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ARTICLE

Nrf-2/ROS/NF-κB pathway is modulated by cynarin in human mesenchymal stem cells in vitro from ankylosing spondylitis

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Abstract

Ankylosing spondylitis (AS) is an immune chronic inflammatory disease, resulting in back pain, stiffness, and thoracolumbar kyphotic deformity. Based on the reported anti-inflammatory and antioxidant capacities of cynarin (Cyn), this study explored its protective role and molecular mechanisms in mesenchymal stem cells (MSCs) from AS. The target pathways and genes were verified using Western blotting, quantitative real-time polymerase chain reaction, and immunofluorescent staining, while molecular docking analysis was conducted. In AS-MSCs, we found that the expression levels of p-NF-κB, IL-6, IL-1β, and TNF-α were higher and IκB-α, Nrf-2, and HO-1 were lower compared with healthy control (HC)-MSCs. With molecular docking analysis, the biding affinities between Cyn and Keap1-Nrf-2 and p65-IκB-α were predicted. The mRNA and protein expression of p-NF-κB, IL-6, IL-1β, and TNF-α and the reactive oxygen species (ROS) generation were downregulated following Cyn administration. Meanwhile, the expression level of IκB-α, Nrf-2, and HO-1 were significantly increased after Cyn pretreatment. The results suggested that the protective mechanisms of Cyn in AS-MSCs were based on enhancing the antioxidation and suppression of excessive inflammatory responses via Nrf-2/ROS/NF-κB axis. Our findings demonstrate that Cyn is a potential candidate for alleviating inflammation in AS.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Oxidative stress was correlated with the intensity of inflammation in patients with ankylosing spondylitis (AS). Cynarin (Cyn) was shown to scavenge reactive oxygen species effectively.

WHAT QUESTION DID THIS STUDY ADDRESS?

This study aimed to investigate whether mesenchymal stem cells (MSCs) participate in oxidative stress regulation in AS and its molecular mechanism.

Chenyu Song and Kaiyang Wang contributed equally to this manuscript.

Meanwhile, this study also explored the mechanisms of Cyn in the treatment of AS.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The abnormal level of inflammation and oxidative stress was verified in AS-MSCs. Moreover, the HO-1 was downregulated and nuclear factor kappa B (NF-κB) was upregulated resulting in chronic persistent inflammation in AS-MSCs. Finally, Cyn could alleviate the inflammation response by Nrf-2/ROS/NF-κB signaling pathway in AS-MSCs.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

A deeper comprehension of the role of Cyn in alleviating inflammation revealed novel prospects for its therapeutic application in the treatment of AS.

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic and systemic immune disease that mainly affects young people which is characterized by inflammation and progressive ossification of the spine.[1–3] The later stage patients with AS may manifest thoracolumbar kyphosis, which could lead to disability and heavy financial burden. Gene polymorphisms and environmental factors have been considered as pathogenesis of AS.[1] HLA-B27 was considered as the strongest gene associated with the incidence of AS. Besides, genes, such as IL-23, ERAP1, and LNPEP, were also believed to be related to the onset of AS.[1] Moreover, microbial infections are linked to chronic inflammation in AS.[2] Extensive evidence suggested that Klebsiella pneumoniae was the perpetuating factor in the pathogenesis of AS.[2] However, none of these theories can perfectly explain the occurrence and progression molecular mechanisms of AS.

Abnormal levels of inflammation and oxidative stress in the serum microenvironment of patients with AS has been reported.[4] Compared with normal controls, the total antioxidant capacity significantly decreased in patients with AS, whereas reactive oxygen species (ROS) were significantly increased.[5] Obviously, oxidative stress has a strong correlation with inflammation. The overexpression of ROS could cause active inflammatory processes leading to the secretion of pro-inflammatory factors.[4] In addition, oxidative stress motivated the nuclear factor kappa B (NF-κB) pathway, which activated macrophages and lymphocytes to augment ROS production.[6] To sum up, the interaction between inflammation and oxidative stress was closely related to the pathogenesis of AS.

Heme oxygenase 1 (HO-1) was encoded by the gene HMOX1, which was a stress-inducible isozyme, and had been verified as a therapeutic target for autoimmune diseases.[7] The expression of HO-1 was largely controlled by nuclear factor erythroid 2-related factor 2 (Nrf-2), which could bind to the promoter region of antioxidant genes, including HMOX1.[8] The decrease of Nrf-2 will increase the activity of NF-κB, resulting in cytokine production to increase the oxidative stress level.[9] Under the physiology condition, the production and removal of ROS are dynamically balanced. However, when the balance between oxidation and anti-oxidation was disrupted, it may contribute to the progression of inflammation.

