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Isofraxidin inhibits the proliferation and differentiation of the osteoblast MC3T3-E1 subclone 14 cell line

Junliang Xu, Wei Ji*

Department of Rheumatology and Immunology, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, 210004, Jiangsu Province, China

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ABSTRACT

Ankylosing spondylitis (AS) is an autoimmune inflammatory disease associated with joint inflammation and destruction. Current treatment modalities alleviate symptoms; however, they cannot cure the disease and are associated with significant side effects. Thus, we aimed to confirm the inhibitory effect of isofraxidin, a herbal extract, on pathological osteogenesis in ankylosing spondylitis to better treat patients affected by the disease. Mouse preosteoblast MC3T3-E1 subclone 14 cells were used in vitro to establish control and isofraxidin intervention groups. Cell viability was then determined using the MTT assay; the expression of osteogenic factors, including Runx2, OSX, collagen I, and ALP was measured using qRT-PCR and western blotting. Final osteogenic mineralization was performed by alizarin red staining. The results showed that isofraxidin could inhibit osteoblast viability; however, this effect was nullified at concentrations of 0-20 μM after adding 1% serum. Gene and protein expression of the osteogenic factors RUNX2, OSX, Collagen I, and ALP was inhibited, and a similar trend was exhibited at 7, 14, and 21 days after isofraxidin treatment. This trend was further verified by alizarin red staining of the final osteogenic mineralized nodules on days 7, 14, 21, and 35. Isofraxidin inhibits MC3T3-E1 subclone 14 proliferation and differentiation and may be considered a potential drug therapy for treating pathological osteogenesis in ankylosing spondylitis.

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Introduction

Ankylosing spondylitis (AS) is a family of spondyloarthropathies characterized by radiographic sacroiliitis often accompanied by back pain and joint stiffness. Its pathological mechanism involves autoimmunity, inflammation, bone remodeling, and ankylosis (1). AS has paradoxical clinical manifestations characterized by pathological osteogenesis and osteoporosis. The clinical symptoms of this disease include spinal stiffness and decreased range of motion caused by spinal inflammation, structural damage, and bone remodeling (2). Inflammation also plays a crucial role in abnormal bone metabolism. Relevant studies have reported persistent inflammatory infiltration in the spinal joints of patients with AS (including long-term patients) (3), with osteoclasts being the key cell type mediating inflammatory bone loss (4). The exact mechanism of osteogenesis remains poorly understood. However, it involves inflammation and spontaneous osteogenesis (5).

Non-steroidal anti-inflammatory drugs are generally used to alleviate symptoms of patients with AS by regulating bone metabolism and reducing prostaglandin synthesis through cyclooxygenase activity inhibition (6); however, studies have found that non-steroidal anti-inflammatory drugs negatively regulate fracture healing (7,8). In contrast, biological agents are the primary therapeutic modalities for treating AS. Anti-TNF-α therapy is a long-term and stable biological agent (9); however, studies documenting its effect on alleviating radiographic progression are lacking. Another biological drug, anti-IL-17, which is known to produce immunosuppression and requires constant monitoring for infection, requires more clinical research to demonstrate its efficacy (10-12). Although AS symptoms can be alleviated by non-steroidal anti-inflammatory drugs and biological agents, the disease remains incurable (13). Therefore, novel drug therapies that can treat AS are needed.

Isofraxidin is a traditional Chinese herbal extract from *Acanthopanax senticosus* and has been used in Chinese traditional medicine for treating rheumatism and joint pain. Isofraxidin modulates several key inflammatory factors, including inducible nitric oxide lyase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and tumor necrosis factor-α (TNF-α), and can suppress proliferation and induce apoptosis in human lung cancer cells, with minimal side effects (14). Collectively, isofraxidin is a safe and effective therapeutic agent for treating AS, a disease characterized by concurrent bone erosion and pathological osteogenesis, and involves TNF-α (15), COX-2, and PEG2 pathway (16) activation. Therefore, this drug has various therapeutic properties, including anti-inflammatory (17-23), anti-cancer (14,24,25), heart protective (26), anti-oxidant (27), and anti-osteoclastogenic (28) effects. However, most studies have focused on its role in suppressing inflammation and treating cancer. Besides that, previous research on the use of traditional Chinese medicine has confirmed that isofraxidin has anti-inflammatory and anti-osteoclastogenic properties and that it shows low

