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Original Article

Isorhamnetin Downregulates MMP2 and MMP9 to Inhibit Development of Rheumatoid Arthritis through SRC/ERK/CREB Pathway*

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ABSTRACT **Objective**: To investigate the effect of isorhamnetin on the pathology of rheumatoid arthritis (RA). **Methods**: Tumor necrosis factor (TNF)-α-induced fibroblast-like synoviocytes (FLS) was exposed to additional isorhamnetin (10, 20 and 40 μmol/L). Overexpression vectors for matrix metalloproteinase-2 (MMP2) or MMP9 or SRC were transfected to explore their roles in isorhamnetin-mediated RA-FLS function. RA-FLS viability, migration, and invasion were evaluated. Moreover, a collagen-induced arthritis (CIA) rat model was established. Rats were randomly divided to sham, CIA, low-, medium-, and high-dosage groups using a random number table (n=5 in each group) and administered with normal saline or additional isorhamnetin [2, 10, and 20 mg/(kg·day)] for 4 weeks, respectively. Arthritis index was calculated and synovial tissue inflammation was determined in CIA rats. The levels of MMP2, MMP9, TNF-α, interleukin-6 (IL-6), and IL-1β, as well as the phosphorylation levels of SRC, extracellular regulated kinase (ERK), and cyclic adenosine monophosphate response element-binding (CREB), were detected in RA-FLS and synovial tissue. Molecular docking was also used to analyze the binding of isorhamnetin to SRC. **Results**: In *in vitro* studies, isorhamnetin inhibited RA-FLS viability, migration and invasion (P<0.05). Isorhamnetin downregulated the levels of MMP2, MMP9, TNF-α, IL-6, and IL-1β in RA-FLS (P<0.05). The overexpression of either MMP2 or MMP9 reversed isorhamnetin-inhibited RA-FLS migration and invasion, as well as the levels of TNF-α, IL-6, and IL-1β (P<0.05). Furthermore, isorhamnetin bound to SRC and reduced the phosphorylation of SRC, ERK, and CREB (P<0.05). SRC overexpression reversed the inhibitory effect of isorhamnetin on RA-FLS viability, migration and invasion, as well as the negative regulation of MMP2 and MMP9 (P<0.05). In *in vivo* studies, isorhamnetin decreased arthritis index scores (P<0.05) and alleviated synovial inflammation. Isorhamnetin reduced the levels of MMP2, MMP9, TNF-α, IL-6, and IL-1β, as well as the phosphorylation of SRC, ERK, and CREB in synovial tissue (P<0.05). Notably, the inhibitory effect of isorhamnetin was more pronounced at higher concentrations (P<0.05). **Conclusion**: Isorhamnetin exhibited anti-RA effects through modulating SRC/ERK/CREB and MMP2/MMP9 signaling pathways, suggesting that isorhamnetin may be a potential therapeutic agent for RA.

KEYWORDS rheumatoid arthritis, isorhamnetin, SRC/ERK/CREB, matrix metalloproteinase

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is clinically characterized by synovial inflammation and joint damage.(1) As RA progresses, there is a marked increase in the number of fibroblast-like synoviocytes (FLS); these become an important component of the invasive hyperplastic mass known as pannus.(2) The hyperproliferation of RA-FLS, along with apoptotic defects, are generally considered the pathological basis of RA.(3) RA-FLS can also secrete inflammatory cytokines and exacerbate the inflammatory response, ultimately promoting the loss

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of joint function.[4] Therefore, inhibiting the aggressive behavior of RA-FLS is believed to ameliorate the pathogenesis of RA. Matrix metalloproteinases (MMPs) are key enzymes that degrade the extracellular matrix (ECM) and are known to be secreted by RA-FLS. Evidence indicates that the upregulation of MMP2 and MMP9 promotes RA-FLS migration and invasion,[5,6] thus suggesting that MMPs could be targeted for the regulation of RA-FLS migration and invasion.

Isorhamnetin is a natural flavonoid compound found in abundance in the fruits of *Hippophae rhamnoides* L. and *Ginkgo biloba* L. Previous studies have demonstrated that isorhamnetin exhibits excellent pharmacological properties for the treatment of cardiovascular diseases and various types of tumors because of its anti-inflammatory, anti-oxidation, anti-apoptosis, anti-proliferation, and anti-migration activities.[7,8] A previous study reported that isorhamnetin significantly reduced the secretion of inflammatory cytokines in collagen-induced arthritis (CIA) mice and alleviated the inflammatory phenotype of these mice.[9] However, the capability of isorhamnetin to protect against RA remains largely unknown.

SRC/extracellular regulated kinase (ERK) pathway is involved in various physiological cell processes and tumor progression.[10,11] For example, activated SRC/ERK1/2 signaling upregulates the expression of MMP14 to promote the migration and invasion of human osteosarcoma cells.[12] SRC/ERK pathway has also been shown to mediate the proliferation and apoptosis of RA-FLS.[13] Interestingly, isorhamnetin has been shown to inhibit the activity of SRC to prevent the occurrence of colorectal cancer.[14] Isorhamnetin can also reduce the phosphorylation level of ERK to control the proliferation, differentiation, and apoptosis of pancreatic cancer cells.[15] In addition, cyclic adenosine monophosphate response element-binding (CREB) is widely known to regulate the expression of inflammatory cytokines.[16] Previous study has shown that the p-CREB/CREB ratio is significantly upregulated in the CIA model.[17] Isorhamnetin has been shown to partially restore the phosphorylation level of CREB in the high-fat and high-fructose diet model.[18] Thus, ERK/CREB signaling pathway holds significant potential for protecting against cardiovascular disease and neuroinflammation.[19-21] However, the role of isorhamnetin in protecting against RA by modulating the SRC/ERK/CREB pathway has yet to be investigated.

In the present study, we investigated the potential role of isorhamnetin in the development of RA. Our study highlighted the potential involvement of the SRC/ERK/CREB and MMP2/MMP9 signaling pathways in the progression of RA in a manner that was regulated by isorhamnetin. These findings suggest a possible new approach for treating RA.

METHODS

Drugs and Reagents

Isorhamnetin was product of Aladdin, Shanghai, China (Cat No. 109591). To prepare the bovine type Ⅱ collagen emulsion, bovine type Ⅱ collagen (Cat No. 20022, Chondrex, USA) was completely dissolved in a 0.05 mol/L solution of acetic acid to a final concentration of 2.0 mg/mL by stirring overnight at 4 ℃; this was followed by mixing with Freund's complete adjuvant (Cat No. 7001, Chondrex, USA) in an ice bath at a ratio of 1:1 and thorough emulsification.