Mesenchymal stem cells (MSCs) are capable of trilineage differentiating, which can be isolated from bone marrow, fat, and muscle.[10,11] Moreover, MSCs also play an important role in immune regulatory.[10] Recently, MSC dysfunction was involved in many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, immune thrombocytopenic purpura, and AS.[4,12–14] Several studies have demonstrated that MSCs from AS exhibited reduced immunomodulatory capacity, abnormal osteogenic differentiation, and abnormal inhibition of osteoclastogenesis.[2,15,16] However, whether MSCs participate in oxidative stress regulation in AS and its molecular mechanism remain to be investigated.

Cynarin (Cyn), also known as 1,3-O-dicaffeoylquinic acid, a phenolic compound, is derived from artichoke leaves and heads, which has anti-inflammatory, anti-oxidative, anti-HIV, anti-carcinogenic, and antimicrobial properties.[17,18] Previously, Cyn could effectively scavenge ROS and reactive nitrogen species in vitro.[17] Besides, a previous study also demonstrated that Cyn played an anti-inflammatory role by inhibiting NF-κB pathway in human leukemic cells.[19] However, up to date, little was known about how Cyn affected oxidative stress and inflammatory responses in MSCs from AS (AS-MSCs). Therefore, this study aimed to explore the mechanisms of Cyn in the treatment of AS-MSCs-mediated inflammation.

MATERIALS AND METHODS

Patient and control recruitment

The collection and experiment of bone marrow samples from patients were already approved by the Ethics Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, and all procedures have followed the Declaration of Helsinki. Four human bone marrow samples were acquired from patients with AS-related thoracolumbar kyphotic deformity who received pedicle subtraction osteotomy. The bone marrow samples of the healthy control group ($n=4$) were taken from patients with spinal fractures requiring surgery.

Reagents

Cyn was supplied by MedChemExpress (USA). Dimethyl sulfoxide (DMSO) and t-butylhydroperoxide (TBHP) purchased from Sigma (USA).

Cell isolation and culture

MSCs were isolated from bone marrow samples using density gradient centrifugation. Then, the MSCs were resuspended in alpha-modified Eagle's medium (α-MEM; Meilun, China) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin–streptomycin (Gibco, USA), and then seeded into flasks and cultured at 37°C with 5% CO_2. After 2 days, the medium was replaced to remove the suspended cells. Thereafter, the medium was replaced every 2 days. When the cells reached 80–90% confluence, the MSCs were digested by 0.25% trypsin containing 0.53 mM EDTA and reseeded in three new culture flasks. The MSCs in the third through fourth passages were used for the following experiments.

Cell viability assay

MSCs were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h, the medium was replaced by complete α-MEM with Cyn (0, 50, 100, 150, 200, and 250 μmol/L) or TBHP (0, 25, 50, 75, 100, 125, and 150 μmol/L). After incubating for 24, 48, and 72 h, the medium was again replaced by complete α-MEM containing 10% cell counting kit-8 (CCK-8; Meilun, China). After culturing for 2 h, the cell viability was measured using a microplate reader (Thermo Multiskan GO, USA).

Measurement of reactive oxygen species

The 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Beyotime, China) probe was added to the serum-free α-MEM in a ratio of 1:1000 to determine the intracellular ROS content. MSCs were treated with 100 μmol/L TBHP after Cyn (0, 50, and 100 μmol/L) treatment for 24 h. The MSCs were incubated with DCFH-DA for 20 min at 37°C away from light and then washed three times using serum-free α-MEM. Finally, the fluorescence microscope (Zeiss, Germany) was used to perform imaging of cells. The Image J software (National Institutes of Health [NIH], USA) was used to analyze the fluorescence intensity.

Western blotting

The protein of MSCs in 6-well plates were extracted by radioimmunoprecipitation assay lysis buffer (RIPA; Solarbio, China) with 1 mmol/L protein phosphatase inhibitor (CST, USA) at 4°C for 20 min. SDS–polyacrylamide gels (SDS-PAGE; EpiZyme, China) was used to separate proteins and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). Then, the PVDF membranes were blocked by quickBlock Blocking Buffer (Beyotime, China) for 30 min and transferred to primary antibodies (diluted 1:1000) at 4°C overnight. After washing three times, the PVDF membranes were immersed in secondary antibodies (diluted 1:3000) for 2 h at room temperature. All of the images were detected by ChemiDocXRS + Imaging System (Tanon, China). Finally, the Image J software was used to measure the greyscale values of each protein band.

