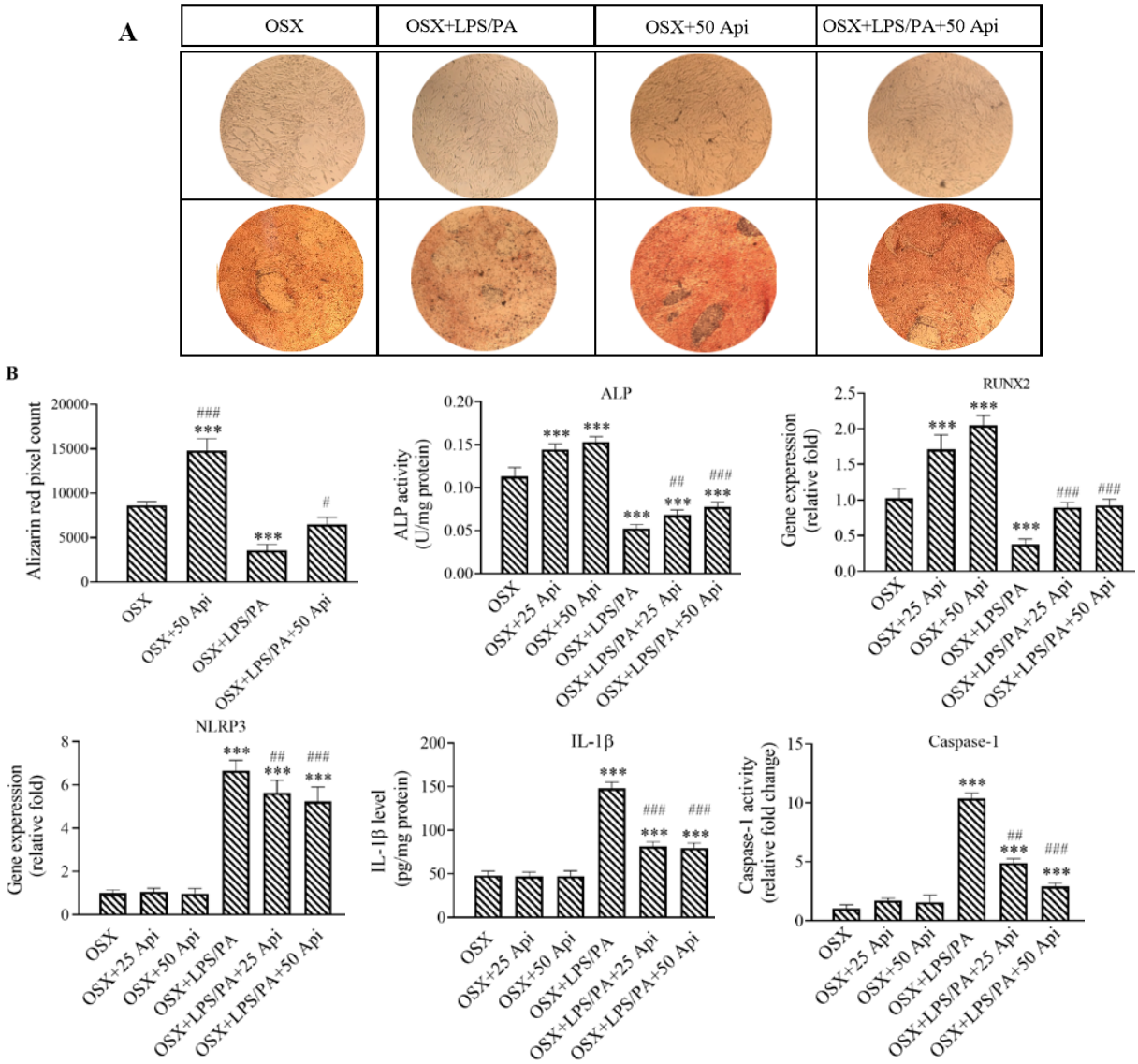

Fig. 3. Inflammatory and osteogenic changes of LPS/PA-exposed hMSCs treated with apigenin. The test was performed for three independent experiments (n = 3) and in duplicate. (A) microscopic evaluation of hMSCs in the osteogenesis process for 21 days. Osteogenic differentiation of unstained hMSCs (top row) and alizarin red stained hMSCs (bottom row) is observed.; (B) represent the quantitative analysis of changes in osteogenesis by alizarin red staining, the activity of ALP, and gene expression of RUNX2. Moreover, it represents gene expression of NLRP3 and protein level of IL-1β as well as activity of caspase 1 in cultured hMSCs in the osteogenic medium in the presence of LPS/PA and treated with apigenin. All the groups were cultured in an osteogenic medium for 21 days and then treated with two concentrations of apigenin (25 µM and 50 µM) for 14 days after exposure to LPS/PA. OSX was considered the control group. Data represent the means ± SD. ***P < 0.001 Indicates significant differences in comparison with the control group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, and $^{\#\#\#}P$ < 0.001 versus OSX + LPS/PA. LPS, Lipopolysaccharide; PA, palmitic acid; hMSCs, human mesenchymal stem cells; ALP, alkaline phosphatase; RUNX2, Runt-related transcription factor 2; NLRP3, leucine-rich repeat and pyrin domain containing 3; IL-1β, interleukin-1 beta; Api, apigenin, OSX, osterix.