Cell Culture and Treatment

RA-FLS (Cat No. AW-CNH477) were purchased from Abiowell (Changsha, China) and cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 ℃ in a CO_2 incubator. As previously described,[25,26] cells were stimulated with 10 ng/mL of tumor necrosis factor (TNF)-α for 24 h to establish an *in vitro* model of RA. Subsequently, RA-FLS were divided to low-, medium-, and high-dosage groups and treated with 10, 20, and 40 μmol/L of isorhamnetin, respectively.[27] The control group received no treatment. The optimal treatment concentration for isorhamnetin was found to be 40 μmol/L, which was used for subsequent experiments.

RA-FLS were treated with 40 μmol/L of isorhamnetin to form the isorhamnetin (Iso) group. In accordance with the manufacturer's instructions, lipofectamine 2000 reagent (Cat No. 11668-019, Invitrogen, CA, USA) was used to transfect MMP2-overexpressed (oe-MMP2) or oe-MMP9 plasmids (HonorGene, Changsha, China) into RA-FLS to explore the regulatory effects of MMP2/MMP9 on isorhamnetin-mediated RA progression.

RA-FLS were also treated with 40 μmol/L of isorhamnetin to form the Iso group. In addition, oe-NC and oe-SRC plasmids (HonorGene, Changsha, China)

were transfected into RA-FLS to evaluate the effect of SRC on isorhamnetin-mediated RA.

Cell Counting Kit-8 Assay

RA-FLS viability was assessed by cell counting kit-8 (CCK-8) assay in accordance with the manufacturer's instructions (CK04, Dojindo, Japan). A total of 5×10^3 cells per well were seeded in a 96-well plate in a volume of 100 μL and each well was treated with 10 μL of CCK-8. Cells were then incubated at 37 ℃ for 4 h and optical density (OD) values were determined at 450 nm.

Wound Healing Assays

RA-FLS were digested with trypsin and added to a 6-well plate with horizontal lines (5×10^5 cells/well). Once the cells completely covered the bottom of the well, a sterile pipette tip was used to create 2 straight scratches along the horizontal lines. The scratched cells were washed off with phosphate buffer solution (PBS) and replaced with low-serum DMEM medium for incubation at 37 ℃. Images were captured at 0, 24, and 48 h, and the width of the area not covered by cells (defined as the width value, in μm) was recorded.

Transwell Assay

Transwell chambers (Cat No. 3428, Corning, USA) were coated with 200 μg/100 μL of Matrigel (Cat No. 354262, Corning, USA) and incubated at 37 ℃ for 30 min. The supernatant was then removed, and the lower chamber was filled with 500 μL of culture medium. Then, a total of 2×10^6 cells/100 μL of RA-FLS were added to the chamber and incubated for 48 h at 37 ℃. Subsequently, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min, and then stained with 0.1% crystal violet (Cat No. AWC0333, Abiowell) for 5 min. Invasive cells were observed under a microscope, and the absorbance was detected and recorded at 550 nm after decolorization.

Western Blot

Total protein was extracted from RA-FLS and synovial tissue of CIA rats using RIPA (Cat No. AWB0136, Abiowell, Changsha, China) and quantified using a bicinchoninic acid assay kit (Cat No. AWB0104, Abiowell, Changsha, China). The extracted protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight at 4 ℃ with primary antibodies, including MMP2 (1:500, Cat No. 10373-2-AP, Proteintech, USA), MMP9 (1:5,000, Cat No. ab76003, Abcam, UK), p-SRC (Cat No. 1:5,000, ab185617, Abcam), SRC (Cat No. 1:500, Cat No. ab47405, Abcam), p-ERK (1:3,000, Cat No. 28733-1-AP, Proteintech), ERK (1:2,000, Cat No. 16443-1-AP, Proteintech), p-CREB (1:5,000, Cat No. ab32096, Abcam), CREB (1:1,500, 12208-1-AP, Proteintech), and β-actin (1:5,000, Cat No. 66009-1-Ig, Proteintech). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, including anti-mouse (1:5,000, Cat No. SA00001-1, Proteintech) and anti-rabbit (1:6,000, Cat No. SA00001-2, Proteintech) for 90 min at room temperature. Following this, the membrane was co-incubated with enhanced chemiluminescence Plus (Cat No. AWB0005, Aiowell Changsha, China) for 1 min, and protein bands were visualized and analyzed by a gel imaging system (ChemiScope6100, CLiNX, Shanghai, China).

Immunofluorescence Analysis

RA-FLS were fixed with 4% paraformaldehyde and permeabilized by 0.3% Triton X-100 at 37 ℃ for 30 min. After blocking with 5% bovine serum albumin for 1 h, primary antibodies including MMP2 (1:50) and MMP9 (1:50) were added and incubated overnight at 4 ℃. The cells were then incubated with anti-rabbit antibody (1:100, Cat No. SA00013-2, Proteintech) for 90 min, followed by 4',6-diamidine-2-phenylindole dihydrochloride to stain the nuclei. Representative images of cells were then captured using a fluorescent microscope (BA210T, Motic, Xiamen, China).

Molecular Docking

The structure of SRC protein was downloaded from the Protein Data Bank database (https://www.rcsb.org/), and the three-dimensional (3D) structure of isorhamnetin was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/compound/5281654). The binding of isorhamnetin and SRC was investigated using Autodock Vina software, which uses semi-empirical free energy fields to predict the binding energies of receptors and ligands. Water molecules were removed, non-polar hydrogens were added, Gasteiger charges were calculated, and AutoDock 4 types were assigned. The total charge of the ligand molecule was adjusted, and the rotatable bonds by the ligand were selected. Docking information files were created, energy_range was set to 4, and the software was used to determine the docking result. Subsequently, Discovery Studio was used to visualize

two-dimensional (2D) and 3D images.