Quantitative real-time polymerase chain reaction

The cellular mRNA from MSCs was extracted using the total RNA extraction kit (ES Science, China) and complementary DNA was synthesized using reverse transcription reagents (Vazyme Biotech, USA). According to the protocol, quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the SYBR Green qPCR kit (Accurate Biotechnology, China) on a LightCycler 480 II (Roche, Switzerland). Primer sequences used to test MSCs genes are shown in the supplementary material. The gene expressions were calculated by the comparative threshold cycle technique ($2^{-\Delta\Delta Ct}$ method).

Cell immunofluorescent staining

The MSCs were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum album (BSA; Aladdin, China) for 1 h. Following incubation with primary antibodies overnight at 4°C, the cells were incubated with Alexa Fluor 488 goat antirabbit IgG (Abcam, Britain) for 1 h at 37°C. The cytoskeleton was stained by phalloidin for 10 min and nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime, China) for 5 min. Each well was observed by fluorescence microscope (Zeiss, Germany).

Osteogenic differentiation and alizarin red S staining

The osteogenic differentiation medium (OM) contained b-glycerol phosphate (Sigma, USA), dexamethasone (Sigma, USA), and ascorbate (Sigma, USA). The medium was replaced every 2 days and the MSCs were cultured in OM for up to 21 days. After being cultured for 21 days, the cells were fixed in 4% paraformaldehyde (PFA) and stained with alizarin red S (ARS) for 20 min. Finally, the stained cells were observed by a microscope (Leica, Germany).

Adipogenic differentiation and oil red O staining

The adipogenic differentiation medium supplemented with dexamethasone (Sigma, USA), insulin (Solarbio, China), 1-methyl-3-isobutylxanthine (Solarbio, China), and indomethacin (Solarbio, China). After 21 days, the cytoplasmic lipid vacuoles were visualized by oil red O staining. In brief, the plates were fixed with 4% PFA for 20 min and stained in oil red O solution for another 20 min. After washing five times, the plates were visualized by a microscope (Zeiss, Germany).

Chondrogenic differentiation and alcian blue staining

The chondrogenic differentiation of MSCs is stimulated by incubation of cells in a medium including ascorbate (Sigma, USA), dexamethasone (Sigma, USA), TGF-β3 (Yeasen, China), sodium pyruvate (Solarbio, China), selenous acid (Solarbio, China), transferrin (Solarbio, China), proline (Solarbio, China), and L-glutamine (Solarbio, China). The medium was replaced every third day until day 21 and then the pellets were harvested for further experiments. The glycosaminoglycan content was measured by alcian blue staining. The slides were fixed with 4% PFA and stained with alcian blue for 15 min. Finally, the slides were detected by a microscope (Zeiss, Germany).

Immunohistochemical staining

The sections were dewaxed in dimethyl benzene and dehydrated with graded ethanol. Then the endogenous peroxidase was inactivated by 3% H_2O_2 for 20 min, followed by blocking with 5% BSA for 1 h. After that, the sections were probed with primary antibody against IL-1β (diluted 1:50) and HO-1 (diluted 1:100) at 4°C overnight. After rewarming for 30 min and washing three times, the specimens were incubated with secondary antibody (diluted 1:200) for 1 h at room temperature. Then, the sections were incubated with 3,3′-diaminobenzidine (DAB; Typing, China) and counterstained with hematoxylin. Finally, the sections were detected by a microscope (Zeiss, Germany).

Molecular docking

The docking process between Cyn and Keap1-Nrf-2 and p65-IκB-α was conducted by Autodock tools (version 4.2.6). The 3D structures of Keap1-Nrf-2 (PDB ID:5CGJ) and p65-IκB-α (PDB ID:1OY3) were obtained from Protein Data Bank (PDB) and the SDF 2D format of Cyn (PubChem ID: 5281769) was acquired from PubChem database which was converted to 3D structure by Open Babel software (version 2.3.2). The Autodock program was utilized to remove water, add hydrogens, carry out energy minimization, conduct semiflexible docking, and calculate the grid-based score. The model with the lowest binding energy was considered as the best docking poses. Finally, the image results were generated by Python molecule (Pymol, version 2.4).

Antibodies

The antibodies used were: rabbit anti-Nrf-2 (#16396-1-AP; Proteintech, China), rabbit anti-HO-1 (#10701-1-AP; Proteintech, China), rabbit anti-IκB-α (#10268-1-AP, Proteintech, China), rabbit anti-p-NF-κB (#AP0445; ABclonal, China), rabbit anti-IL-1β (#ab9722, Abcam, Britain), mouse anti-TNF-α (#60291-1-ig; Proteintech, China), rabbit anti-IL-6 (#bs-0782R; Bioss, China).