The gene expression of NLRP3, as well as the protein activity of caspase-1 and the level of IL-1, were measured to corroborate the inflammatory alterations of these cells throughout osteogenesis and treatment. As expected, inflammatory changes in the process of osteogenesis of hMSCs exposed to LPS/PA led to a significant increase in these three factors compared with the control group. The anti-inflammatory effects of apigenin at both concentrations were also observed as a substantial drop in NLRP3 gene expression and caspase-1 activity and IL-1β protein level when compared to OSX+LPS/PA. These findings revealed that inflammation inhibits osteogenesis and apigenin has an osteogenic effect by suppressing inflammation.

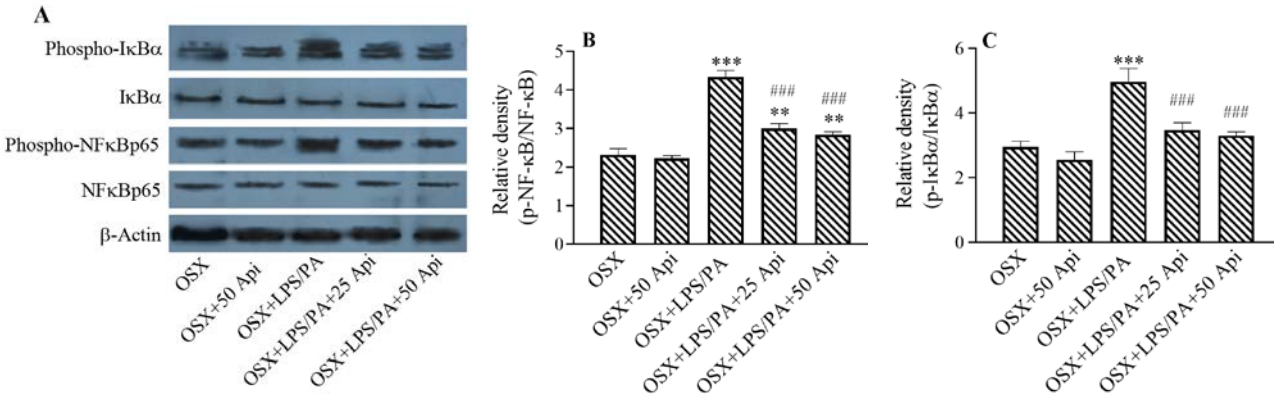

Fig. 4. (A) Western blot density change of p-NF-kB/NF-kB and p-IkBa/IkBa in hMSCs cultured in osteogenic medium and exposed to LPS/PA and apigenin. This experiment was performed in the cells of three donors (n = 3) and each experiment was performed in duplicate. It represents the changes in the protein expression of (B) NF-κB and (C) IkBa in cultured hMSCs in the osteogenic medium in the presence of LPS/PA and treated with apigenin. All the groups were cultured in an osteogenic medium for 21 days and treated with apigenin at 25 and 50 μM for 14 days after exposure to LPS/PA. Data represent the means ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to OSX group; $^{###}P < 0.001$ versus1 OSX + LPS/PA. LPS, Lipopolysaccharide; PA, palmitic acid; hMSCs, human mesenchymal stem cells; Api, apigenin, OSX, osterix; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B.

Investigation of NF-κB/IκBα pathway by western blot analysis

Next, we examined the changes in the expression of NF-κB and IκBα proteins during the osteogenesis process by western blotting (Fig. 4). Accordingly, hMSCs treated with LPS/PA dramatically promoted phosphorylated expression form of NF-κB and IκBα in the osteogenic process when compared with the control group (OSX group). The findings showed that activation of the NF-κB and IκBα pathways might increase inflammation, which is linked to osteogenesis inhibition. The inhibitory impact of apigenin on inflammation was also detected by lowering the phosphorylated levels of NF-κB and IκBα (OSX + LPS/PA + 25/50 Api *vs* OSX + LPS/PA). Inhibition of the active forms of NF-κB and IκBα by apigenin showed that this compound could also increase bone formation by inhibiting inflammation through this pathway.

DISCUSSION

The study's premise was that there is a connection between inflammation and osteoporosis. Osteoporosis is a chronic illness of old age caused by reduced estrogen secretion, which is linked to bone loss and increased fat penetration into the bone marrow, especially in postmenopausal women (24). Also, the role of inflammation on bone turnover and osteoporosis has been identified (25,26), which is also strongly associated with menopause (27).