Animal Modeling and Grouping

Twenty-five Wistar male rats, weighing 170–200 g, were procured from CSTQSW Co., Ltd. [Changsha, China, SCXK (Xiang) 2022-0011)] and housed at constant room temperature (22±2 ℃) and humidity (60%±5%) under a 12-h light/dark cycle with standard rodent food and water. Rats were randomly grouped into sham, CIA, low-, medium-, and high-dosage groups using a random number table (n=5 in each group). As previously reported,[22] rats were injected with 300 μL of collagen type Ⅱ emulsion at the base of tail to establish the CIA model and mimic the characteristics of RA. On day 7 after the initial injection, 300 μL of bovine type Ⅱ collagen emulsion was injected to reinforce the intervention. Rats in the low-, medium-, and high-dosage groups received an additional intraperitoneal injection of isorhamnetin at doses of 2, 10, and 20 mg/(kg·d), respectively, once a day for 4 weeks;[23] rats in the sham group received the same dose of normal saline. Joint swelling in rats was monitored every 4 days during modeling, and the arthritis score (scored as 1–8 points on 2 hind limbs) was used as the assessment criterion.[24] The suffering of animals was minimized during the operation. After modeling, the rats were injected intraperitoneally with 50 mg/kg sodium pentobarbital and euthanized by cervical dislocation, and the knee joints and synovial tissues were collected for analysis.

This study was approved by the Animal Experiment Ethics Committee of Hunan University of Chinese Medicine (No. LL2022061401) and conducted in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

Hematoxylin-Eosin Staining

Synovial tissues were collected from CIA rats and subsequently processed by embedding, dewaxing and sectioning. Hematoxylin-eosin (HE) staining was then performed on the tissues. Next, the sections were immersed in xylene, sealed with neutral gum, and assessed for morphological changes by microscopy.

Enzyme-Linked Immunosorbent Assay

The operating instructions were followed to measure the levels of TNF-α, interleukin (IL)-6, and IL-1β in cell supernatant and synovial tissue. enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-6, IL-1β, and TNF-α were used in this study (Batch Nos. CSB-E04740h, CSB-E04638h, CSB-E08053h, CSB-E11987r, respectively, Cusabio, Wuhan, China), and rat IL-6 kit (Batch No. R6000B, R&D systems, USA), and rat IL-1β kit (Batch No RLB00, R&D systems).

Statistical Analysis

Data analysis was performed using GraphPad 8.0 software (USA). The experimental data are presented as mean ± standard deviation ($\bar{x}\pm s$) and statistical difference between the two groups was evaluated by the t-test. Analysis of variance (ANOVA) was used to compare differences among 3 or more groups, followed by Tukey's test for multiple comparisons. P<0.05 was considered statistically significant.

RESULTS

Isorhamnetin Regulates Viability, Migration, and Invasion of RA-FLS

The model group exhibited a significant increase in RA-FLS viability compared to the control group, while isorhamnetin decreased cell viability after 24 and 48 h of treatment (P<0.05, Figure 1A). Specifically, the medium group showed reduced cell viability at 48 h when compared to the low-dosage group, while the high-dosage group exhibited reduced cell viability at both 24 and 48 h (P<0.05). There was no significant difference in OD values between the high- and medium-dosage groups at 24 and 48 h (Figure 1A).

After 24 h of treatment, the migration ability of RA-FLS did not significantly change with different interventions. After 48 h of treatment, additional isorhamnetin significantly reversed the increased RA-FLS migration (P<0.05, Figures 1B and 1C). The medium and high- groups showed a decreasing trend in migration ability at 48 h when compared to the low-dosage group (P>0.05), and the migration ability of the medium-dosage group did not differ significantly from that of the high-dosage group at 24 and 48 h (Figures 1B and 1C).

Similarly, the invasive ability of RA-FLS was enhanced in the model group (P<0.05). In contrast, isorhamnetin resulted in decreased RA-FLS invasion with increasing concentration (P<0.05, Figure 1D). Specifically, RA-FLS invasion was inhibited in the medium- and high-dosage groups when compared to the low-dosage group (P<0.05). The high-dosage group exhibited reduced cell invasion when compared

to the medium-dosage group (P<0.05, Figure 1D). Additionally, elevated levels of TNF-α, IL-6, and IL-1β were observed in the model group, while isorhamnetin reversed the levels of these factors (P<0.05, Figure 1E). Specifically, the medium-dosage group exhibited reduced levels of TNF-α, IL-6, and IL-1β when compared to the low-dosage group, and the levels of these cytokines were further reduced in the high-dosage group (P<0.05, Figure 1E).

Isorhamnetin Inhibits Abundance of MMP2 and MMP9

Western blotting assay showed that TNF-α induced a markedly increase in the levels of MMP2 and MMP9 in RA-FLS (P<0.05, Figure 2A). In contrast, isorhamnetin gradually inhibited the expression of MMP2 and MMP9 in a dose-dependent manner. IF analysis also confirmed that isorhamnetin inhibited the expressions of MMP2 and MMP9 (P<0.05, Figures 2B and 2C). Specifically, in the medium- and high-dosage groups, MMP2 expression was inhibited when compared to the low-dosage group, while MMP9 expression was only inhibited in the high-dosage group (P<0.05). Moreover, there was no significant difference in protein expression between the high- and medium-dosage groups (Figures 2B and 2C).

Isorhamnetin Suppresses Viability, Migration, and Invasion of RA-FLS via MMP2 and MMP9

The results showed that treatment with isorhamnetin led to a reduction in the levels of MMP2 and MMP9 proteins when compared to the model group (P<0.05). Notably, compared to the Iso+oe-NC group, oe-MMP2 and oe-MMP9 increased the protein abundance of MMP2 and MMP9, respectively (P<0.05, Figure 3A). Compared to the model group, isorhamnetin inhibited cell migration and invasion (P<0.05). Conversely, either oe-MMP2 or oe-MMP9 promoted cell migration and invasion when compared to the Iso+oe-NC group (P<0.05, Figures 3B and 3C). In addition, the Iso group exhibited reduced levels of TNF-α, IL-6, and IL-1β when compared to the model group (P<0.05). However,