Statistical analysis

Data analyses were performed with GraphPad Prism software (version 9.5) and all values are presented as means

± SD. The unpaired Student's *t*-test was performed to analyze double groups variables and a one-way analysis of variance was performed with multiple groups for statistical significance calculation. Any *p* value less than 0.05 was considered statistically significant.

Ethics statement

The Nanjing Drum Tower Hospital's review board approved this study.

RESULTS

The level of inflammation and oxidative stress in bone marrow of the facet joints in AS

Using immunohistochemical staining, in bone marrow of the facet joints, the expression level of IL-1β was higher (Figure 1a,c) and HO-1 was lower (Figure 1b,d) significantly in patients with AS compared with healthy controls (HCs). Meanwhile, the ROS production was highly elevated in AS-MSCs (Figure 1e,f). These findings indicated that the level of inflammation and oxidative stress was abnormal.

NF-κB pathway was highly elevated and Nrf-2 pathway was inhibited in AS-MSCs compared with HC-MSCs

To verify whether NF-κB and Nrf-2 pathways were changed in AS-MSCs, the expression level of the above two pathway were compared between AS-MSCs and HC-MSCs by Western blot and qRT-PCR. Compared with HC-MSCs, the expression of Nrf-2 and HO-1 were downregulated (Figure 2a–c,i,j), which indicated that Nrf-2/HO-1 pathway was weakened in AS-MSCs. Moreover, the expression of p-NF-κB was higher (Figure 2a,d,k) and IκB-α was lower (Figure 2a,e,l) in AS-MSCs implying that NF-κB pathway was activated. Moreover, the expression of pro-inflammatory factors, including IL-6, IL-1β, and TNF-α was also significantly higher (Figure 2a,f–h,m–o) in AS-MSCs.