It has been found that the ability of mesenchymal stem cells in osteogenic differentiation is negatively affected by inflammation (7). Therefore, targeting inflammation is one of the potential strategies to optimize bone repair in the elderly (9).

As a result, mesenchymal stem cells were inflamed with LPS/PA throughout the osteogenic differentiation process in the current investigation, and their behavior was studied under the impact of inflammation. As expected, inflammation disrupted the process of this differentiation. This was in line with prior research on the link between aging and inflammation, as well as the development of osteoporosis (25). Other investigations have properly described the effect of lipopolysaccharide-induced inflammation on inhibiting osteogenesis (7,28). Inflammation was also linked to the activation of the NF-κB/IκBα pathway in our research which is consistent with previous knowledge about the role of the NF-κB pathway in inflammation, which influences osteogenic capacity (28). Our findings revealed a link between the activation of the NLRP3/caspase-1 pathway and up-regulation of NF-κB, which is consistent with previous knowledge. Indeed, the transcriptional

activation of NLRP3, pro-IL-1, and pro-IL-18 is subsequently mediated by NF-κB-dependent pathways following activation of the NLRP3 inflammasome, which typically requires activation of Toll-like receptor 4 or TNF-α (29,30).

However, the new finding in our study is that LPS/PA inhibited osteogenic differentiation *via* NF-κB/IκBα and NLRP3/caspase1 axis and partially identified the role of activation of this pathway in suppressing osteogenesis.

These alterations in osteogenesis were verified using ALP activity, alizarin red staining, and gene expression of RUNX2.

RUNX2, an osteogenic and nuclear transcription factor, regulates bone development (31). Several molecular and genetic investigations have demonstrated the critical function of the RUNX2 transcription factor in this process (32). ALP is an early marker of osteoporosis that is important for bone development and its maximal activity suggests differentiation (33). As a result, the decline in their expression and activity, together with the reduction in calcium deposition seen during staining, supports the hypothesis that inflammation inhibits osteogenesis.

The anti-inflammatory properties of apigenin were investigated in this study. When the stem cells were inflamed with LPS/PA, the anti-inflammatory effects of this flavonoid were seen by considerable down-regulation of NF-κB/IκBα and NLRP3/caspase-1, and IL-1β when compared to the non-treatment group.

Flavonoids are secondary herbal metabolites and phenolic chemicals; some of which have anti-inflammatory properties (34). Structurally related flavonoids such as apigenin, luteolin, and kaempferol showed immunomodulatory and anti-inflammatory effects (35,36)

Due to their intricate biological anti-inflammatory compound, certain flavonoids reduce the activity of pro-inflammatory enzymes such as cyclooxygenase-2 and lipoxygenases (37). Above all, many anti-inflammatory flavonoids exert their effect by altering the expression of transcription factors of inflammatory cytokines and chemokines like IL-1, IL-18, and TNF-α and in fact, they prevent the activation of transcription factors such as NF-κB, activator protein 1, signal transducer and activator of transcription (STAT), and also NLRP3 inflammasome (38). The anti-inflammatory effect of apigenin was exerted through the NF-κB/IκBα pathway (14,39). In the human mast cell line, apigenin decreased the production of IL-8, IL-6, TNF-α, granulocyte-macrophage colony-stimulating factor, and cyclooxygenase-2 *via* reducing intracellular Ca^{2+} levels and blocking NF-κB activation (14). The effect of apigenin in inhibiting epithelial-mesenchymal transition in hepatocellular carcinoma was also observed by blocking the NF-κB/snail pathway (39). The anti-inflammatory effect of this compound was raised to such an extent that it can even be compared to prednisolone (34). Moreover, this flavonoid has been shown to have anti-inflammatory properties in several investigations (38-41), which are achieved by inhibiting the NLRP3 inflammasome (38,40,42), and the NF-κB/IκBα pathway (39, 41).

Apigenin has also been shown to have osteogenic effects as a plant flavone by inhibiting osteoclasts, preventing bone loss, and stimulating osteogenic differentiation of hMSCs (17,43,44). The difference in our study was the investigation of osteogenic effects of apigenin in inflammatory conditions.

CONCLUSION

In the present study, the effect of inflammation on bone differentiation and the effect of apigenin on osteogenesis in inflammatory circumstances were investigated. These effects were attributed to changes in NF-κB/IκBα/NLRP3 expression, caspase-1 activity, and IL-1β levels, along with ALP activity, RUNX2 gene expression, and alizarin red staining. As a result, apigenin's stimulatory impact on osteogenesis can be exerted in two ways: by lowering inflammation and by apigenin's intrinsic effect on differentiation. The importance of flavonoids was also highlighted in this study, with an emphasis on their anti-inflammatory properties. Because of the limitations of the current study in terms of evaluating adipogenic pathways and utilizing pathway inhibitors, more extensive assessments are being investigated in future studies to acquire more complete data.

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