Coix Seed Oil ameliorates synovial angiogenesis in collagen-induced arthritis rats through inhibiting HIF-1α / VEGF-A pathway via SIRT1

Qiangqiang Xu  
The First Hospital of China Medical University: The First Affiliated Hospital of China Medical University

Hongxi Kong  
The First Hospital of China Medical University: The First Affiliated Hospital of China Medical University

Shuang Ren  
The First Hospital of China Medical University: The First Affiliated Hospital of China Medical University

Fanyan Meng  
The First Hospital of China Medical University: The First Affiliated Hospital of China Medical University

Ruoshi Liu  
The First Hospital of China Medical University: The First Affiliated Hospital of China Medical University

Hongxin Jin  
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine

Jie Zhang (✉ zhangjie945@126.com)  
The First Hospital of China Medical University: The First Affiliated Hospital of China Medical University

https://orcid.org/0000-0003-2972-3870

Research Article

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Abstract

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by symmetric arthritis. Coix Seed Oil (CSO) has been shown to reduce inflammation in rheumatoid arthritis (RA). However, the CSO impact on synovial angiogenesis in RA remains unclear. Therefore, this study aimed to study the CSO inhibitory impact on RA synovial angiogenesis and elucidate the corresponding mechanisms.

Methods

Collagen-induced arthritis (CIA) rat model was established by male Sprague-Dawley rats and then treated with different doses of CSO for four weeks. Arthritis Index (AI), paw swelling, and weight of rats were recorded to assess their clinical symptoms. Hematoxylin and Eosin (H&E) staining, immunohistochemical, and immunofluorescence staining were performed to observe changes in synovial tissues. The serum HIF-1α and VEGF-A protein were evaluated through enzyme-linked immunosorbent assay (ELISA). The FLS was stimulated with TNF-α for developing an in vitro inflammatory model. Cytotoxicity and cell viability were measured using a CCK8 test. Wound healing and Transwell migration experiments were employed to determine FLS migratory ability, together with Immunofluorescence was utilized to assess HIF-1α nuclear translocation within FLS. The SIRT1, HIF-1α, VEGF-A, together with CD31 expression profiles were assessed through Western blot assays. The isolated aortic rings were extracted to observe the CSO inhibitory impact on angiogenesis.

Results

CSO inhibited angiogenesis, together with HIF-1α and VEGF-A expression within CIA rat synovial tissue. Mechanistically, CSO regulated the HIF-1α / VEGF-A pathway by mediating SIRT1 expression, exerting anti-angiogenic effects in vitro and in vivo.

Conclusions

This study indicated that CSO could ameliorate synovial angiogenesis in CIA rats through inhibiting HIF-1α / VEGF-A pathway via SIRT1.

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by symmetric arthritis [1]. The pathogenesis and pathophysiology of RA remain largely unknown. Previous studies have demonstrated increased angiogenesis within synovial membrane in RA, angiogenesis supports the proliferation of inflammatory synovial pannus and allows inflammatory leukocytes to reach synovial tissues [2, 3].
Vascular endothelial growth factor (VEGF) acts on vascular endothelial cells (VECs). VEGF-A secreted by fibroblast-like synoviocytes (FLS) can specifically bind to VEGFR and stimulate the proliferation and activation of VECs, thereby participating in synovial angiogenesis [4]. Many investigations have demonstrated a substantial correlation between higher VEGF-A levels and the disease activity of RA [5, 6]. Therefore, targeted inhibition of VEGF-A expression may be an effective treatment for RA.

With the continuous development in metabolomics techniques, researchers have found that dysfunctional FLS energy metabolism is a fundamental pathogenic factor in all the stages of RA [7]. within synovial hypoxic microenvironment caused by the proliferation of FLS [8], mitochondrial oxidative phosphorylation cannot meet the cellular metabolic demand. Joint inflammation is exacerbated by FLS mitochondrial malfunction, excessive reactive oxygen species (ROS) production, mitochondrial DNA generation, and enhanced oxidative damage [9]. Hypoxia is a key driving force in angiogenesis [10, 11]. HIF is an oxygen sensor. Previous studies showed that RA synovium had greater levels of HIF-1α than healthy individuals [12]. HIF-1α breakdown was dramatically decelerated in a hypoxic environment, resulting in HIF-1α nuclear accumulation, which ultimately caused the transcription of downstream VEGF-A target genes [13].

SIRT1 is a protein deacetylation-dependent kinase that is dependent on nicotinamide adenine dinucleotide (NAD+). Its transcriptional regulation is correlated with multiple metabolic pathways, including nutrient deprivation, DNA damage, and oxidative stress [14]. Within synovial hypoxic microenvironment, the mRNA levels of SIRT1 decreased, thereby facilitating FLS proliferation, migration, and invasion[15, 16]. Recent studies have elucidated the interconnections between SIRT1 and HIF-1α. SIRT1 is a crucial HIF-1α regulator [17]. HIF-1α revealed itself as a target of SIRT1 deacetylation, which plays a role in maintaining HIF-1α stability. SIRT1 can acetylate HIF-1α at lysine709, thereby enhancing its hypoxic stability and inhibiting its activity. The interaction of SIRT1 with HIF-1α plays a crucial role in synovial angiogenesis [18, 19]. Therefore, SIRT1-mediated HIF-1α / VEGF homeostasis is an important regulatory pathway in RA pathogenesis.