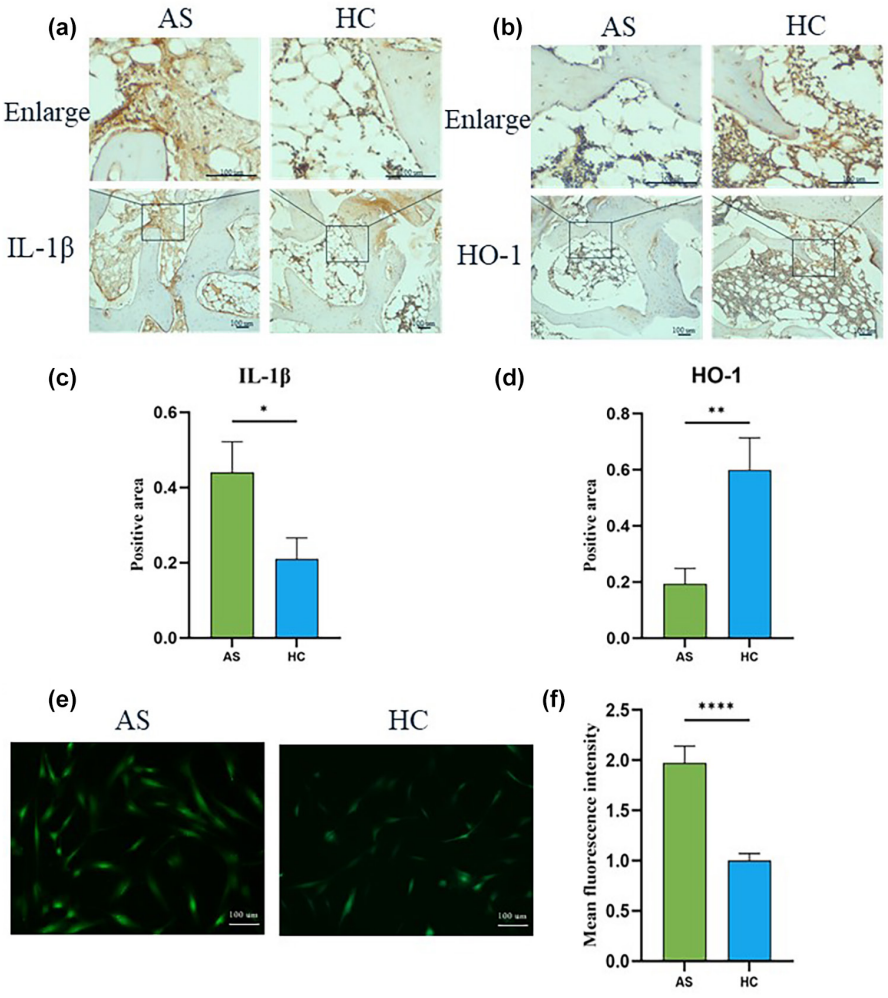

FIGURE 1 Comparison of inflammatory state in bone marrow of the facet joints between AS and HC. (a) Immunohistochemical staining of the IL-1β expression in facet joints (scale bar: 100 μm). (b) Immunohistochemical demonstration of HO-1 (scale bar: 100 μm). (c, d) Percentages of IL-1β- and HO-1-positive area in bone marrow. (e) Comparison of ROS production between AS-MSCs and HC-MSCs. (f) Quantitative analysis of the mean fluorescence intensity of DCFH-DA ($n = 3$ per group). All data are presented as means ± SD and comparisons are performed by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ compared with the HC group. AS, ankylosing spondylitis; HC, healthy control; MSC, mesenchymal stem cell; ROS, reactive oxygen species.