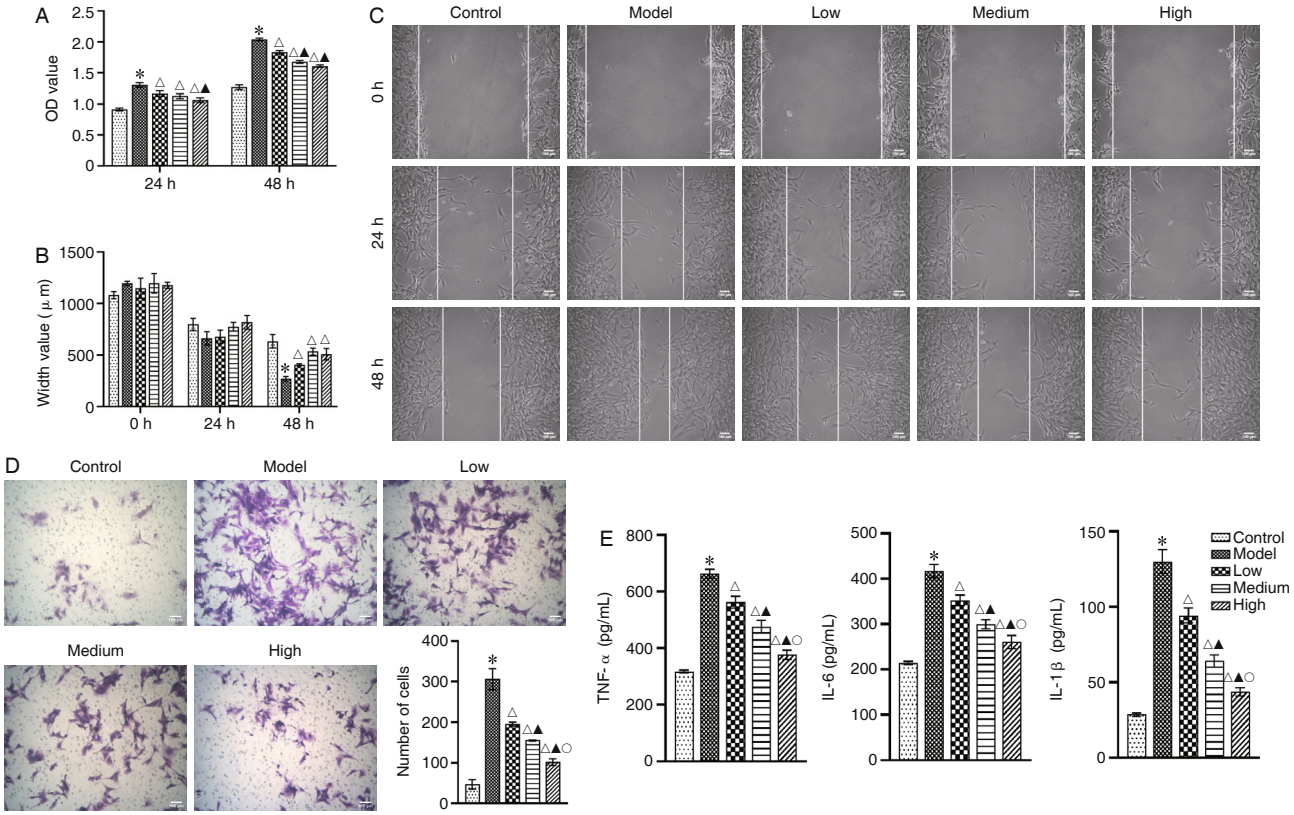

Figure 1. Isorhamnetin Inhibites RA-FLS Viability, Migration, and Invasion

Notes: (A) The CCK8 assay was used to evaluate the effect of different concentrations of isorhamnetin (10, 20, 40 μmol/L) on RA-FLS viability after 24 and 48 h. (B) The effect of isorhamnetin (10, 20, 40 μmol/L) on RA-FLS migration was evaluated by wound healing assay at 0, 24, and 48 h. (C) Representative images obtained from the wound healing assay. (D) Transwell chamber was applied to analyze the invasion ability of RA-FLS. (E) The levels of TNF-α, IL-6, and IL-1β were determined using ELISA. *P<0.05 *vs*. control group, △P<0.05 *vs*. model group, ▲P<0.05 *vs*. low dosage group, ○P<0.05 *vs*. medium dosage group; data are presented as $\bar{x} \pm s$, n=3 in each group; the same in Figure 2

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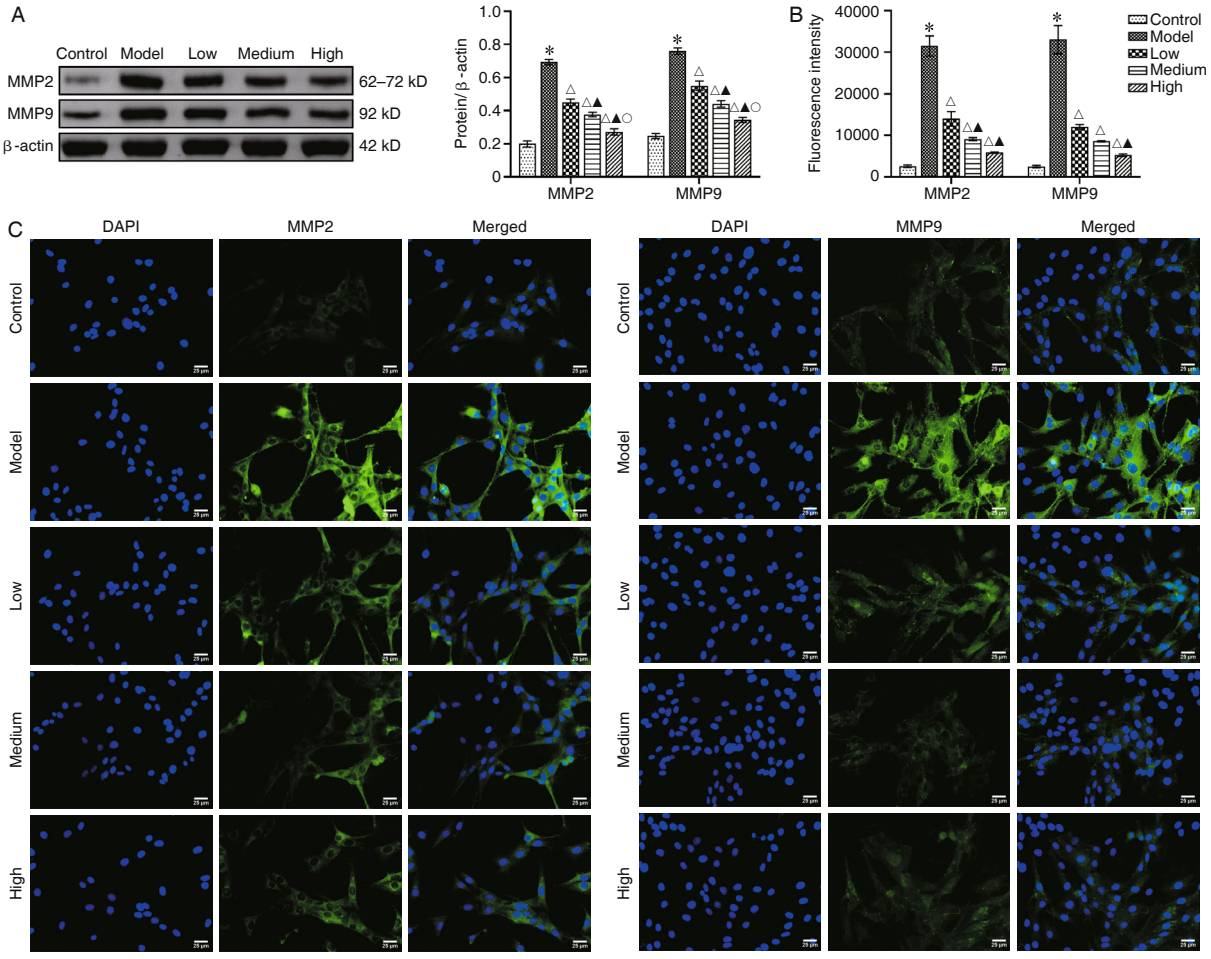