* Corresponding author. Email: xiejiaoxi6733@163.com

toxicity, which supports its use in the treatment of inflammation, osteoporosis, and osteogenesis associated with AS. Thus, isofraxidin is thought to suppress AS inflammation, relieve bone erosion, and inhibit osteogenic proliferation and differentiation with minimal toxicity. Despite this information, its effect on osteogenesis remains poorly understood.

Previous studies used MC3T3-E1 cells, a precursor osteoblast, to create an AS model due to its similarity with human osteogenesis (29,30). Herein, MC3T3-E1 subclone 14 cells were used to mimic pro-osteoblast proliferation, morphological differentiation to osteoblasts, and the osteogenic mineralization process. Furthermore, we investigated the effect of isofraxidin on the proliferation and differentiation of MC3T3-E1 subclone 14 cells.

Materials and Methods

Cell culture and osteogenic induction

Isofraxidin (structural formula in Figure 1) was obtained from Solarbio (China), andMC3T3-E1 subclone 14 cells were purchased from the Chinese Academy of Cell Sciences, which cultured in the Minimum Essential Medium Alpha (MEM-α) (VivaCell, China), containing 10% fetal bovine serum (VivaCell, China) and 5% CO_2, at 37°C for 24 h. The culture medium was then changed to MEM-α containing 5 mM β-glycerophosphate (Sigma, Merck KGaA Germany) and 50 μg/mL vitamin C (Sigma, Merck KGaA) to induce osteogenesis.

MTT assay

MTT (Sangon Biotech, China) was dissolved in PBS at a concentration of 5 mg/mL. Cells (5×10^3) were seeded in 96-well plates and cultured at 37 °C in a 5% CO_2 incubator. They were then treated with MEM-α without FBS for 24, 48, and 72 h, after which the medium was removed. The initial MTT solution (5 mg/mL) was diluted 10-fold to a concentration of 0.5 mg/mL. The MTT working solution was added in 200 μL aliquots to each well and incubated for 2 h at 37 °C in a 5% CO2 incubator. The MTT working solution was discarded, and 100 μL DMSO was added to each well and incubated at 37 °C for 10 min. The absorbance at 570 nm was measured via a microplate reader.

Alizarin red staining

To assess calcium deposition in bone nodules in the extracellular matrix, alizarin red (Beyotime, China) was used to stain the mineralized nodules in the cellular matrix. Cells were seeded in 6-well plates and treated with 0 μM and 20 μM isofraxidin for 7, 14, 21, 28, and 35 days. The medium was then removed, and the cells were washed twice with PBS, fixed in an absolute ethanol fixative for 20 min, and then removed. The fixed cells were washed twice with ionized water, and 1 mL of alizarin red was added to each well for 30 min. The solution was then rinsed twice with PBS. Mineralization results were observed under an inverted microscope (Lecia DMI5000M, Germany).

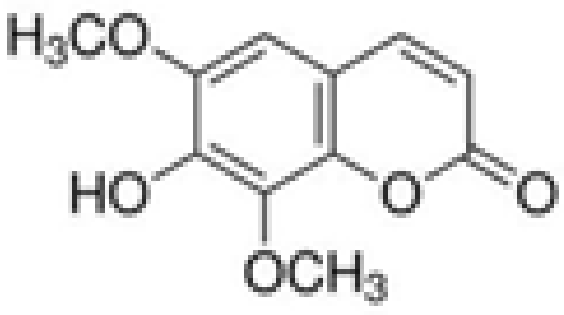

Figure 1. Structure of isofraxidin.

qRT-PCR

The cells were treated with isofraxidin for 7, 14, and 21 days. RNA samples were isolated by the TRIzol reagent (Life Technologies, US) and reverse transcribed into cDNA by a reverse transcription kit (Yisheng, China). The SYBR Green PCR Master Mix (Yisheng, China) was used to amplify cDNA. Expression levels of the four genes *Runx2*, *OSX*, collagen I, and *ALP* were quantified using a real-time system. The primer sequences for genes are listed in Table 1.