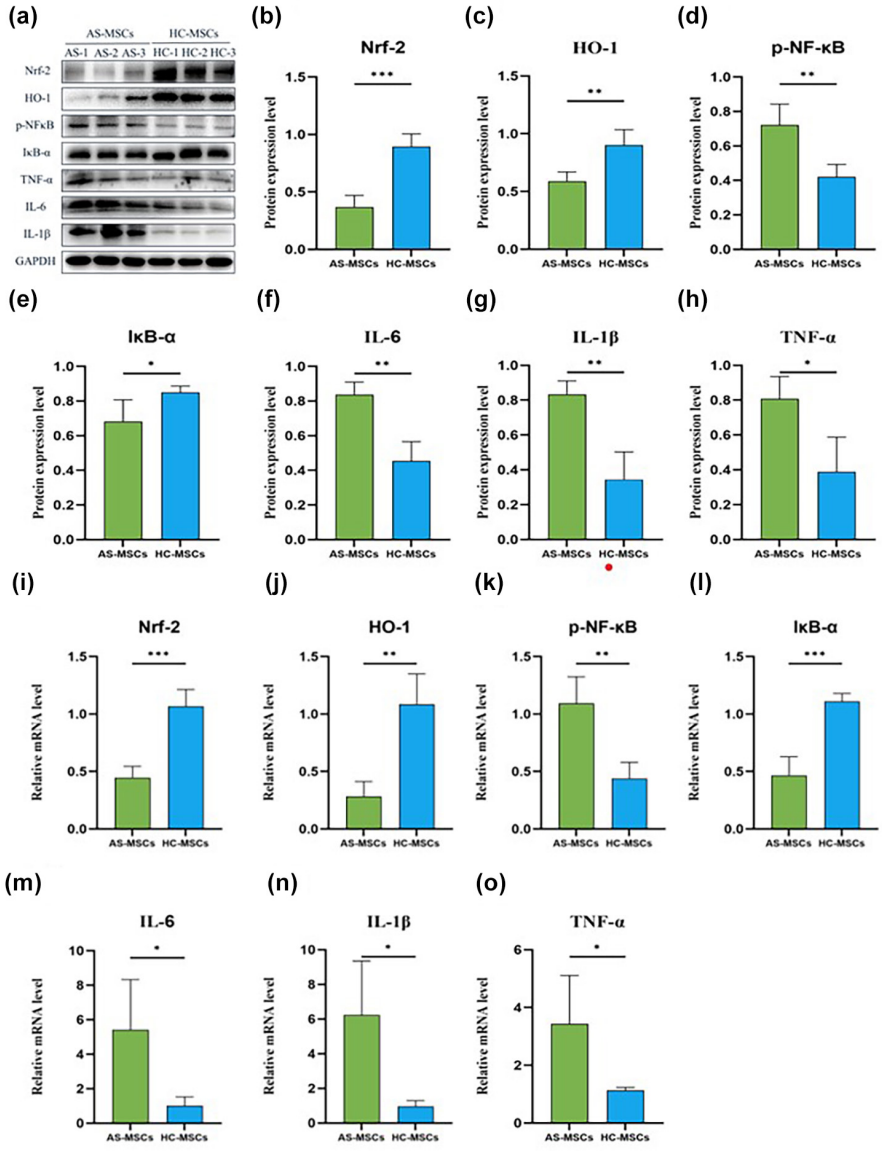

FIGURE 2 Comparison of expression levels of Nrf-2 and NF-κB pathways between AS-MSCs and HC-MSCs. (a) Western blot analysis of Nrf-2, HO-1, p-NF-κB, IκB-α, IL-6, IL-1β, and TNF-α. Each lane represents one patient. (b–h) Quantitative analysis of protein expression of Nrf-2, HO-1, p-NF-κB, IκB-α, IL-6, IL-1β, and TNF-α. Each lane represents one patient ($n = 3$ per group). (i–o) The mRNA expression levels of Nrf-2, HO-1, p-NF-κB, IκB-α, IL-6, IL-1β, and TNF-α were detected by qRT-PCR. All data are presented as means ± SD and comparisons are performed by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the HC group. AS, ankylosing spondylitis; HC, healthy control; MSC, mesenchymal stem cell; qRT-PCR, quantitative real-time polymerase chain reaction.

Cytotoxicity of Cyn and TBHP, molecular docking analysis of Cyn with Keap1-Nrf-2 and p65-IκB-α, and the characterization of MSCs

The chemical structure of Cyn was shown in Figure 3a. As demonstrated in Figure 3b,c, Cyn (≤100 μM) and TBHP (≤100 μM) were safe for use in MSCs by cell counting kit-8. Molecular docking pattern maps between Cyn and the two targets (Keap1-Nrf-2 and p65-IκB-α) were shown in Figure 3d,e. Cyn exhibited the binding energy of −4.46 kcal/mol with Keap1-Nrf-2 and -2.72 kcal/mol with p65-IκB-α. Cyn could form hydrogen bonds with amino acid residues VAL606, ARG470 of Keap1-Nrf-2 and GLN287, GLU203, and ALA123 of p65-IκB-α. These results indicated that Cyn can bind with Keap1-Nrf-2 and p65-IκB-α. The isolated AS-MSCs and HC-MSCs were both exhibited a spindle-shaped morphology (Figure 3f). Their osteogenic, adipogenic, and chondrogenic differentiation potentials were confirmed by positive ARS, Oil red O, and alcian blue staining (Figure 3g), respectively.

Cyn enhanced antioxidant capacity dependent on Nrf-2 pathway in AS-MSCs

The effect of Cyn on Nrf-2 pathway was tested by Western blot and qRT-PCR and ROS production was detected by DCFH-DA probe. TBHP led to a significant increasing production of ROS, whereas Cyn pretreatment significantly hampered the increase in intracellular ROS production in a dose-dependent manner in AS-MSCs (Figure 4a,b). TBHP suppressed Nrf-2 and HO-1 expression in vitro. Meanwhile, Cyn significantly increased the expression level of Nrf-2 and HO-1 (Figure 4c–g).