Figure 2. Isorhamnetin Hinders Expression of MMP2 and MMP9

Notes: (A) Western blot analysis was performed to evaluate the effect of isorhamnetin (10, 20, 40 μmol/L) on the abundance of MMP2 and MMP9. (B) IF analysis was applied to detect the fluorescence intensity of MMP2 and MMP9 in RA-FLS *in vitro*. (C) Representative images obtained from the IF assay

either oe-MMP2 or oe-MMP9 reversed the levels of these cytokines (P<0.05, Figure 3D).

Isorhamnetin Inhibits Activation of SRC/ERK/CREB Pathway and Binds to SRC

The ratios of p-SRC/SRC, p-ERK/ERK, and p-CREB/CREB in the model group were higher than that in the control group (P<0.05). However, treatment with 40 μmol/L isorhamnetin suppressed this trend (P<0.05, Figure 4A). Additionally, molecular docking demonstrated that isorhamnetin was able to dock with the protein structure of SRC (Figure 4B).

Isorhamnetin Inhibits Viability, Migration and Invasion of RA-FLS via SRC

Compared to the Iso+oe-NC group, oe-SRC significantly promoted the expression of SRC (P<0.05), thus suggesting that plasmid transfection had been successful. In addition, oe-SRC reversed the isorhamnetin-mediated inhibition of p-ERK/ERK and p-CREB/CREB ratios (P<0.05), thus contributing to the phosphorylation of ERK and CREB (Figure 5A). Compared to the model group, isorhamnetin inhibited the expression of MMP2 and MMP9, while additional oe-SRC led to an increase in the levels of MMP2 and MMP9 (P<0.05, Figure 5B). Furthermore, compared to the Iso+oe-NC group, oe-SRC significantly promoted isorhamnetin-inhibited cell viability and migration after 48 h of treatment (P<0.05, Figures 5C and 5D). Similarly, isorhamnetin-inhibited cell invasion was also reversed by oe-SRC (P<0.05, Figure 5E). In addition, oe-SRC increased the levels of TNF-α, IL-6, and IL-1β when compared to the Iso+oe-NC group (P<0.05, Figure 5F).

Isorhamnosine Alleviates Onset of RA in CIA Rats via SRC/ERK/CREB and MMP2/MMP9 Signaling Pathways