Most current conventional antirheumatic drugs, such as NSAIDs and disease-modifying antirheumatic drugs (DMARDs), do not directly target angiogenesis as their primary mechanism of action. Some biological inhibitors have anti-angiogenesis roles, but long-term administration can cause adverse effects, including gastrointestinal distress and bone marrow suppression [20, 21]. Coix Seed is a traditional Chinese medicine. CSO is an ester extract of the fruit of Coix Seed, and has been widely studied for its pharmacological properties, including anti-inflammatory, anti-tumor, and improved glucolipid metabolism [22–24]. In addition, our previous studies showed that CSO could decrease the levels of HIF-1α mRNA expression in Lewis lung cancer. The results confirmed that CSO could alleviate the development of joint inflammation in CIA rat models [data not shown]. However, it remains unclear whether CSO can ameliorate synovial angiogenesis in CIA by inhibiting the SIRT1-mediated HIF-1α / VEGF-A pathway.
Therefore, in our study, constructed to observe whether CSO could inhibit synovial angiogenesis and further demonstrated whether CSO could participate within anti-angiogenesis process by regulating HIF-1α / VEGF-A pathway via SIRT1.

Methods and Materials

Drugs and reagents

CSO was procured through Zhejiang Kanglaite Pharmaceutical CO., Ltd. Methotrexate (MTX) was purchased from Shanghai Pharma CO., Ltd. CII collagen and Complete Freund's Adjuvant (CFA) were obtained from Chondrex; ELISA kits for HIF-1α and VEGF-A were procured from Shanghai Guido Biotechnology, CO., Ltd. PMSF, RIPA lysis buffer, DAPI, and BCA protein detection kit were purchased from Beyotime Biotechnology, CO., Ltd. TGX Stain-Free™ Acrylamide Kit and protein marker were purchased from Bio-Rad. Anti-SIRT1 antibody was purchased from Abcam. Anti-HIF-1α, Anti-VEGF-A, Anti-CD31, Anti-αSMA, Anti-GAPDH, Goat Anti-Rabbit IgG (H+L) HRP, Goat Anti-Mouse IgG (H+L) HRP, Goat Anti-Rabbit IgG (H+L) CY3-conjugated, and Goat Anti-Mouse IgG (H+L) FITC-conjugated antibody were procured through Affinity. Fetal bovine serum (FBS) was obtained from Clark bioscience. High Dulbecco's modified Eagle's medim (H-DMEM), penicillin-streptomycin, phosphate-buffered saline (PBS), together with 0.25% Trypsin-EDTA were procured through Thermo Fisher. Recombinant rat TNF-α, Recombinant rat VEGF-A165, and Transwell chambers were purchased from PeproTech. Matrigel was purchased from Corning. EX527 was acquired from ChemExpress.

Experimental animals

Six-week-old male Sprague-Dawley rats (200 ± 20 g) were procured through Beijing HFK Bioscience Co., Ltd. and housed within Department of Laboratory Animal Science under specific pathogen-free (SPF) conditions (Humidity 40 – 60 %; 12 / 12h day / night cycle; temperature 24 ± 2°C).

Establishment of CIA models and drug administration

As previously mentioned, [25] the CIA rat models were established. Briefly, 0.1 M acetic acid was used to dissolve CII collagen, and this solution was emulsified with an equivalent proportion of CFA. Intradermal injections (tail-base) immunized the rats, with second immunization given seven days later. Rats were segregated in a randomized manner within six groups: Control group (CON, n = 6), CIA model group (CIA, n = 6), CSO low dose group (CSO-L, 2.1 g/kg/day, n = 6), CSO medium dose group (CSO-M, 4.2 g/kg/day, n = 6), CSO high dose group (CSO-H, 8.4 g/kg/day, n = 6), and MTX group (MTX, 0.5mg/kg, 3 times/week. n = 6). Equal amounts of 0.9% saline were administered to the rats within CON and CIA groups. Following the secondary vaccination, the rats in each group received treatment continuously for four weeks. The day of CFA induction was recorded as day 0, and medication treatments were provided from days 14 to 35.

Assessment of arthritis
The AI of rats was scored (ranging from 0 to 4) every 3 days post-the first immunization (day 0). The specific details of the AI scoring are as follows [26]: (0) without swelling; (1) toe joint having slight swelling; (2) mild swelling extending from hindfoot to ankle; (3) moderate swelling or even erythema of ankle; (4) extreme erythema and swelling, even an ankle-to-toe joint ulcer, and bleeding. Moreover, the width of the rat’s right hind ankle joint was measured using vernier calipers, and the rats were weighed weekly.