Western blotting

Western and IP cell lysis (Beyotime, China) were used to extract total protein, and a BCA kit (Beyotime, China) was used to determine its concentration. Protein samples (50 μg/lane) were separated using 12% SDS-PAGE, then transferred to PVDF membranes and blocked with 5% BSA for 2 h at room temperature (maintained at 24 °C). The membranes were incubated overnight at 4 °C with the primary antibodies against the following proteins: Osterix (ab209484; 1:1000; Abcam, UK), RUNX2 (ab236639; 1:1000; Abcam, UK), ALP (13199-2-AP; 1:1000; Proteintech, China), Collagen I (ab270993; 1:1000; Abcam, UK), and GAPDH (1:1,000; Goodhere Biotechnology, China). Membranes were washed trice with TBS containing 0.05% Tween-20 (TBST). Secondary antibodies anti-rabbit (Lianke, Hangzhou, China) were added for 2 h at room temperature. After washing the trice with TBST, protein bands were observed using ECL, then detected via Tanon 5200 Multi imager (Tanon Science & Technology, China).

Statistical analysis

Data analysis was performed using GraphPad Prism v5.0. Each experiment was performed independently for at least three times, and all results are presented as mean ± SEM. Student's t-test and two-way ANOVA test were used to assess statistical significance. $P<0.05$ was considered significant.

Results

Effects of isofraxidin on the viability and proliferation of MC3T3-E1 cells

MC3T3-E1 subclone 14 cells were treated with dif-

Table 1. Primer sequences of genes Runx2, OSX, collagen I, and ALP.

Gene	Sequence (5'to3')
GAPDH	Forward: GTGTTTCCTCGTAGA
	Reverse: CCTTGACTGTGCCGTTGAAT
ALP	Forward: TCCGTGGGCATTGTGACTAC
	Reverse: TGGTGGCATCTCGTTATCCG
Osterix	Forward: AAACATCAGCGCACCCA
	Reverse: GCAGGCGAAGTGGAAGAT
RUNX2	Forward: CTTCGTCAGCATCCTATCAGTTCC
	Reverse: GTCAGCGTCAACACCATCATTCT
Collagen	Forward: AGAGCATGACCGATGGATTC
	Reverse: CCTTCTTGAGGTTGCCAGTC

ferent isofraxidin (0–225 μM) and serum (0% and 1%) concentrations for 24, 48, and 72 h. Isofraxidin significantly inhibited cell viability in the absence of serum (P<0.01) (Figures 2 and 3). In contrast, 0-20 μM concentrations of isofraxidin had no apparent inhibitory effects following 1% serum administration (Figure 4). An isofraxidin concentration of 20 μM was selected for subsequent experiments to demonstrate the effect of isofraxidin on cells.

Effects of isofraxidin on the expression of bone markers' gene

The transcription factors, OSX, Collagen I, ALP, and Runx2, involved in osteoblast differentiation and bone formation were measured at 7, 14, and 21 days following isofraxidin treatment (Figure 5). The expression of all four genes was inhibited and displayed similar trends (P<0.01).