In vivo study found that compared to the sham

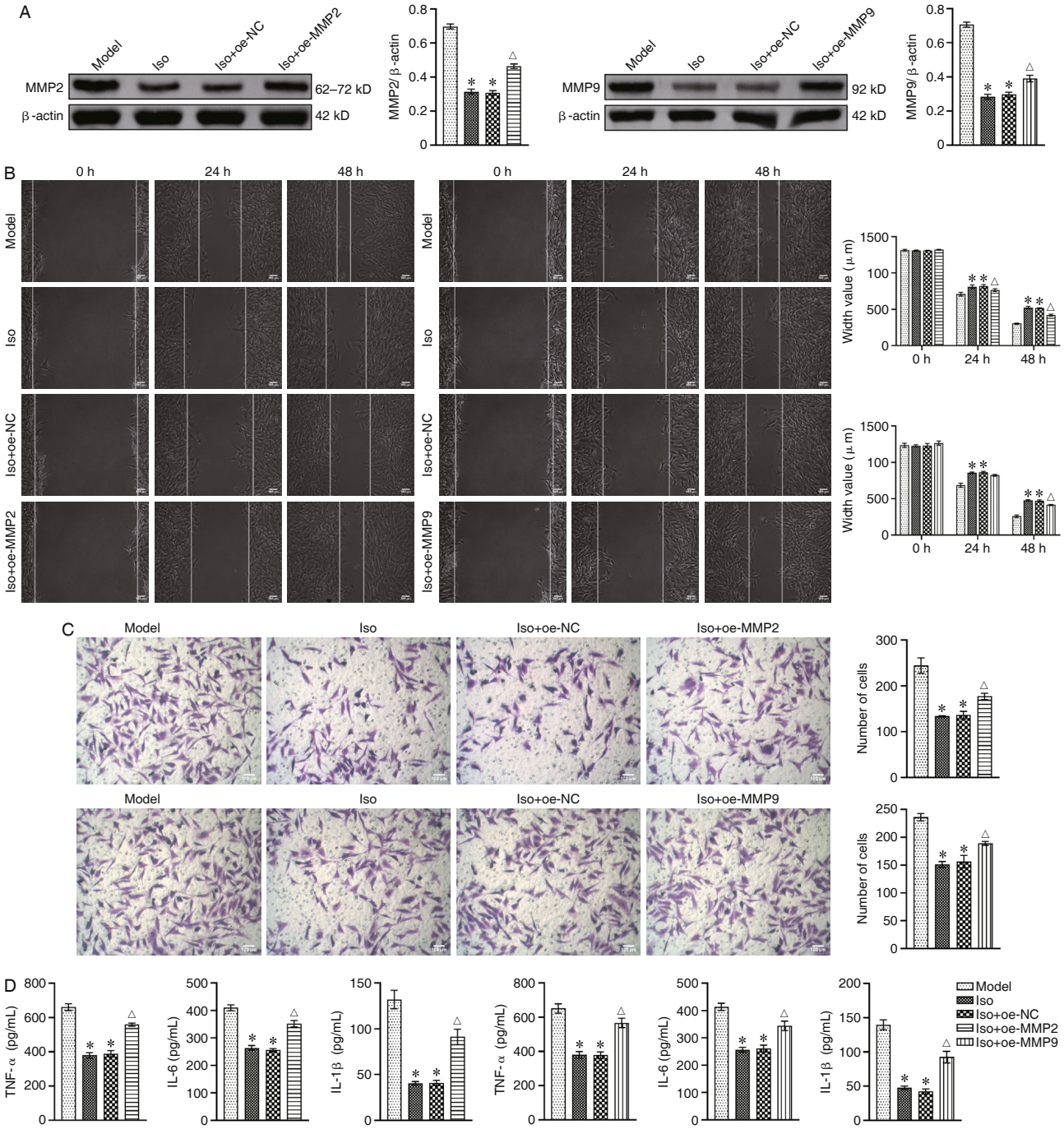

Figure 3. MMP2 and MMP9 Regulates RA-FLS Viability, Migration, and Invasion

Notes: (A) The protein abundance of MMP2 and MMP9 was determined by Western blot. (B) The regulatory effects of MMP2 and MMP9 on RA-FLS migration was evaluated by wound healing assay at 0, 24, and 48 h. (C) Transwell assays were used to assess the regulatory effects of MMP2 and MMP9 on RA-FLS invasion. (D) The regulatory effects of MMP2 and MMP9 on TNF-α, IL-6, and IL-1β levels were examined. $^{*}P<0.05$ *vs*. model group, $^{\triangle}P<0.05$ *vs*. Iso+oe-NC group; data are presented as $\bar{x} \pm s$, n=3 in each group

group, CIA rats exhibited progressively more severe joint swelling ($P<0.05$). Conversely, arthritis scores were lower in the isorhamnetin group than in the CIA group ($P<0.05$). Specifically, medium and high dosages of isorhamnetin (10 and 20 mg/kg) were more effective than low levels (2 mg/kg, $P<0.05$); on day 28 there was a significant difference between rats receiving high dosage of isorhamnetin and those receiving low dosage ($P<0.05$, Figure 6A). We also investigated the synovial tissue of CIA rats and found that it showed synovial hyperplasia and severe inflammatory cell infiltration. However, the administration of isorhamnetin alleviated synovitis in CIA rats. Specifically, the high dosage group exhibited the most significant therapeutic effect; the

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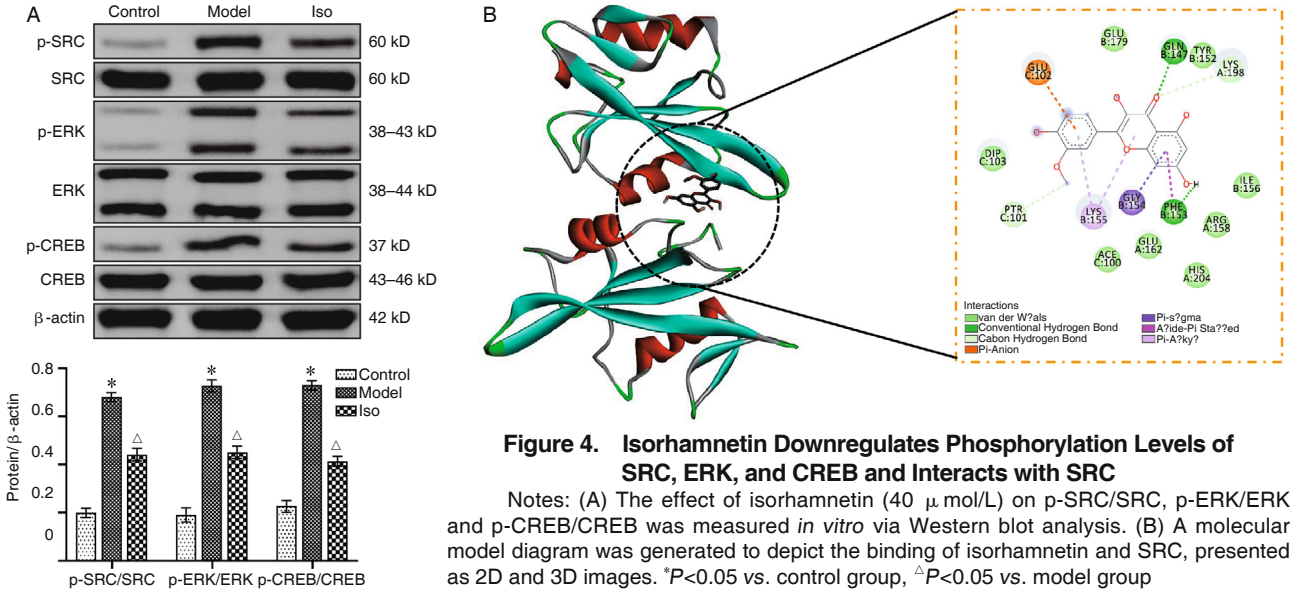

Figure 4. Isorhamnetin Downregulates Phosphorylation Levels of SRC, ERK, and CREB and Interacts with SRC

Notes: (A) The effect of isorhamnetin (40 μmol/L) on p-SRC/SRC, p-ERK/ERK and p-CREB/CREB was measured *in vitro* via Western blot analysis. (B) A molecular model diagram was generated to depict the binding of isorhamnetin and SRC, presented as 2D and 3D images. $^{*}P<0.05$ *vs*. control group, $^{\triangle}P<0.05$ *vs*. model group