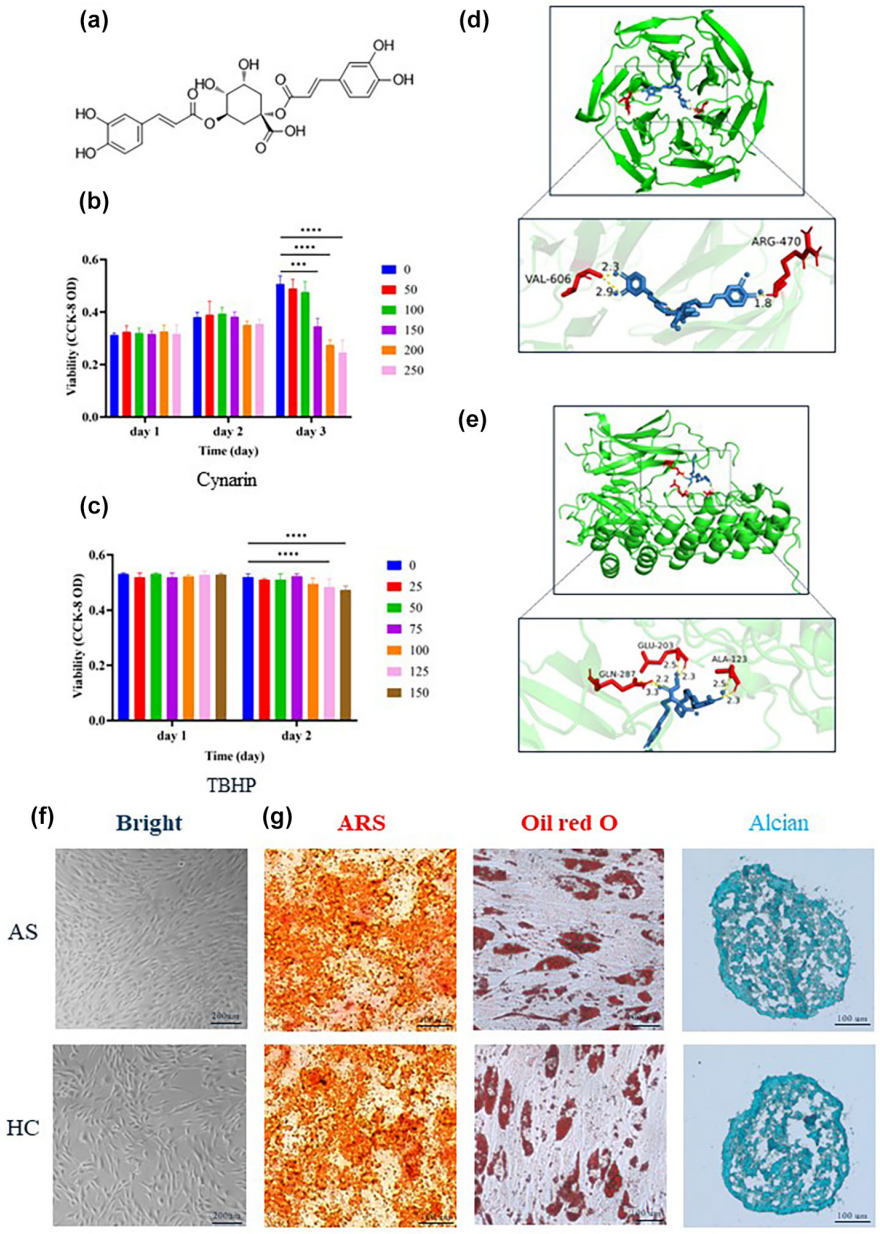

FIGURE 3 Cytotoxicity induced by Cyn and TBHP, molecular docking analysis of Cyn with Keap1-Nrf-2 and p65-IκB-α and trilineage differentiation of MSCs. (a) Chemical structure of Cyn. (b, c) Cell counting kit-8 assay to determine cell viability of MSCs after treatment with different concentrations of Cyn and TBHP. (d) The morphology of isolated MSCs (scale bar: 200 μm). (e) Mineralized nodules were visualized by Alizarin red S staining, Cytoplasmic lipid vacuoles were detected by oil red O staining, and Chondrogenic differentiation of MSCs was assessed by alcian blue staining (scale bar: 100 μm). (f, g) Molecular docking pattern maps of Cyn–Keap1-Nrf-2 and Cyn–p65-IκB-α. Cyn, cynarin; MSC, mesenchymal stem cell.

Above results suggested that Cyn might play an antioxidant role by regulating the Nrf2/HO-1 pathway.

Cyn inhibited NF-κB pathway to alleviate inflammatory response in AS-MSCs

The effect of Cyn on NF-κB pathway was also detected by Western blot and qRT-PCR. TBHP promoted the protein expression level of p-NF-κB and downregulated the protein level of IκB-α. Compared to the TBHP group, Cyn pretreatment markedly inhibited NF-κB phosphorylation (Figure 5a,b,g), whereas evidently raising IκB-α expression (Figure 5a,c,h) in AS-MSCs. Besides, TBHP raised the production of IL-6, IL-1β, and TNF-α; however, Cyn reversed these changes (Figure 5a,d–f,i–k). These results demonstrated that Cyn alleviated inflammatory response induced by TBHP by mediating the NF-κB pathway.

Cyn promoted nuclear translocation of Nrf-2 and suppressed nuclear translocation of p65 in AS-MSCs

Whether Nrf-2 or p65, they both needed migrating into the nucleus to initiate related genes transcription. Next, we used immunofluorescence to examine the effect of Cyn on nuclear translocation of Nrf-2 and p65. As shown in Figure 6a, the nuclear translocation of p65 was suppressed by Cyn. Besides, Cyn promoted nuclear translocation of Nrf-2 (Figure 6b).