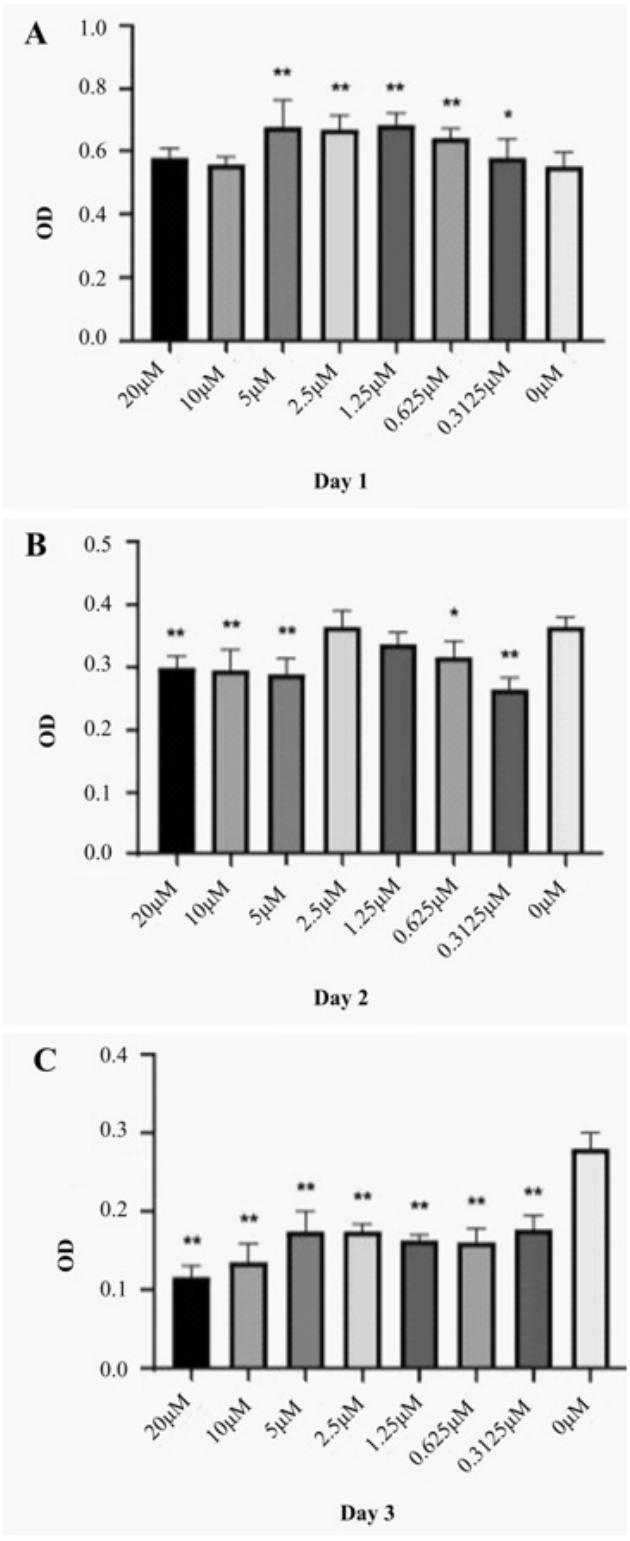

Figure 2. MC3T3-E1 subclone 14 treated with 0–20 μM isofraxidin without serum for 1, 2, and 3 days. *P<0.05; **P<0.01.