**Histological analysis**

The synovial tissues were fixed in 4% formaldehyde and then paraffinized, and sectioned at 4-μm-thick. Histopathological alterations in synovial tissues were evaluated with H&E staining. It has been previously reported [27, 28], that histological differences were scored by independent experts who were unaware of the experimental details.

**ELISA**

The serum protein levels of HIF-1α and VEGF-A in rats were assessed via the ELISA kits (Guduo biotechnology, China), and conducted according to kit protocols.

**Immunohistochemical assessment (IHC)**

Initially, rat synovial tissues were treated with Anti-CD31 primary antibody (1:150 dilution; overnight / 4 °C), and then with Goat Anti-Rabbit IgG (H+L) HRP-conjugated secondary antibody (1:200 dilution) at room temperature for 2 hours. Eventually, the methods previously presented were used to perform a semi-quantitative analysis [28].

**Immunofluorescence analysis (IF)**

Synovial tissue vascular maturity was evaluated through immunofluorescence (IF) analysis. Anti-CD31 (1:150 dilution) and Anti-αSMA (1:200 dilution) primary antibodies were placed into incubation with synovial tissues, followed by Goat Anti-Rabbit IgG (H+L) CY3-conjugated secondary antibody (1:200 dilution) and Goat Anti-Mouse IgG (H+L) FITC-conjugated secondary antibody (1:200 dilution), respectively and stained with DAPI. In addition, nuclear translocation of HIF-1α was observed by fixing FLS with 4% formaldehyde for 30 minutes, permeabilizing through 0.5% Triton-X 100 (30 min), followed by blocking with 5% BSA for 1 h. The blocked tissues were placed into incubation with anti-HIF-1α primary antibody (1:100 dilution) overnight at 4 °C, followed by 2 hours at room temperature with goat anti-mouse IgG (H+L) FITC-conjugated secondary antibody (1:200 dilution).

**Cell culture and treatment**

The synovial tissues of 6-week-old male Sprague-Dawley rats have been extracted according to the study reported previously [29]. Synovial tissues were cut into 1-1.5 mm³ pieces post-the removal of fatty and
fibrous tissues. Tissues were cultured in H-DMEM (3 mL) supplemented with 20% FBS within 5% CO₂ humidified atmosphere at 37°C. Finally, FLS were then passaged 3-6 times.

The cells were separated into five groups, each receiving a different treatment: untreated group (NC, n = 3), TNF-α group (TNF-α, n = 3), TNF-α + CSO group (TNF-α + CSO, n = 3), TNF-α + EX527 group (TNF-α + EX527, n = 3), and TNF-α + EX527 + CSO group (TNF-α + EX527 + CSO, n = 3).

**CCK8 assay**

FLS (5 x 10^3 cells / well) were added into 96-well plates. CSO or TNF-α (100 μL / well of different concentrations) was added to the wells for 24 h or 48h. The CSO concentrations were as follows: 12.5, 25, 50, 100, 200, 400, 800, and 1000 μg/mL. The TNF-α concentrations were as follows: 0, 2.5, 5, 10, together with 20 ng/mL. Following a PBS wash, FLS were cultured in 100 μL of H-DMEM containing CCK8 solution (10 μL) for two hours. An enzyme marker determined absorbance (450 nm).

**Wound healing assay**

In 6-well plates, FLS (1 x 10^5 cells / well) were placed into incubation for 24 hours. Using 200 μL pipette tips scratch lines on the plates. Each well was placed with 2 mL of the corresponding treatment reagent for 48 h. The cell-covered area was calculated using ImageJ software to indicate the wound-healing ability of cells.

**Transwell migration assay**

The Transwell chamber with an 8 μm hole size membrane was used to conduct the Transwell migration assay. Briefly, 300 μL FLS (5 x 10^4 cells / mL) solution, containing CSO or EX527 or CSO+ EX527, were added into the upper chambers; The lower chambers were coated with a chemoattractant consisting of a 600 μL solution of 20% FBS HDMEM, including TNF-α. The migrating cells were fixed and stained with a crystal violet solution after 20 hours of incubation. Optical microscopy was utilized in order to measure the relative quantity of migrating FLS.

