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Avicularin Reduces the Expression of Mediators of Inflammation and Oxidative Stress in Bradykinin-Treated MG-63 Human Osteoblastic Osteosarcoma Cells

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Data Interpretation D
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Background: Avicularin is a plant-derived flavonoid used in traditional Chinese medicine to treat conditions that include ankle fracture. Bradykinin stimulated MG-63 human osteoblastic osteosarcoma cells has previously been studied in an *in vitro* model. This study aimed to investigate the effects of avicularin on bradykinin-treated MG-63 human osteoblastic osteosarcoma cells *in vitro*.

Material/Methods: MG-63 cells were treated with increasing concentrations of avicularin for 48 hours, followed by 1 μ M of bradykinin for 24 h. The MTT assay was used to measure cell viability. Quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to measure the expression of inflammatory mediators, interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF- α) mRNA and protein, respectively. The expression of oxidative stress factors, malondialdehyde (MDA), superoxide dismutase (SOD), and catalase were measured. Western blot and qRT-PCR were performed to determine p38, p65, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) protein levels and mRNA expression, respectively.

Results: Avicularin had no cytotoxic effect on MG-63 cells. Avicularin significantly upregulated the expression levels IL-1 β , IL-6, and TNF- α in the bradykinin treated group in a dose-dependent manner. Avicularin reduced the level of MDA and the activity of SOD and catalase in the bradykinin treated MG-63 cells, reduced p-p38, p-p65, iNOS, and COX-2 expression, and decreased the p-p38/p38 ratio and the p-p65/p65 ratio in bradykinin treated MG-63 cells in a dose-dependent manner.

Conclusions: Avicularin reduced the expression of inflammatory cytokines and the levels of markers of oxidative stress in MG-63 human osteoblastic osteosarcoma cells *in vitro*.

MeSH Keywords: **Ankle Fractures • Bradykinin • Inflammation • Medicine, Chinese Traditional**

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Background

Bone fractures, including ankle fractures, can be a cause of joint damage and are associated with clinical features that include pain, swelling, and bruising of the skin [1]. Currently, the treatment of fractures includes surgical treatment and immobilization with the use of plaster casts [2]. Although most patients receive effective treatment, in some patients, bone fractures fail to set [3]. The majority of fractures occur mainly in older women with osteoporosis [4]. Therefore, timely diagnosis and effective treatment of fractures are essential for relieving pain and improving fracture healing.

Bone formation is a complex process that requires a balance of osteoblast and osteoclast activity, and after fracture, the healing process requires osteoblasts [5]. Previous studies have shown that osteoclast differentiation requires the involvement of inflammatory factors [5,6]. Following a fracture, pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF- α) are associated with the recruitment of osteoclasts [6].

Avicularin (quercetin-3-O- α -L-arabinofuranoside) is a bio-active plant flavonoid that is used in traditional Chinese medicine and is derived from several plant species [7]. Avicularin has antioxidant, anti-allergic, anti-inflammatory, anti-tumor, hepatoprotective activities [8–10]. Guo et al. showed that avicularin might alter multidrug resistance in gastric cancer by increasing the expression of BAX and BOK [11]. Previous studies have shown that avicularin suppressed lipopolysaccharide (LPS)-induced extracellular-signal-regulated kinase (ERK) expression in RAW 264.7 macrophages to exert anti-inflammatory effects [12]. Avicularin has also been shown to inhibit the accumulation of lipid in mouse 3T3-L1 adipocytes [12]. The findings from these previous studies have shown that avicularin has a regulatory effect on the inflammatory response and may inhibit cell growth.

Bradykinin-induced MG-63 human osteoblastic osteosarcoma cells have previously been studied *in vitro* as a cell model of fracture [14–16]. Therefore, this study aimed to investigate the effects of avicularin on bradykinin-treated MG-63 human osteoblastic osteosarcoma cells *in vitro*.