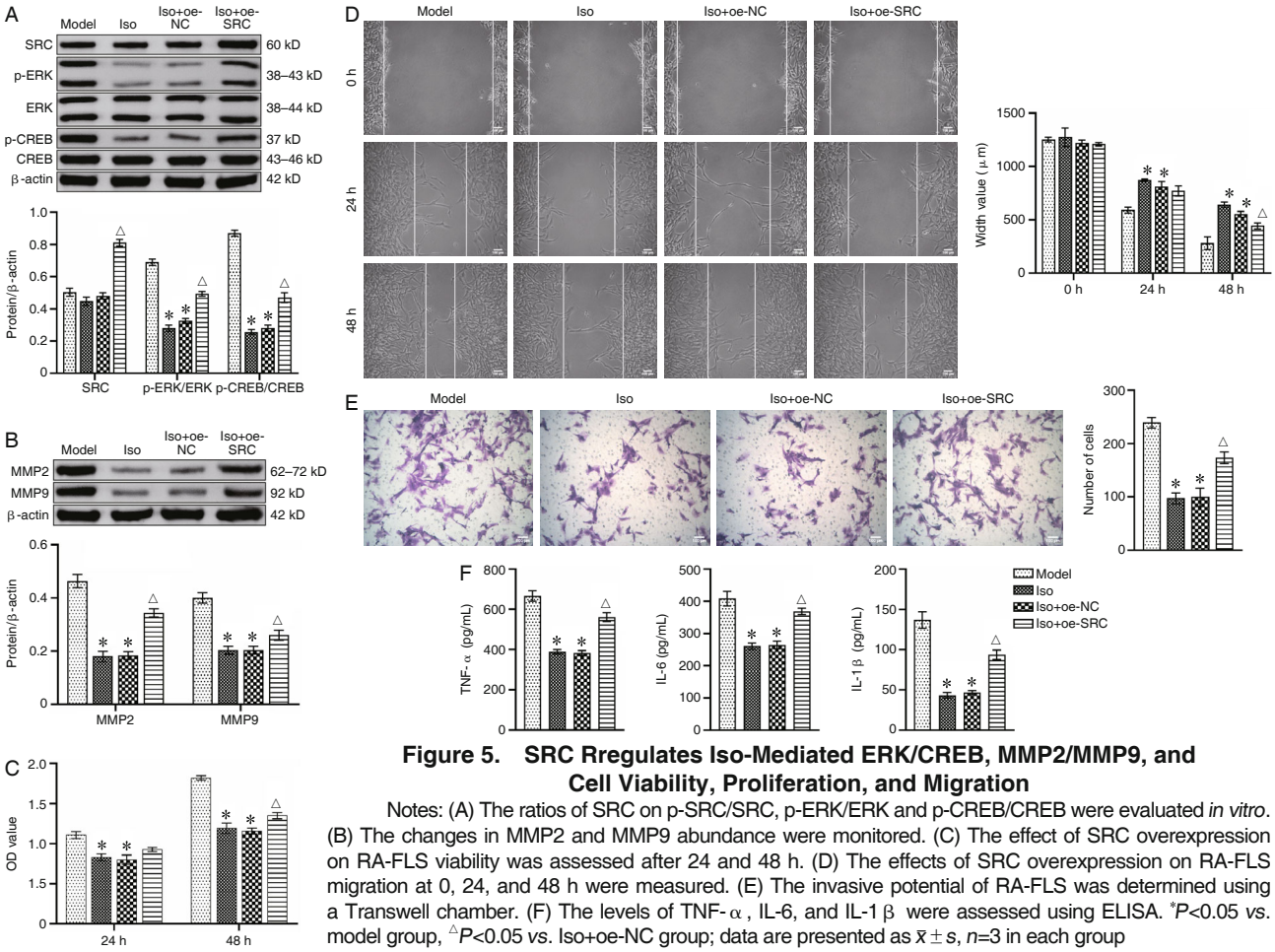

Figure 5. SRC Rregulates Iso-Mediated ERK/CREB, MMP2/MMP9, and Cell Viability, Proliferation, and Migration

Notes: (A) The ratios of SRC on p-SRC/SRC, p-ERK/ERK and p-CREB/CREB were evaluated *in vitro*. (B) The changes in MMP2 and MMP9 abundance were monitored. (C) The effect of SRC overexpression on RA-FLS viability was assessed after 24 and 48 h. (D) The effects of SRC overexpression on RA-FLS migration at 0, 24, and 48 h were measured. (E) The invasive potential of RA-FLS was determined using a Transwell chamber. (F) The levels of TNF-α, IL-6, and IL-1β were assessed using ELISA. $^{*}P<0.05$ *vs*. model group, $^{\triangle}P<0.05$ *vs*. Iso+oe-NC group; data are presented as $\bar{x}\pm s$, $n=3$ in each group

medium dosage group had the second best therapeutic effect while the low dosage group had the least effect (Figure 6B). In addition, compared with the sham group, the protein levels of MMP2 and MMP9 were elevated in synovial tissue from CIA rats ($P<0.05$). However, treatment with isorhamnetin reduced the abundance of MMP2 and MMP9 in a dose-dependent manner ($P<0.05$, Figure 6C).

Additionally, the levels of p-SRC, p-ERK, and

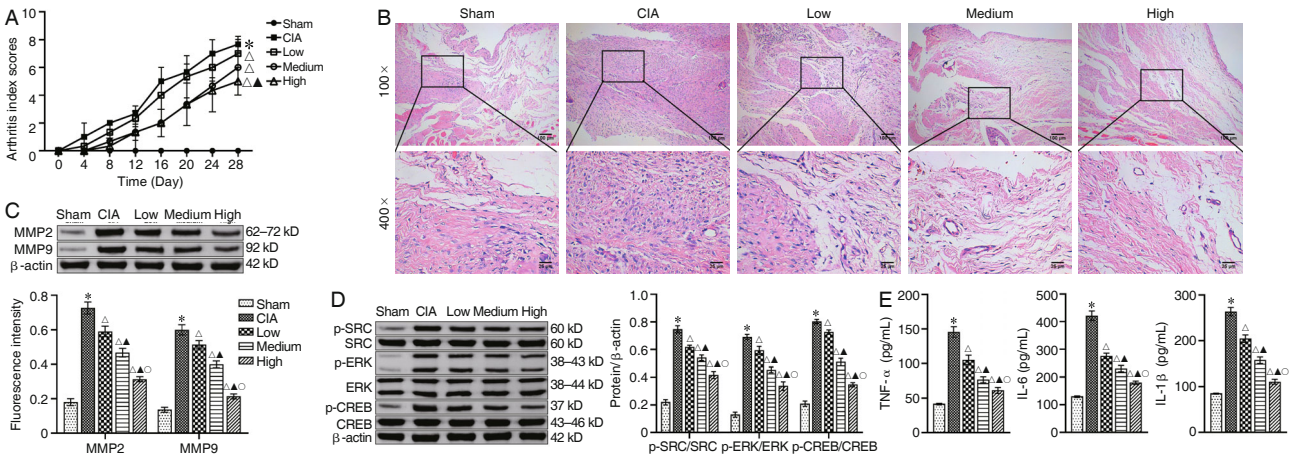

Figure 6. Isorhamnetin Affects Pathogenesis of RA via SRC/ERK/CREB and MMP2/MMP9 Signaling Pathways in CIA Rats

Notes: (A) Within 28 days of modeling, the arthritis index scores of rats in all groups were recorded. (B) HE staining was used to evaluate the pathological changes in the synovial tissues of rats in each group. (C) Abundance changes in MMP2 and MMP9 were examined after treatment with different doses of isorhamnetin (2, 10, 20 mg/kg). (D) The effect of isorhamnetin (2, 10, 20 mg/kg) on ratios of p-SRC/SRC, p-ERK/ERK and p-CREB/CREB was analyzed. (E) The levels of TNF-α, IL-6, and IL-1β were assessed using ELISA. $^{*}P<0.05$ *vs*. sham group; $^{\triangle}P<0.05$ *vs*. CIA group; $^{\blacktriangle}P<0.05$ *vs*. low dosage group; $^{\bigcirc}P<0.05$ *vs*. medium dosage group; data are presented as $\bar{x} \pm s$, n=5 in each group

p-CREB were reduced in synovial tissue from CIA rats following isorhamnetin intervention ($P<0.05$). Specifically, we observed a reduction in the levels of p-SRC, p-ERK, and p-CREB in the medium dosage group when compared to the low dosage group; this trend for reduction was further extended in the high dosage group ($P<0.05$, Figure 6D). Moreover, isorhamnetin reduced the levels of TNF-α, IL-6, and IL-1β in synovial tissue from CIA rats in a dose-dependent manner ($P<0.05$, Figure 6E).