**Western blot analysis**

The synovial tissues of rats or FLS were lysed in RIPA lysis solution comprising 1% PMSF. Moreover, the BCA protein detection kit was utilized to evaluate the protein concentration. Protein lysate (30 μg) was separated on 10-12% SDS-PAGE gel (120V, 90-120 min) and then transferred to PVDF membrane (220 mA, 1 KD/min). The membrane was incubated with the matching primary antibodies overnight 4 °C after being blocked with 5% BSA solution at room temperature (RT) for 30 min. Post-TBST rinsing, membranes were placed into incubation with HRP-conjugated secondary antibodies (RT / 120 min). GAPDH (1:3000 dilution) was an internal control. Western blot analysis was performed using the primary antibodies: Anti-SIRT1 (1: 1000 dilution) Anti-HIF-1α (1: 1000 dilution), Anti-VEGF-A (1: 1000 dilution), and Anti-CD31 (1: 1000 dilution) respectively. Goat Anti-Rabbit IgG (H + L) HRP-conjugated secondary antibodies (1:3000 dilution) were used for detection.
dilution) and Goat Anti-Mouse IgG (H + L) HRP-conjugated secondary antibodies (1:3000 dilution) were the secondary antibodies utilized for the Western blot analysis.

**Rat aortic rings assay**

A 48-well plate was wrapped with matrigel in advance, and the aorta of rats was stripped post-sacrificing. The aorta was cut into 1-1.5 mm rings and placed into 48-well plates. The matrigel was then solidified on the plates by 30 minutes of incubation (37 °C / 5% CO$_2$). 10% FBS, 90% H-DMEM, 20 μg / L VEGF-A, and CSO were added to the wells. Post-7 days, angiogenesis within rat thoracic aortic rings was observed.

**Statistical analyses**

GraphPad Prism 8.4.2 software was employed for analyzing the data of this study. The datasets reflected mean ± standard deviation (SD). One-way analysis of variance (ANOVA) examined the variations across many groups. Whereas, $P < 0.05$ or $P < 0.01$ indicated the statistical significance threshold.

**Results**

**CSO alleviated the clinical symptoms of CIA rats**

CIA models were established, and their clinical symptoms post-treatment with CSO administration were observed. Figure 1A depicted the experimental process. The significant joints swelling within CIA group was reduced through CSO therapy (Fig. 1B). In contrast to the rats within CIA group, the treatment of CSO-M and CSO-H reduced the AI score and paw swelling (Fig. 1C and 1D; $P < 0.01$). However, CSO-L and CIA groups had similar AI scores and paw swelling ($P > 0.05$). The CSO-M therapy substantially reduced weight loss within rats in comparison to CIA group ($P < 0.05$). Such outcomes revealed CSO was able to effectively reduce CIA rats' clinical symptoms.

**CSO improved joint tissue morphology in CIA rats**

In Fig. 2, it is apparent that the rats within CON group had neatly distributed synovial cells without visible inflammatory cellular infiltration, synovial tissue hyperplasia, together with a lower number of micro-vessels. CIA rat synovial tissue, however, had large quantities of micro vessels and an evident infiltration of inflammatory cells. CIA animals had higher H&E pathology scores and synovial tissue micro-vessel counts than CON rats (**$P < 0.01$). In comparison to the rats within CIA group, the synovial histopathology scores within CSO-H and MTX groups decreased statistically significantly (##$P < 0.01$). Despite a decrease in micro-vessels within synovial tissues of the CSO-L group, with no major variation between them and CIA group ($P > 0.05$). In contrast, the CSO-M, CSO-H, and MTX groups' synovial tissues had considerably lower micro-vessels than the CIA group's (##$P < 0.01$).

**CSO reduced the serum levels of HIF-1α and VEGF-A in CIA rats**
ELISA indices were used to measure rats’ serum HIF-1α and VEGF-A levels. Figure 3 shows that the CIA group had higher HIF-1α and VEGF-A levels than the CON group (**$P<0.01$). The CSO-L and CSO-M groups had lower HIF-1α levels to CIA group, although the variation had no statistical significance ($P>0.05$). CSO-H group had significantly lower HIF-1α levels than the CIA group ($#P<0.05$). This showed that high-dose CSO could inhibit HIF-1α increases in CIA rats. The CSO-H group had the greatest serum VEGF-A differential from the CIA group ($#P<0.05$). This showed that high CSO dosages might significantly reduce VEGF-A serum levels in CIA rats.

**CSO inhibited angiogenesis in synovial tissue of CIA rats**

CD31 level expression, a commonly used marker to measure angiogenesis, was identified using an IHC test in order to evaluate the anti-angiogenesis effect of CSO [30]. According to Fig. 4, CD31 expression level in synovial tissues increased significantly within CIA group compared to CON group (**$P<0.01$). Positive CD31 staining rate in synovial tissues decreased significantly within CSO group in comparison with CIA (#$P<0.01$), and the inhibition effect had positive correlation to dose; Additionally, rats within CIA group had more immature vasculature that was CD31$^+$ / $\alpha$SMA$^-$ labeled in their synovial tissues than did rats within CON group (**$P<0.01$). Furthermore, in comparison to CIA group, CSO-L, CSO-M, CSO-H, and MTX groups showed substantially fewer CD31$^+$ / $\alpha$SMA$^-$ labeled immature capillaries in synovial tissues (#$P<0.01$); the inhibitory impact of CSO on angiogenesis was dose-dependent. The overall number of vessels and mature vessels that were CD31$^+$ / SMA$^+$ labeled increased considerably in all modeled rats as compared with CON group (**$P<0.01$), though no variation having statistical significance was observed across all modeled groups ($P>0.05$). Such dataset outcomes suggested CSO to dramatically reduce angiogenesis in CIA rats.