Material and Methods

Cell culture and treatment

MG-63 human osteoblastic osteosarcoma cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) (Cat. No. CRL-1427). The MG-63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan,

UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 50 μ g/ml penicillin, and 50 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were cultured overnight at 37°C and 5% CO₂ in multiwell culture plates until the cells reached 80–90% confluence. MG-63 cells were washed twice in PBS and once in serum-free DMEM. MG-63 cells were treated with increasing doses of avicularin (10 μ M, 30 μ M, 100 μ M, and 300 μ M) for 48 h [17], and 1 μ M of bradykinin for 24 h [14–16].

MTT assay

Cell viability was assessed using the MTT assay to investigate the cytotoxic effect of avicularin on MG-63 cells. MG-63 cells were stimulated with increasing concentrations of avicularin at 10 μ M, 30 μ M, 100 μ M, and 300 μ M for 48 h. Then, 20 μ l of MTT solution (5 mg/ml) was added to each well and incubated for a further 4 h, followed by the addition of 150 μ l of dimethyl sulfoxide (DMSO). The absorbance was measured at 490 nm.

Enzyme-linked immunosorbent assay (ELISA)

Levels of inflammatory cytokines in the cell culture supernatant were detected using an ELISA kit with primary antibodies to tumor necrosis factor- α (TNF- α) (Cat. No. E-EL-H0109c; Elabscience Biotechnology Co., Ltd., Wuhan, Hubei, China), interleukin-1 β (IL-1 β) (Cat. No. E-EL-H0149c; Elabscience Biotechnology Co., Ltd.), and IL-6 (Cat. No. E-EL-H0102c; Elabscience Biotechnology Co., Ltd.).

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

An RNA Rapid Extraction Kit (Takara, Nanjing, China) was used to obtain the total RNA from the MG-63 cells. The cells were lysed with 800 μ l of TRIzol lysis buffer. After centrifugation, the liquid phase was mixed with 1 ml of isopropanol and centrifuged at 12,000 \times g for 10 minutes, and 30 μ l of diethylpyrocarbonate (DEPC) was added to dissolve the RNA precipitate. Then, cDNA was synthesized by RNA using a cDNA reverse transcription kit (Vazyme, Nanjing, Jiangsu, China). The reaction conditions were: 70°C for 5 min, 37°C for 5 min, and 42°C for 60 min. Then, qRT-PCR was performed using the SYBR Green PCR Master kit (Vazyme, Nanjing, Jiangsu, China) in 20 μ l reaction system. Then, transcription was performed with GAPDH as internal control. The primer sequences used were as follows: iNOS, forward: 5'-CACCTTGGAGTTACCCAGT-3'; iNOS, reverse: 5'-ACCACTCGTACTGGGATGC-3'; COX-2, forward: 5'-TCCATTGACCAGAGCAGAGA-3'; COX-2, reverse: 5'-TCTGGACGAGGTTTTCCAC-3'; IL-1 β , forward: 5'-TGTGAAATGCCACCTTTTGA-3'; IL-1 β , reverse: 5'-TGAGTGATACTGCCTGCCTG-3'; TNF- α , forward: 5'-GAACTGGCAGAAGAGGCACT-3';

TNF- α , reverse: 5'-GGTCTGGCCATAGA ACTGA-3';
IL-6, forward: 5'-CCGGAGAGGAGACTTCACAG-3';
IL-6, reverse: 5'-CAGAATTGCCATTGCACA-3';
p38, forward: 5'-GACGAATGGAAGAGCCTGAC-3';
p38, reverse: 5'-AGATACATGGACAAACGGACA-3';
p65, forward: 5'-ACCAACACAGACCCAGGGAGT-3';
p65, reverse: 5'-CAGTACCAGCGAGTTATAG-3';
GAPDH, forward: 5'-GGCATTGCTCTCAATGACAA-3';
GAPDH, reverse: 5'-TGTGAGGGAGATGCTCAGTG-3'.
The $2^{-\Delta\Delta Cq}$ method [18] was used for the quantification of relative gene expression.