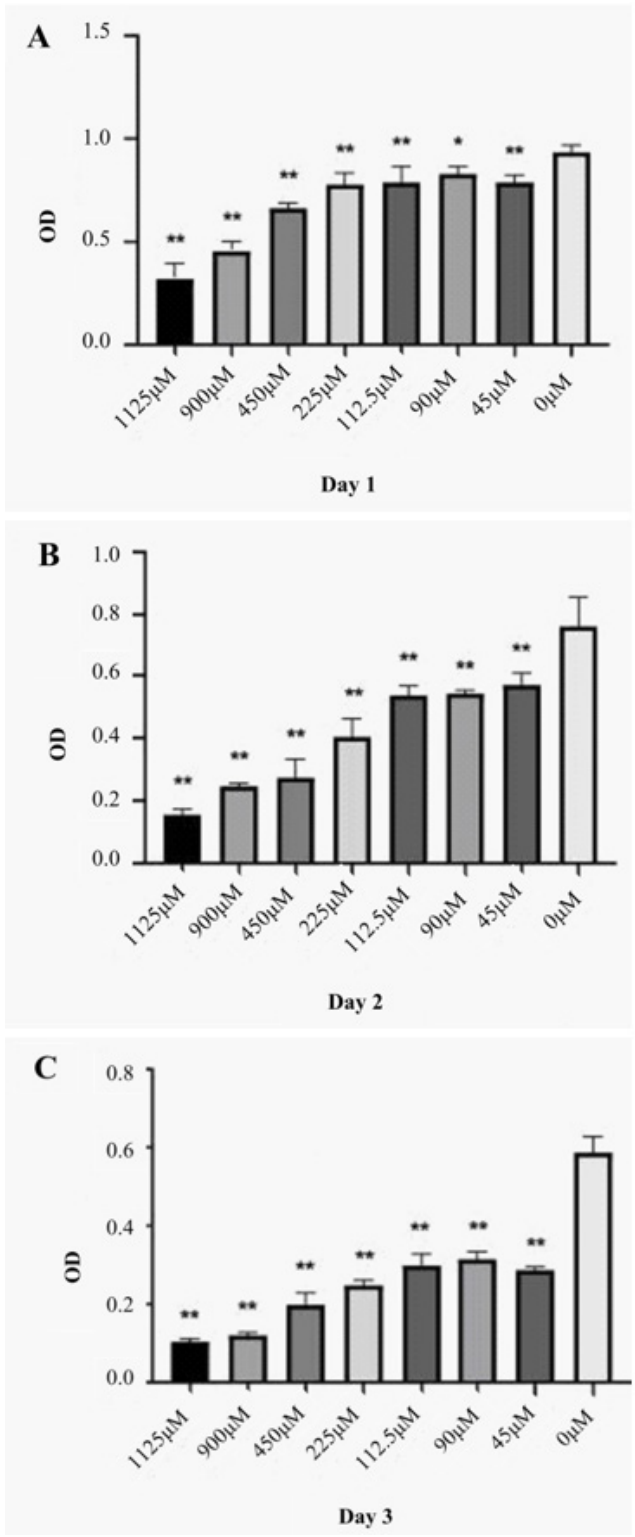

Figure 3. MC3T3-E1 subclone 14 treated with 0 μM and 45–225 μM isofraxidin without serum for 1, 2, and 3 days. *P<0.05; **P<0.01.

Effects of isofraxidin on mineralization

To examine the effect of isofraxidin on osteoblast mineralized nodules, MC3T3-E1 subclone 14 cells were cultured in an osteogenic inducing medium containing 0 μM and 20 μM isofraxidin. Changes in calcium deposition were displayed by alizarin red staining at 7, 14, 21, 28, and 35 days. The staining intensity increased in a time-