DISCUSSION

RA is a severe autoimmune condition that predominantly affects the joints of the hands and feet; this condition results in cartilage destruction, bone erosion, and potential disability.[28] Patients with RA are also known to exhibit symptoms of systemic disease which is associated with an increased mortality rate.[29] While some analgesics, anti-inflammatory, and anti-rheumatic drugs have been developed, their clinical application is limited due to potential side effects and constraints, thereby yielding limited therapeutic effectiveness.[30] In light of these limitations, there is a pressing demand to develop novel therapeutic approaches to address RA.

Natural plant compounds, derived from herbs or plants, are popular therapeutic agents for diseases such as cancer, malaria, and acquired immunodeficiency syndrome due to their low toxicity and side effects.[31] Study has shown that natural compounds from plants can effectively alleviate the development of RA by interfering with the expression levels of pro-inflammatory factors.[32] Flavonoid compounds are known for their strong anti-inflammatory activity and shown significant potential for treating autoimmune diseases.[33] Recently, the potential of flavonoids such as baicalin, icariin and oroxylin A, has been demonstrated for the treatment of RA.[34-36] In the present study, we found that isorhamnetin effectively inhibited the viability, migration, and invasion of RA-FLS. In a rat model of CIA, isorhamnetin reduced arthritis scores and attenuated the inflammatory response in synovial tissue; these findings are consistent with a previous report.[9] In a previous study, Wang, et al[9] found that isorhamnetin alleviated joint inflammation and damage, reduced the levels of IL-1β, IL-6, and TNF-α, and regulated oxidative stress in CIA mice and lipopolysaccharide-induced FLS. These findings suggest that isorhamnetin could exert a protective role in RA.

The pathogenesis of RA is closely linked to MMPs at the molecular level.[37] The abnormal proliferation of RA-FLS induces the secretion of pro-inflammatory cytokines, which in turn increases the expression levels of MMPs. This upregulation of MMPs leads to the degradation of proteins in the extracellular matrix, ultimately damaging the integrity of the articular cartilage.[38,39] In the present study, increased levels of MMP2 and MMP9 was observed in both TNF-α-induced RA-FLS and CIA rat models,

although treatment with various concentrations of isorhamnetin significantly reduced their expression; notably, higher concentrations exhibite the strongest effects. Furthermore, overexpression of either MMP2 or MMP9 reversed the inhibitory effects of isorhamnetin on the migration and invasion of RA-FLS. In addition, the supplementation of TNF-α-induced RA-FLS with MMP2 or MMP9 also reversed the inhibitory effects of isorhamnetin on the levels of IL-1β, IL-6, and TNF-α. These findings suggest that isorhamnetin protects against RA by regulating the expression levels of MMP2 and MMP9.

Previous report described the activation of SRC kinase signaling in osteoclasts during the early stages of RA development.[40] Previous research has shown that SRC/ERK signaling inhibits the proliferation of RA-FLS and induces apoptosis.[13] Elevated levels of ERK1/2 and CREB phosphorylation have also been reported in RA.[17] In this study, we observed a significant increase in the p-SRC/SRC, p-ERK/ERK and p-CREB/CREB ratios in RA models. Molecular docking further revealed that isorhamnetin bound to SRC, thus indicating that SRC is a target of isorhamnetin. Treatment with isorhamnetin blocked the phosphorylation of SRC, ERK, and CREB in RA models. Furthermore, the supplementation of SRC reserved the inhibitory effects of isorhamnetin on the viability, migration, and invasion of RA-FLS. These results suggest that isorhamnetin inhibits MMP2 and MMP9 by supressing the activating the SRC/ERK/CREB signaling pathway, ultimately alleviating the onset of RA (Figure 7).

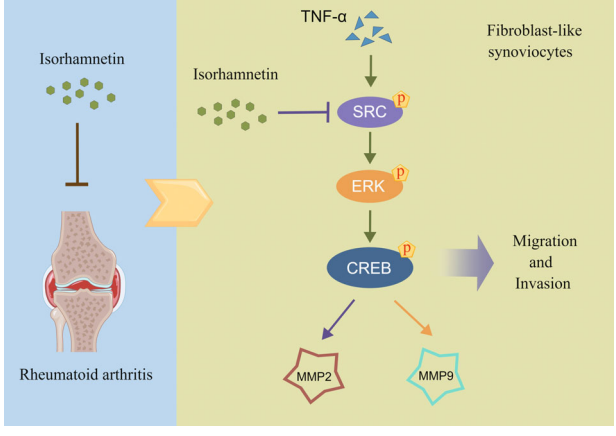

Figure 7. A Potential Mechanism Diagram of Isorhamnetin in Inhibiting Development of Rheumatoid Arthritis

However, our study has some limitations that should be considered. We were unable to apply intervention agents targeting MMPs and SRC in animal models to further validate their roles. In addition, further exploration of other MMPs besides MMP2 and MMP9 is needed. The direct regulatory mechanism between SRC/ERK/CREB and MMPs also requires further investigation. These limitations will be addressed in our future research endeavors.

In conclusion, our study highlights the potential therapeutic value of isorhamnetin for the treatment of RA, along with the underlying regulatory mechanism involved. Isorhamnetin effectively reduced the accumulation of MMP2 and MMP9, thereby ameliorating the pathology of RA; these actions involved modulation of the SRC/ERK/CREB signaling pathway. These findings provide new insights into the use of natural plant extracts or compounds for treating autoimmune diseases, and offer possible strategies for managing RA.

Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

Liu XR contributions to conceptualization, data curation, investigation, methodology, funding acquisition, and writing of the original draft. Li SF and Mei WY contributed to conceptualization, formal analysis, validation, investigation, software, and validation. Liu XD and Zhou RB contributed to conceptualization, project administration, supervision, and review. All authors read and approved the final manuscript.

Availability of Data and Material

The datasets used and analyzed during the current study available from the corresponding author on reasonable request.

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