Western blot

MG-63 cells were lysed with RIPA lysis buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min to isolate the cellular proteins. The protein concentration was determined using a BCA kit (Pierce Biotechnology, Rockford, IL, USA), and the proteins were stored at -20°C . The proteins were then separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% dried skimmed milk powder in TBS containing 0.1% Tween for 2 h, the membranes incubated overnight at 4°C with the primary antibodies. The primary antibodies were to p38 (1: 1,000) (Cat No. Ab170099; Abcam, Cambridge, MA, USA), p-p38 (1: 1,000) (Cat No. Ab4822; Abcam, Cambridge, MA, USA), p65 (1: 1,000) (Cat No. Ab16502; Abcam, Cambridge, MA, USA), p-p65 (1: 1,000) (Cat No. Ab86299; Abcam, Cambridge, MA, USA), iNOS (1: 1,000) (Cat No. Ab213987; Abcam, Cambridge, MA, USA), and COX-2 (1: 1,000) (Cat No. Ab15191; Abcam, Cambridge, MA, USA). The membranes were incubated with the secondary horseradish peroxidase (HRP)-conjugated antibody (1: 2,000) (Cat No. 7074; Cell Signaling Technology, Danvers, MA, USA) at room temperature for 2 h. The protein bands were detected by enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA). GAPDH (1: 1,000) (Cat No. Ab181602; Abcam, Cambridge, MA, USA) was used as a normalized control. The band density was quantified using Gel-Pro Analyzer densitometry software.

Measurement of markers of oxidative stress

Superoxide dismutase (SOD) activity in the MG-63 was measured using the SOD assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions. The activity of malondialdehyde (MDA) in the MG-63 cells was using an MDA assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions. Catalase was measured in the MG-63 cells using a catalase assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions.

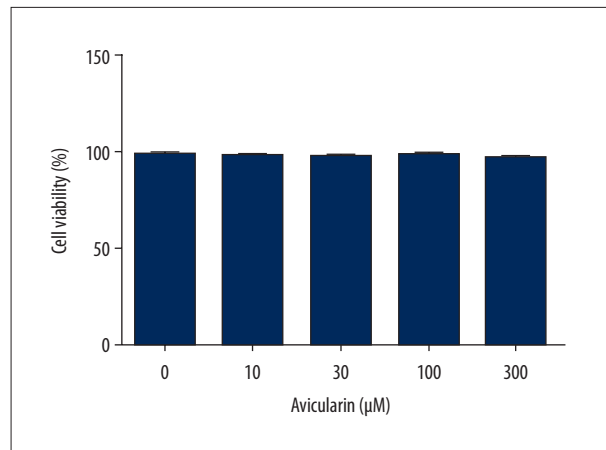


Figure 1. The effects of avicularin on the viability of MG-63 human osteoblastic osteosarcoma cells. MG-63 cells were treated with increasing concentrations of avicularin (10 μM , 30 μM , 100 μM , and 300 μM) for 48 h. Cell viability was measured using the MTT assay. Data are presented as the mean \pm standard deviation (SD) from three independent experiments.

Statistical analysis

Data were expressed as the mean \pm standard deviation (SD) from three independent experiments. Data were analyzed using SPSS version 16.0 statistical software (IBM, Chicago, IL, USA). Student's t-test, one-way analysis of variance (ANOVA), and Tukey's post hoc test were performed. A P-value <0.05 was considered to be statistically significant.

Results

Avicularin had no cytotoxic effect on MG-63 human osteoblastic osteosarcoma cells

MG-63 cells were treated with increasing concentrations of avicularin for 48 h, followed by the MTT assay for cell viability. The viability of MG-63 cells in different groups showed no significant changes, indicating that avicularin did not have a cytotoxic effect on MG-63 cells (Figure 1).