**CSO regulated the expression levels of SIRT1, HIF-1α, VEGF-A, and CD31 in the synovial tissues of CIA rats**

According to Fig. 5, CIA rats had considerably lower levels of SIRT1 expression in their synovial tissues than did the rats within CON group. HIF-1α, VEGF-A, and CD31 expression levels were increased considerably (**$P<0.01$). SIRT1 expression did not have major variation across CSO-L and CIA groups ($P>0.05$), but it was significantly higher within CSO-M, CSO-H, and MTX groups in comparison with CIA group (#$P<0.01$), and HIF-1α expression was significantly reduced within CSO-H and MTX groups (#$P<0.05$). Compared to CIA group, CSO and MTX groups rats displayed reduced synovial VEGF-A expression (#$P<0.01$). CSO-L rats had no difference in CD31 expression in their synovial tissues compared to CIA rats ($P>0.05$). In comparison with CIA group, CD31 expression profiles within synovial membranes fell considerably within CSO-M, CSO-H, and MTX groups (#$P<0.01$). In summary, high-dose CSO may considerably increase SIRT1 levels while lowering HIF-1α, VEGF-A, and CD31 levels within synovial tissues of CIA rats.
Effects of different CSO concentrations on the viability of FLS

To determine the optimal CSO dosage concentration, FLS were cultured with 10 different CSO dosages for 48 hours. The cell viability of FLS constantly reduced as CSO concentration increased, as indicated in Fig. 6A. The estimated value for CSO's half maximum inhibitory concentration (IC50) is 625 µg / mL. The final concentration of CSO chosen for the following dose was 500µg / mL.

Effects of different concentrations of TNF-α on cell proliferation ability and SIRT1 expression

For 24 hours, five TNF-α doses (0, 2.5, 5, 10, and 20 ng / mL) were used to induce FLS. As demonstrated in Figs. 6B–6D, FLS within 2.5, 5, 10, and 20 ng / mL TNF-α treatment groups proliferated more rapidly than cells within 0 ng / mL groups (**P < 0.01). However, SIRT1 expression within FLS did not alter between the 2.5/5 ng / mL treatment groups and the 0 ng / mL TNF-α group (P > 0.05). In comparison with 0 ng / mL TNF-α group, FLS SIRT1 expression reduced considerably within 10 and 20 ng / mL treatment groups, but cell proliferation rates did not vary (P > 0.05). SIRT1 expression did not change between 10 and 20 ng / mL (P > 0.05). Eventually, post-integrating these data with CCK-8 and Western blot results, 10 ng / mL TNF-α was selected for further investigations.

CSO inhibited the migration ability of TNF-α-induced FLS proliferation

The CSO impact on the capacity for horizontal and vertical migration of TNF-α-induced FLS was examined using wound healing and Transwell migration assays (Fig. 7). In comparison with NC group, the TNF-α group’s cell-covered rate and Transwell migration cells dramatically enhanced post-CSO treatment for 48 hours (**P < 0.01). Additionally, the cell-covered rate and Transwell migration cells within TNF-α + CSO group were considerably reduced compared to within TNF-α group (##P < 0.01), suggesting CSO may inhibit FLS’s capacity to migrate following TNF-α activation. Additionally, it was discovered that as compared to the TNF-α group, the migratory capacity of FLS was significantly enhanced within TNF-α + EX527 and TNF-α + CSO + EX527 groups (##P < 0.01). The capacity of FLS to migrate across the TNF-α + EX527 and TNF-α + CSO + EX527 groups, however, did not vary significantly (P > 0.05). This suggested that the capacity of FLS to migrate laterally and vertically in response to TNF-α stimulation may be increased by inhibiting SIRT1 expression. At this stage, CSO administration did not reduce FLS’s capacity to migrate, confirming that CSO may control FLS’s ability to migrate post-TNF-α induction by controlling SIRT1 expression.

CSO inhibited the TNF-α-induced nuclear translocation of HIF-1α in FLS
Figure 8 depicted NC group's FLS HIF-1α expression was low. In comparison with NC group, HIF-1α expression and nuclear translocation within TNF-group were higher (**P < 0.01). HIF-1α nuclear translocation was significantly lower within TNF-α + CSO group (##P < 0.01). TNF-α + EX527 group had significantly higher HIF-1α expression and nuclear translocation in FLS than TNF-α group (##P < 0.05). TNF-α + EX527 and TNF-α + EX527 + CSO groups had similar nuclear HIF-1α levels (P > 0.05). This suggested that CSO might control the enhanced expression of SIRT1 in FLS to prevent HIF-1α nuclear translocation.