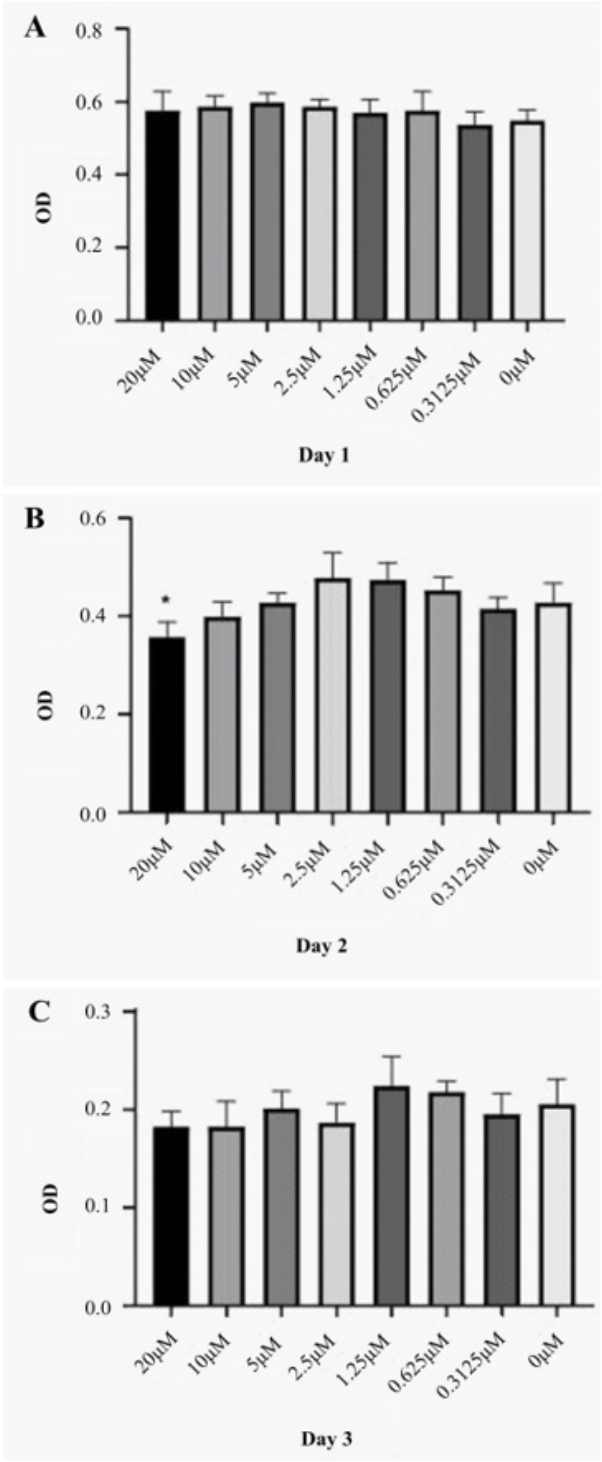

Figure 4. MC3T3-E1 subclone 14 treated with 0–20 μM isofraxidin with 1% serum for 1, 2, and 3 days. *P<0.05; **P<0.01.

dependent manner, and it was weaker in the 20 μM group than in the 0 μM group. Thus, isofraxidin inhibits cell differentiation and mineralization (Figure 6).

Effects of isofraxidin on the expressions of osteogenic proteins of MC3T3-E1 cells

ALP, Collagen I, OSX, and Runx2 protein expression was determined in the control and isofraxidin intervention groups at 7, 14, and 21 days, all of which were weaker in the 20 μM group than in the control group (Figure 7).

Discussion

The anti-inflammatory, anti-cancer, and anti-osteoclastogenic (28) effects of isofraxidin, coupled with its minimal side effect profile (14), have been reported in previous studies. However, its role in regulating osteoblast activity has not yet been elucidated. In this study, MC3T3-E1 subclone14 cells were treated with 0–225 μM isofraxidin, and isofraxidin could inhibit cell activity at concentrations of 0–20 μM. This trend gradually decreased as the concentration increased from 20–225 μM and was completely lost following 1% serum administration. In the following experiments, the 20 μM and 225 μM concentrations were used to examine and detect. Runx2, as it is highly expressed in the bone tissue of AS patients and examining it at minimum and maximum concentrations will reveal its comprehensive profile (31). Runx2 plays a major role in osteogenic differentiation, which can directly regulate OSX expression. OSX is a downstream osteogenic differentiation factor and regulates collagen I expression, a bone matrix protein gene, through p38 and ERK phosphorylation (32-34). In this study, Runx2, OSX, collagen

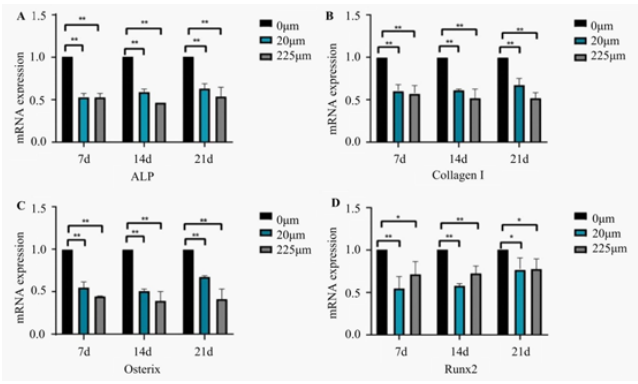

Figure 5. Alkaline phosphatase, collagen I, osterix, and *Runx2* gene expression in MC3T3-E1 cells after isofraxidin treatment for 7, 14, and 21 days. *P<0.05; **P<0.01.