Avicularin reduced the expression of inflammatory cytokines in bradykinin-treated MG-63 human osteoblastic osteosarcoma cells

Enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR) were used to investigate the effect of avicularin on inflammatory cytokines in bradykinin-treated MG-63 cells. The ELISA showed that compared with the untreated group, bradykinin treatment significantly increased the expression levels of interleukin-1 β (IL-1 β),

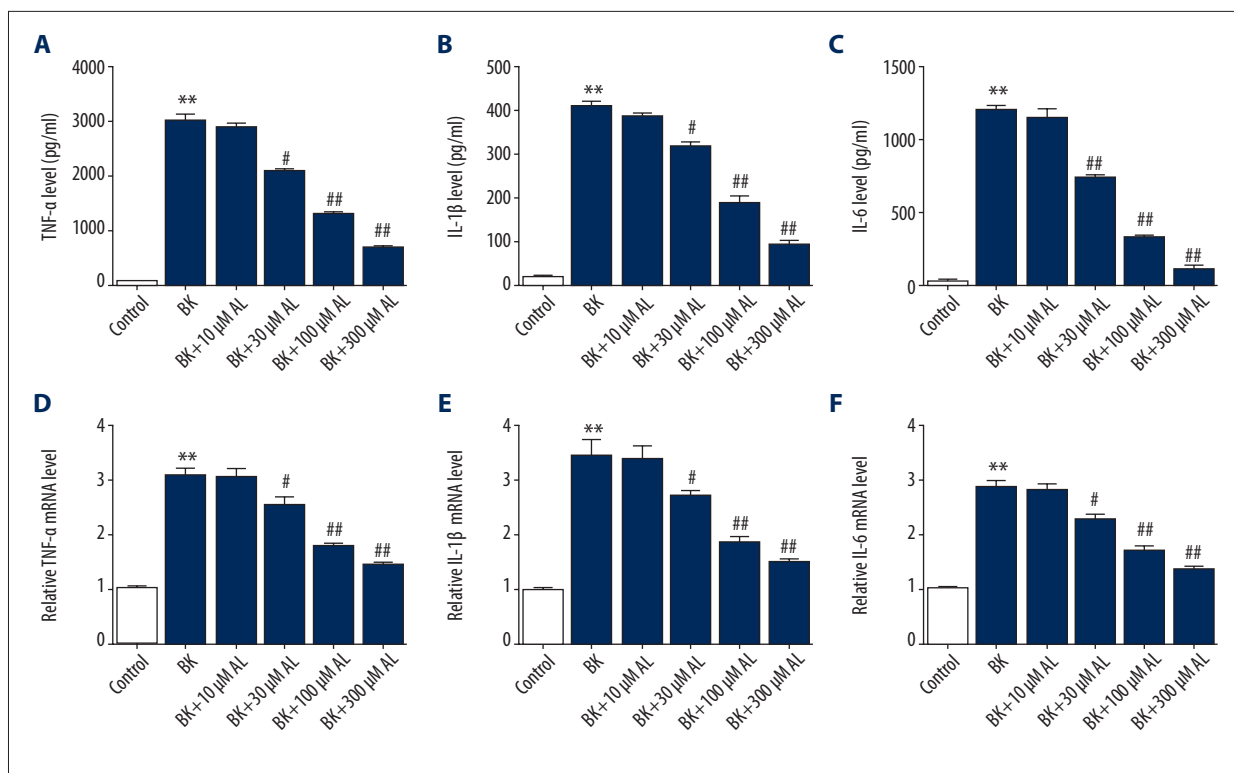


Figure 2. The effects of avicularin on the expression of inflammatory factors in bradykinin-treated MG-63 human osteoblastic osteosarcoma cells. MG-63 cells were treated with increasing concentrations of avicularin (10 μ M, 30 μ M, 100 μ M, and 300 μ M) for 48 h, then treated with 1 μ M bradykinin for 24 h. Enzyme-linked immunosorbent assay (ELISA) was used to measure the expression levels of tumor necrosis factor- α (TNF- α) (A), interleukin-1 β (IL-1 β) (B), and IL-6 (C) in MG-63 cells from the control groups and bradykinin-treated groups. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of TNF- α (D), IL-1 β (E), and IL-6 (F) mRNA expression in MG-63 cells from the control groups and bradykinin-treated groups. Data were presented as the mean \pm SD from three independent experiments. ** p <0.01 vs. the control group; #, ## p <0.05, p <0.01 vs. the bradykinin group.