CSO regulated the expression levels of SIRT1, HIF-1α, and VEGF-A in TNF-α-induced FLS

The TNF-α group’s SIRT1 expression level in FLS was considerably lower (**P < 0.01) in comparison with NC group, but it was greater (##P < 0.01) within TNF-α + CSO group relative to TNF-α group. Furthermore, VEGF-A and HIF-1α expression levels within TNF-α group were substantially lower in comparison to within NC group (##P < 0.01), while the HIF-1α expression was consistent with the trend of HIF-1α nuclear translocation shown in earlier investigations. Additionally, HIF-1α and VEGF-A expression were considerably reduced within TNF-α + CSO group in comparison to TNF- group (##P < 0.01). This suggested that CSO might increase SIRT1 protein expression while downregulating HIF-1α and VEGF-A. EX527 was added for downregulating SIRT1 in order to further investigate whether CSO may control HIF-1α and VEGF-A protein levels via SIRT1. The findings demonstrated major upregulation for HIF-1α / VEGF-A relative to TNF-α group, whereas SIRT1 expression within TNF-α + EX527 group further reduced relative to within TNF-α group (##P < 0.01). This showed that SIRT1 regulated HIF-1α and VEGF-A expression in FLS, while decreasing SIRT1 expression in FLS might considerably enhance these levels. When SIRT1, HIF-1α, and VEGF-A expression levels from the TNF-α + EX527 and TNF-α + EX527 + CSO groups were evaluated, there was no discernible change (P > 0.05). This suggested that CSO may control SIRT1 protein expression in TNF-α-induced FLS, which in turn might regulate the level at which HIF-1α and VEGF-A were expressed.

CSO inhibited the isolated aortic rings' angiogenesis

The rat-isolated aortic ring angiogenesis experiment was done in order to further confirm the anti-angiogenic effects of CSO in vitro. As shown in Fig. 10, there was no micro-vessels formation around the aortic rings within NC group; the VEGF-A_{165} group, however, had much more micro-vessels grown around the aortic rings than the NC group (**P < 0.01). Additionally, VEGF-A_{165} + CSO administration substantially reduced the quantity and length of micro-vessels in comparison to VEGF-A_{165} administration (##P < 0.01). This further confirmed that CSO also exhibited an anti-angiogenesis effect in vitro.
Discussion

As an important early event in RA, synovial angiogenesis is involved within initiation of inflammatory response before inflammatory cell infiltration in RA synovium [31]. Therefore, targeted inhibition of synovial angiogenesis might be a new direction within current treatment approaches for RA.

Angiogenesis is a complex process, in which VECs form new capillaries in a sprouting manner based on pre-existing vessels, and is tightly regulated by pro / inhibitory angiogenic factors[32]. In RA synovial tissues, the balance of pro / anti-angiogenic factors is disrupted. FLS secretes a large amount of VEGF-A, fibroblast growth factor (FGF), angiopoietin (Ang), and other pro-angiogenic cytokines. VEGF-A, the most important regulator of vascular neogenesis, induces endothelial cell activation, increases microvascular permeability, and promotes angiogenesis [33]. Our current study showed that the synovial VEGF-A expression was significantly higher within CIA group relative to CON group. CSO significantly downregulated VEGF-A and CD31, mitigated synovial angiogenesis within CIA rats, and reduced VEGF-A165-induced micro-vessels in isolated aortic rings.

Studies have shown that hypoxia contributes to the inflammatory and immune response [34]. The proliferation of FLS in RA synovial tissues consumes a large amount of oxygen and energy, leading to local hypoxia in synovial tissues, thereby causing the formation of abnormal blood vessels in order to adapt to the hypoxic environment. Due to the functional abnormality of the neovascular network, the synovial oxygen partial pressure is not effectively restored, while oxygen is delivered to the proliferating inflammatory cells, thereby increasing inflammation within RA synovium and further reducing the oxygen partial pressure in synovial tissues [35]. These low oxygen partial pressure conditions block the FLS mitochondrial oxidative phosphorylation, which increases the NADH / NAD⁺ ratio, leading to loss of mitochondrial membrane potential, abnormal increase in ROS, mutations in mitochondrial DNA, and ultimately FLS mitochondrial dysfunction [9]. Studies have shown that mitochondrial function is regulated by various signaling molecules, among which SIRT1 and HIF-1α are the two critical metabolic sensors within cellular metabolic pathway [17].