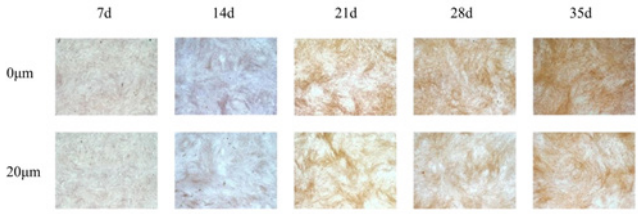

Figure 6. Alizarin Red staining (A) of MC3T3-E1 subclone 14 cells after isofraxidin treatment for 7, 14, 21, 28, and 35 days.

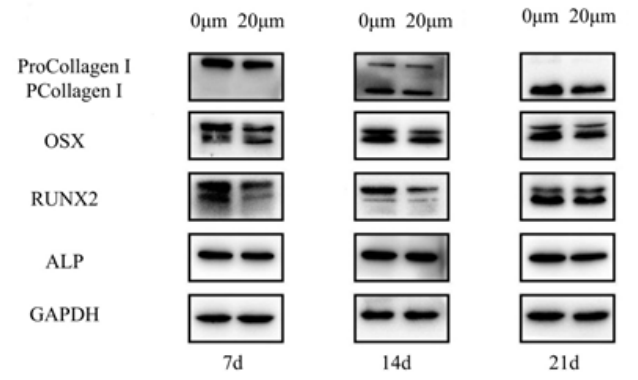

Figure 7. Collagen I, osterix, alkaline phosphatase, and Runx2 protein expression in MC3T3-E1 cells after isofraxidin treatment for 7, 14, and 21 days..

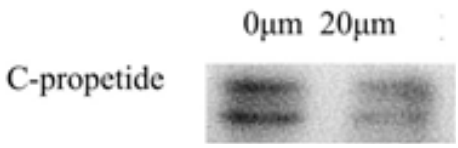

Figure 8. C-propeptide, which is cleaved by collagen I, protein expression in MC3T3-E1 cells after isofraxidin treatment for 14 days.

I, and osteogenic biomarker ALP expression levels were inhibited following isofraxidin treatment, as detected by qRT-PCR and western blotting. Moreover, through MTT and qRT-PCR, isofraxidin was shown to inhibit osteoblasts. The 225 μM concentration group was considered toxic to cells. Thus, alizarin red staining and western blotting were not explored deeply.

The osteogenic marker protein collagen I is a triple helix molecule. The N-terminal can be divided into N-terminal peptide and N-propeptide, whereas the C-terminal can be divided into C-terminal peptide and C-propeptide. Collagen I secreted by osteoblasts is rapidly cleaved in the matrix, shedding N-terminal and C-terminal peptides. Mature collagen I then recombine in the cell matrix (35,36). Different collagen I states were detected on days 7, 14, and 21 of osteogenesis, whereby collagen I was uncleaved at day 7 (proCollagen I), and pCollagen I (N front-end peptide) was found at the vigorous stage of osteogenesis on day 14. Cells were in a mature state at 21 days, wherein collagen I had recombined in the matrix, and uncleaved proCollagen I was not detected in cell lysate. The above process is consistent with osteoblast differentiation. In addition, C-propeptide, which could not be cleaved by collagen I, was not detected on day 7. On days 14 and 21, only low C-propeptide expression and a miscellaneous band were observed, which may have been caused by incomplete collagen I processing between osteoblasts. There was not enough repeated data to accurately and comprehensively explore this entity, and C-propeptide was only present on day 14 during peak osteoblast differentiation (Figure 8).

This study has certain limitations. It does not incorporate sufficient in vitro analysis. Also, the specific role of isofraxidin in osteogenesis under the inflammatory microenvironment is not completely understood, and in vivo studies are required to comprehensively explore this mechanism.

Nevertheless, based on the presented results coupled with the chronic inflammation, pathological osteogenesis, and osteoporosis associated with AS, isofraxidin may be a potential therapeutic drug for treating this disease in the future.

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The authors declare no conflict of interest.

Consent for publication
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Authors' contributions
Substantial contributions to conception and design: WJ, JX
Data acquisition, data analysis and interpretation: WJ
Drafting the article or critically revising it for important intellectual content: WJ, JX
Final approval of the version to be published: All authors
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors

Data Availability
All data generated or analyzed during this study are included in this published article.

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