IL-6, and tumor necrosis factor- α (TNF- α) in MG-63 cells, and avicularin reduced the levels of these inflammatory cytokines in a dose-dependent manner (Figure 2A–2C). Consistent with ELISA results, the qRT-PCR findings showed that the mRNA expression of the inflammatory cytokines were increased in the bradykinin-treated group. The expression of these inflammatory cytokines was reduced with the increasing concentrations of avicularin (Figure 2D–2F).

Avicularin reduced the levels of malondialdehyde (MDA) and increased superoxide dismutase (SOD) and catalase activity in the bradykinin-treated MG-63 human osteoblastic osteosarcoma cells

When compared with the untreated MG-63 cell group, the MDA levels in the bradykinin group were significantly increased, and the SOD and catalase activities were significantly reduced. Avicularin reduced the levels of MDA in a dose-dependent manner (Figure 3A) and increased the activities of SOD and catalase activities in a dose-dependent manner (Figure 3B, 3C).

Avicularin inhibited p38MAPK/NF- κ B pathway activation in the bradykinin-treated MG-63 human osteoblastic osteosarcoma cells

Compared with the untreated group, the protein levels of p-p38 and p-p65 were significantly upregulated in the bradykinin group (Figure 4A), and the p-p38/p38 ratio and p-p65/p65 ratio were reduced (Figure 4B, 4C). Avicularin reduced the protein expression of p-p38 and p-p65 and reduced the ratio of p-p38/p38 and p-p65/p65 in a dose-dependent manner. Also, qRT-PCR showed no significant difference in p38 and p65 mRNA expression between the groups (Figure 4D, 4E).

Avicularin inhibited the expression levels of inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) in bradykinin-treated MG-63 human osteoblastic osteosarcoma cells

Quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to

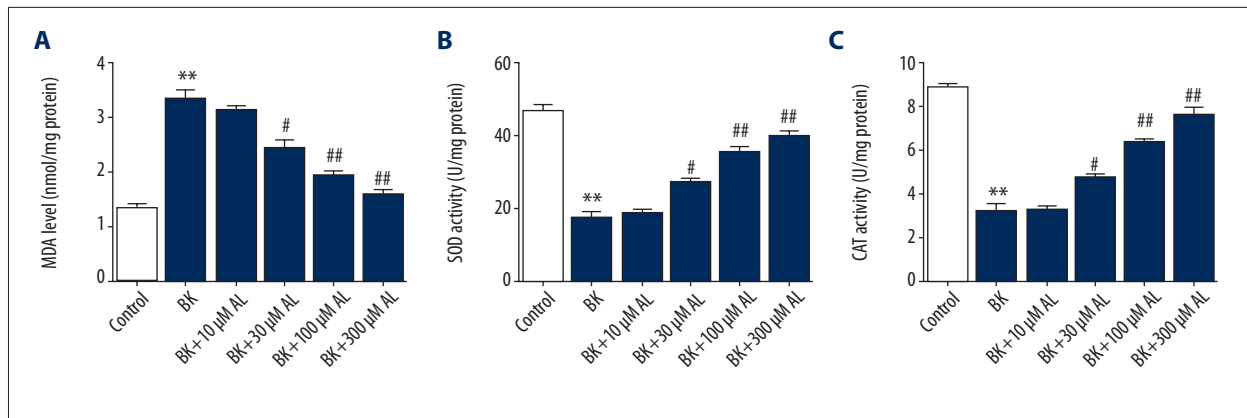


Figure 3. The effects of avicularin on the levels of MDA, SOD, and catalase in bradykinin-treated MG-63 human osteoblastic osteosarcoma cells. Increasing concentrations of avicularin treated MG-63 cells for 48 h, then we used 1 µM bradykinin to treat the cells for 24 h. **(A)** The malondialdehyde (MDA) content was determined by MDA assay kit. **(B)** Superoxide dismutase (SOD) activity was determined by the SOD assay kit. **(C)** Catalase activity was measured using a catalase assay kit. Data are presented as the mean±standard deviation (SD) from three independent experiments. ** $p < 0.01$ vs. the control group; #, ## $p < 0.05$, $p < 0.01$ vs. the bradykinin group.