To adapt to hypoxia, HIF-1α is a crucial regulator [36]. HIF-1α is made up of two subunits, HIF-1 α, and β, with HIF-1α being the sole subunit that regulates oxygen levels. Under the normoxic condition, prolyl hydroxylase (PHD) hydroxylates the ODD structural domain of HIF-1α, and the hydroxylated residue is then degraded by E3 ubiquitin ligase [37]. However, PHD activity is inhibited under hypoxic conditions in RA synovial tissue, allowing HIF-1α to accumulate in the FLS cytoplasm and translocate to the nucleus, where it forms the activated HIF-1α heterodimer with HIF-1β. It then binds to hypoxia response elements (HRE) on the promoter regions of target genes, causing angiogenesis, migration, and apoptosis [38, 39]. The present research demonstrated a statistically significant rise in HIF-1α expression in CIA group in comparison with the CON group (P< 0.01). Within TNF-α induced FLS inflammatory cell model, HIF-1α expression increased significantly as compared to that within NC group; moreover, IF indicated a large amount of HIF-1α aggregation into the nucleus within TNF-α group (P< 0.01). CSO administration significantly decreased the HIF-1α expression (P< 0.01) as well as the HIF-1α nuclear translocation (P< 0.01). Additionally, the expression trend of VEGF-A, a downstream target protein of HIF-1α, was consistent
with that of HIF-1α. This suggested that CSO could exert an anti-inflammatory effect and inhibit synovial angiogenesis by regulating the HIF-1α / VEGF-A pathway.

In addition, studies have shown that HIF-1α is activated under hypoxic conditions and stably expressed under the regulation of other molecules, such as ROS, NF-κB, nitric oxide, and SIRT1 [40–42]. SIRT1 is a NAD-dependent deacetylase with anti-inflammatory, antioxidant, anti-angiogenic, and metabolic regulatory effects [43, 44]. It has been demonstrated that SIRT1 expression decreased within synovial tissues of RA patients as compared to that in Osteoarthritis patients. The overexpression of SIRT1 could alleviate RA joint inflammation [45]. The current study showed that the synovial tissue of CIA rats had considerably lower SIRT1 protein expression than for CON group (P< 0.01). CSO-M and CSO-H had greater SIRT1 expression than the CIA group (P< 0.01). The TNF-α group had substantially lower SIRT1 expression in FLS than the NC group (P< 0.01), whereas the TNF-α + CSO group had considerably greater SIRT1. This suggested that CSO could alleviate synovial angiogenesis in CIA rats by modulating the SIRT1 expression.

The correlation between SIRT1 and HIF-1α is still controversial [18, 46]. Under hypoxic conditions, HIF-1α can bind to the HRE on the SIRT1 promoter and upregulate SIRT1 expression, while the high SIRT1 expression can inhibit HIF-1α expression. Another researcher found that SIRT1 could inhibit the recruitment of cofactor P300 / CBP through HIF-1α, thereby downregulating HIF-1α [47]. This demonstrates the lack of clarity within interplays across SIRT1 and HIF-1α.

In order to verify the interplays across HIF-1α and SIRT1 in FLS, the SIRT1 expression was inhibited by adding EX527, and the migration ability, HIF-1α nuclear translocation, and levels for HIF-1α / VEGF-A protein expression were observed. Dataset outcomes showed inhibiting the SIRT1 expression could significantly enhance the cell migration ability of FLS (P< 0.05) as well as significantly increase the nuclear translocation of HIF-1α (P< 0.05). Conversely, VEGF-A and HIF-1α expression increased significantly (P< 0.01). This data revealed that SIRT1 in TNF-α-induced FLS might control the HIF-1α and VEGF-A expression.

To further clarify whether CSO could regulate levels of VEGF-A and HIF-1α protein via SIRT1, TNF-α-induced FLS, treated with EX527, were further treated with CSO. The TNF-α + EX527 + CSO group had higher HIF-1α and VEGF-A levels than the TNF-α group post-48 hours (P< 0.01). HIF-1α, VEGF-A, cell migration, and nuclear translocation did not differ between the TNF-α + EX527 + CSO and TNF-α + EX527 groups (P> 0.05). This demonstrated that CSO might reduce HIF-1α and VEGF-A in TNF- induced FLS via SIRT1.

**Conclusions**

This study confirmed that CSO could effectively attenuate synovial tissue angiogenesis of CIA rats. Mechanistically, CSO exerted anti-angiogenic effects by mediating SIRT1 expression and negatively regulating the HIF-1α / VEGF-A pathway. Notably, the effects of CSO on other cell models, such as cobalt chloride-induced hypoxia model of HUVECs and co-culture model of RA FLS and HUVECs, have not been
further validated. In conclusion, CSO could serve as a novel drug for RA therapy due to its ability to reduce angiogenesis in CIA rats.