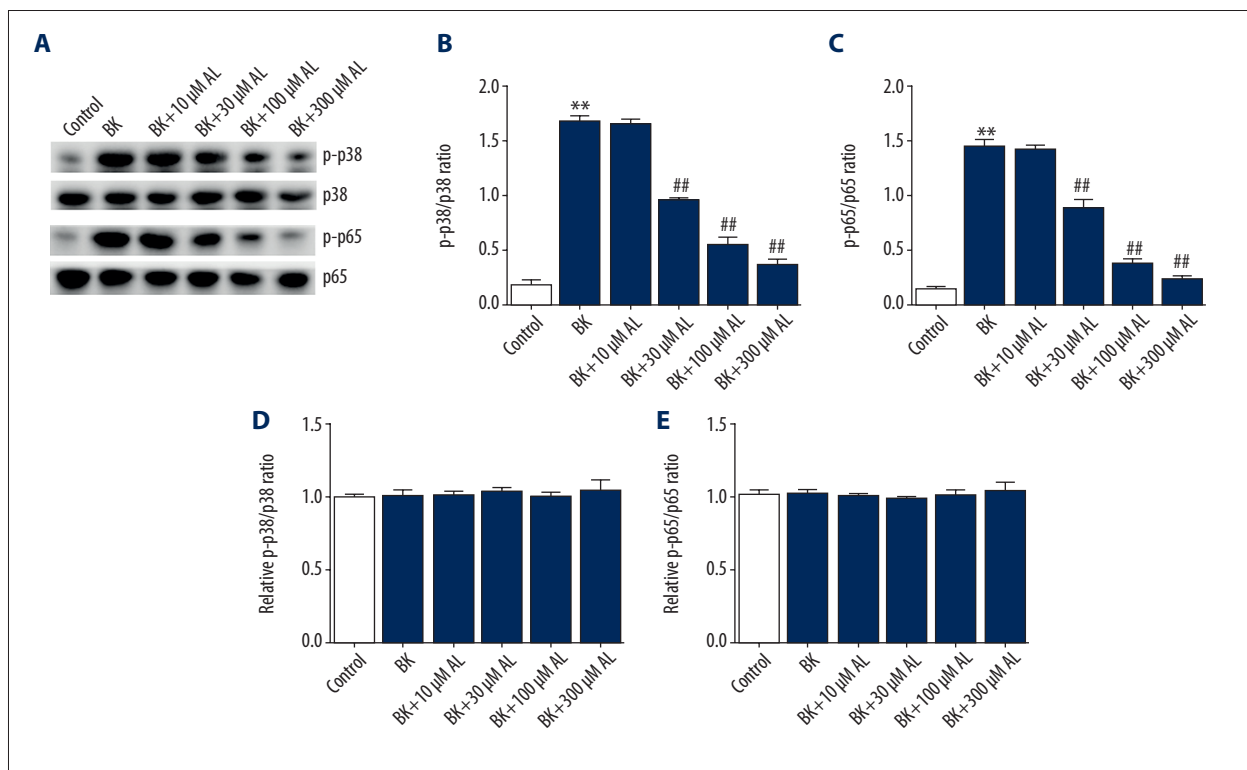


Figure 4. The effects of avicularin on p38MAPK/NF-κB pathway in bradykinin-treated MG-63 human osteoblastic osteosarcoma cells. MG-63 cells were treated with increasing concentrations of avicularin for 48 h, and 1 µM of bradykinin was used to treat the cells for 24 h. **(A)** Western blot detected the expression of p-p38 and p-p65 at the protein level. **(B)** The ratio of p-p38/p38. **(C)** The ratio of p-p65/p65. Quantitative real-time polymerase chain reaction (qRT-PCR) measured the mRNA expression level of p38 **(D)** and p65 **(E)** at the mRNA level. Data are presented as the mean±SD from three independent experiments. ** $p < 0.01$ vs. the control group; #, ## $p < 0.05$, $p < 0.01$ vs. the bradykinin group.