**Abbreviations**

RA: rheumatoid arthritis; CSO: Coix Seed Oil; CIA: Collagen-induced arthritis; MTX: Methotrexate; FLS: Fibroblast-like synoviocytes; SIRT1: Silent Information Regulator of Transcription 1; HIF-1α: Hypoxia Inducible Factor-1α; VEGF-A: Vascular Endothelial Growth Factor-A; IC50: The half maximal inhibitory concentration; TNF-α: Tumor necrosis factor-α; WB: Western blot; IHC: Immunohistochemical; IF: Immunofluorescence; CCK8: Cell Counting Kit

**Declarations**

**Acknowledgments**

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**Author contributions**

Qiangqiang Xu and Hongxi Kong performed the experiments, analyzed the data, and wrote the manuscript. Shuang Ren, Fanyan Meng, Ruoshi Liu and Hongxin Jin helped in performing the experiments. Jie Zhang conceived and supervised the project.

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**Availability of data and materials**

The datasets generated for this study are available on request to the corresponding author.

**Ethics approval and consent to participate**

The animal experiments were approved by the Ethics Committee for Animal Experimentation of China Medical University. All the animal experiments were conducted in accordance with the Animal Care and Use Committee guidelines of China Medical University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
References


Figures
Figure 1

CSO alleviated the clinical symptoms of CIA rats. A Experimental procedure. B Representative imaging for ankle joints within individual groups. C Arthritis index of rats. D Paw swelling of rats (mm). E Weight of rats (g). Datasets reflected mean ± SD. *P < 0.05, **P < 0.01 vs. CON group; #P < 0.05, ##P < 0.01 vs. CIA group, n = 6.
CSO improved joint tissue morphology in CIA rats. A H&E staining of synovial tissues (40X). B The number of vessels in synovial tissues. C H&E score of synovial tissues. Datasets reflected mean ± SD.*P <0.05, **P <0.01 vs. CON group; #P <0.05, ##P <0.01 vs. CIA group, n = 6.
CSO reduced the serum levels of HIF-1α and VEGF-A in CIA rats. A Serum level of HIF-1α in rats. B Serum level of VEGF-A in rats. Datasets reflected mean ± SD. *$P < 0.05$, **$P < 0.01$ vs. CON group; #$P < 0.05$, ##$P < 0.01$ vs. CIA group, $n = 6$. 
Figure 4

CSO inhibited angiogenesis in synovial tissue of CIA rats. A IHC staining of CD31 and IF staining of CD31/αSMA (40X). B CD31 Positive ratio (%). C CD31⁺/αSMA⁻ IOD. D CD31⁺/αSMA⁺ IOD. E Total vessel IOD. Datasets reflected mean ± SD. *P<0.05, **P<0.01 vs. CON group; #P<0.05, ##P<0.01 vs. CIA group, n = 3.

Figure 5

CSO regulated the expression levels of SIRT1, HIF-1α, VEGF-A, and CD31 in the synovial tissue of CIA rats. A The representative photos of Western blot. B SIRT1 / GAPDH. C HIF-1α / GAPDH. D VEGF-A / GAPDH. E CD31 / GAPDH. Datasets reflected mean ± SD. *P < 0.05, **P < 0.01 vs. CON group; #P<0.05, ##P<0.01 vs. CIA group, n = 3.
**Figure 6**

Effects of different concentrations of CSO and TNF-α on cell proliferation ability and SIRT1 expression. **A** FLS cell survival rate post-CSO treatment (n = 6). **B** Proliferation of FLS post-TNF-α treatment (%). **C** Expression level of SIRT1. **D** SIRT1 / GAPDH. Datasets reflected mean ± SD. \(*P < 0.05, **P < 0.01\) vs. 0 ng / mL TNF-α group; n = 3.
Figure 7

CSO inhibited the migration ability of TNF-α-induced FLS proliferation. A Wound-healing assay (40X). B Transwell migration (100X). C FLS-covered area (%). D Relative number of migration cells. Datasets reflected mean ± SD. *P < 0.05, **P < 0.01 vs. untreated group (NC); #P < 0.05, ##P < 0.01 vs. TNF-α group, n = 3.
Figure 8

CSO inhibited TNF-α-induced nuclear translocation of HIF-1α in FLS.

A HIF-1α translocation (100X). B Nuclear HIF-1α ratio. Datasets reflected mean ± SD. *P < 0.05, **P < 0.01 vs. untreated group (NC); #P < 0.05, ##P < 0.01 vs. TNF-α group, n = 3.
Figure 9

CSO modulated SIRT1, HIF-1α, and VEGF-A expression within TNF-α-induced FLS. **A** Western blot analysis. **B** SIRT1 / GAPDH. **C** HIF-1α / GAPDH. **D** VEGF-A / GAPDH. Datasets reflected mean ± SD. **## P < 0.01, # P < 0.05 vs. TNF-α group; **P < 0.01, * P < 0.05 vs. untreated group (NC); n = 3
CSO inhibited the isolated aortic rings’ angiogenesis. A Isolated aortic rings angiogenesis experiment (40X). B Numbers of micro-vessels. C Micro-vessel length (pixels). Datasets reflected mean ± SD. n = 3, *P < 0.05, **P < 0.01 vs. untreated group (NC), and #P < 0.05, ##P < 0.01 vs. VEGF-A165 group.