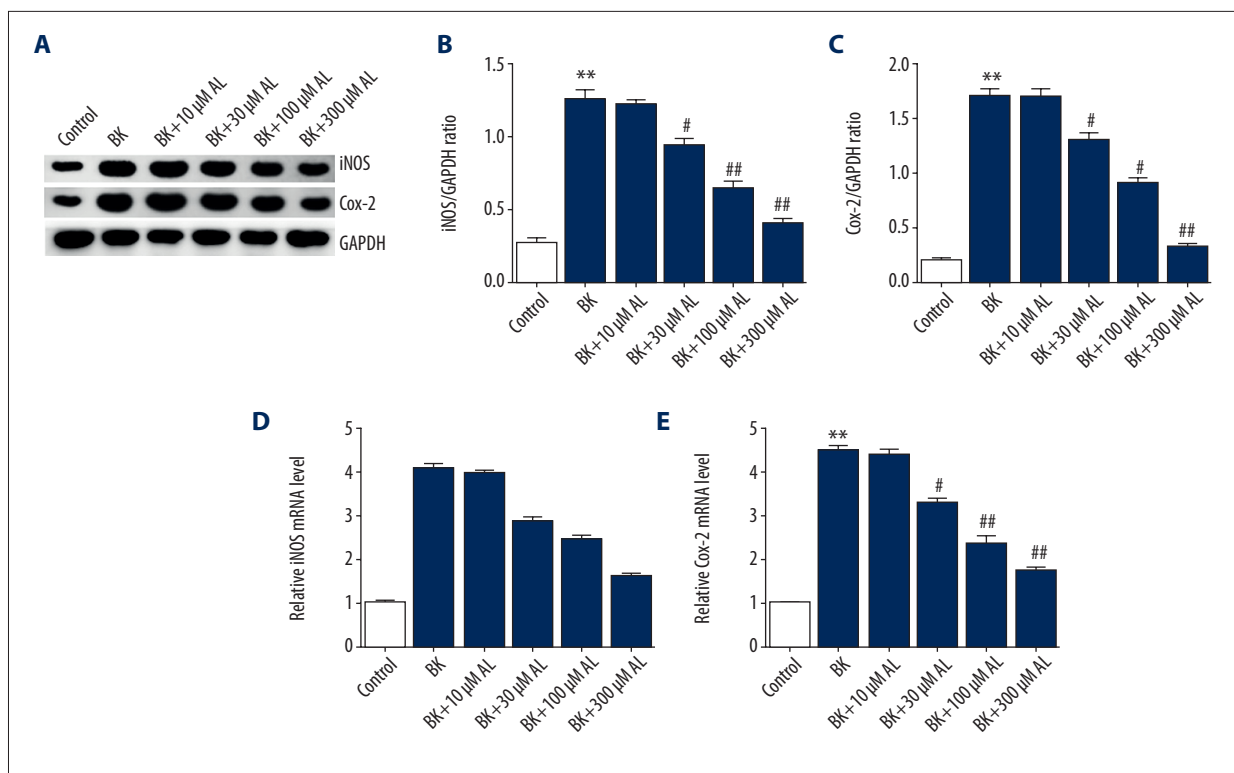


Figure 5. The effects of avicularin on the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in bradykinin-treated MG-63 human osteoblastic osteosarcoma cells. MG-63 cells were treated with increasing concentrations of avicularin for 48 h, and 1 μ M of bradykinin was used to treat the cells for 24 h. (A) Western blot assay was performed to measure the protein expression of iNOS and COX-2. (B) The ratio of iNOS/GAPDH is presented. (C) The ratio of COX-2/GAPDH is presented. Quantitative real-time polymerase chain reaction (qRT-PCR) detected the expression level of iNOS (D) and COX-2 (E) at the mRNA level. Data are presented as the mean \pm standard deviation (SD) from three independent experiments. ** $p < 0.01$ vs. the control group; #, ## $p < 0.05$, $p < 0.01$ vs. the bradykinin group.

detect the expression of iNOS and COX-2. Compared with the untreated group, iNOS and COX-2 proteins were significantly increased in the bradykinin-treated group (Figure 5A–C), and the mRNA expression levels were increased (Figure 5D, 5E). Avicularin reduced the protein and mRNA expression of iNOS and COX-2 in a dose-dependent manner.

Discussion

This study aimed to investigate the effects of avicularin (quercetin-3-O- α -L-arabinofuranoside) on bradykinin-treated MG-63 human osteoblastic osteosarcoma cells *in vitro*. The findings showed that 1 μ M of bradykinin significantly increased the expression levels of inflammatory cytokines in MG-63 cells. However, the levels of the inflammatory cytokines were down-regulated by treatment with avicularin. Avicularin reduced the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and catalase in a dose-dependent manner. Avicularin reduced the increase of p-p38, p-p65, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) following bradykinin treatment.

Fractures of the lower limbs, including ankle fracture, are common and increase in incidence with age [19,20]. Flavonoid compounds have been shown to have multiple biological functions, including neuroprotective, anti-tumor, antioxidant, and anti-inflammatory effects [21,22]. Suematsu et al. showed that quercetin inhibited neuronal apoptosis [23]. Pan et al. showed that quercetin had a protective role in rheumatoid arthritis [24]. Avicularin has previously been shown to suppress intracellular lipid accumulation by reducing CCAAT/enhancer-binding protein (C/EBP) α , and aP2 (fatty acid-binding protein 4) associated with glucose uptake in adipocytes [13]. Shen et al. showed that avicularin reduced neuroinflammation and hippocampal cell apoptosis [25].

Previous studies have shown that inflammatory cytokines, including interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF- α) induced osteoclast differentiation [26–29]. In the present study, the levels of inflammatory cytokines were determined before and after avicularin treatment. The enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR) showed that avicularin

reduced the upregulation of bradykinin-induced inflammatory cytokines. Avicularin reduced the level of malondialdehyde (MDA), superoxide dismutase (SOD), and catalase activities.

Also, inducible nitric oxide synthase (iNOS) that leads to the production of NO, has an important role in several inflammatory diseases [30]. Cyclooxygenase-2 (COX-2) is an inducible enzyme that is expressed at low levels in normal tissues [31]. COX-2 is abundantly expressed during inflammation, responds rapidly to a variety of pro-inflammatory cytokines, and has a key role in the pathogenesis of inflammation. Brechter et al. showed that bradykinin stimulated COX-2 mRNA expression in calvarial bones [32]. In the present study, iNOS and COX-2 were expressed by MG-63 cells following bradykinin treatment, which were significantly suppressed by treatment with avicularin. Also, avicularin reduced p-p38 and p-p65 protein expression and reduced the p-p38/p38 ratio and the p-p65/p65 ratio. These results showed that the mechanism of avicularin in bradykinin-induced MG-63 cells might be associated with the mitogen-activated protein kinase (MAPK) and nuclear

factor- κ B (NF- κ B) signaling pathways. However, this was a preliminary *in vitro* study of avicularin on MG-63 human osteoblastic osteosarcoma cells. The preliminary findings from this study should be supported by further *in vitro* studies using more cell lines, including benign osteoblast cell lines and normal control cell lines, such as fibroblasts.

Conclusions

This study aimed to investigate the effects of avicularin on bradykinin-treated MG-63 human osteoblastic osteosarcoma cells *in vitro*. Avicularin reduced the expression of inflammatory cytokines and the levels of markers of oxidative stress in MG-63 human osteoblastic osteosarcoma cells.

Conflict of interest

None.

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