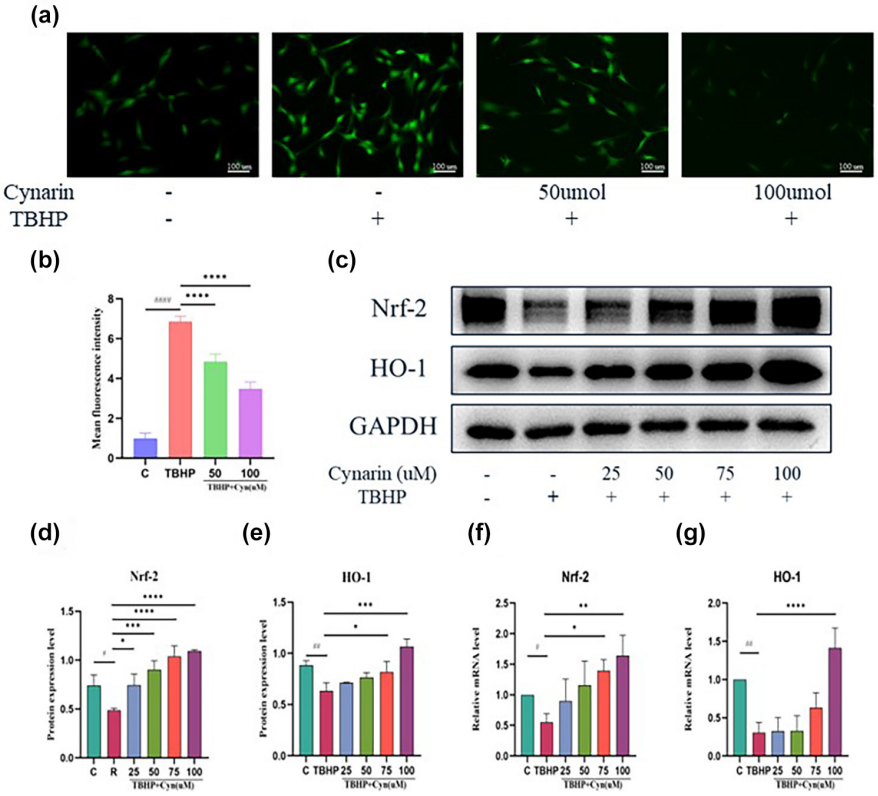

FIGURE 4 Effects of Cyn on the Nrf-2 pathway in AS-MSCs. The AS-MSCs were incubated with the indicated different doses of Cyn. (a) Effect of Cyn on ROS production induced by TBHP (100 μm, scale bar: 100 μm). (b) Quantitative analysis of the mean fluorescence intensity of DCFH-DA. (c) Western blot analysis of Nrf-2 and HO-1. (d, e) Quantitative analysis of protein expression of Nrf-2 and HO-1. (f, g) The mRNA levels of Nrf-2 pathway were detected by qRT-PCR. All data are presented as means ± SD and comparisons are performed by one-way analysis of variance. #$p < 0.05$, ##$p < 0.01$ compared with control group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with model group, $n = 3$. AS, ankylosing spondylitis; Cyn, cynarin; MSC, mesenchymal stem cell; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species.

DISCUSSION

AS is a kind of autoimmune inflammatory arthritis along with new bone formation which leads to spinal kyphotic deformity.[1–4,20,21] A previous study has reported that the damage of facet joints may be consisted in impairment of spinal mobility.[3] Appel et al. and Qian et al. reported that persistent inflammation and the levels of CD4+ T cells and CD20+ B cells were higher in facet joints of AS.[3,22] In this study, we found that the expression level of IL-1β was higher and HO-1 was lower in facet joints of AS compared with HC. As the main protein of anti-oxidative stress, the low expression of HO-1 indicated that the oxidative stress level was abnormal in patients with AS.

Recently, several studies have demonstrated that oxidative stress was correlated with the intensity of inflammation in patients with AS,[23,24] which were consistent with our findings. Compared with the healthy groups, the plasma total antioxidant status was lower and the oxidative stress index was higher in AS.[20] Additionally, a significantly higher lipid peroxidation product was reported, as well as lower values of glutathione peroxidase activity in erythrocyte in patients with AS.[21] MSCs not only have the ability of trilineage differentiating, including osteogenesis, but also can regulate immune, which suggest that MSCs may play an important role in pathogenesis of AS. Although studies have shown MSCs exhibited different functional abnormalities in AS previously, the underlying reasons remained to be elucidated. Up to date, only a few reports have been published focusing on the prooxidant-antioxidant environment of patients with AS, but there is little literature to explore the underlying molecular mechanisms. In this study, we found that the expressions of Nrf-2 and HO-1 were decreased in AS-MSCs compared with HC-MSCs. These results suggested that the antioxidant ability was impaired, which was related to the pathogenesis of AS.

In addition to its well-known ability to resist oxidative stress, the HO-1 system also has anti-inflammatory capability.[8] These abilities are verified by human and animal models that are HO-1-deficient, which are characterized by increasing sensitivity to oxidative stress and

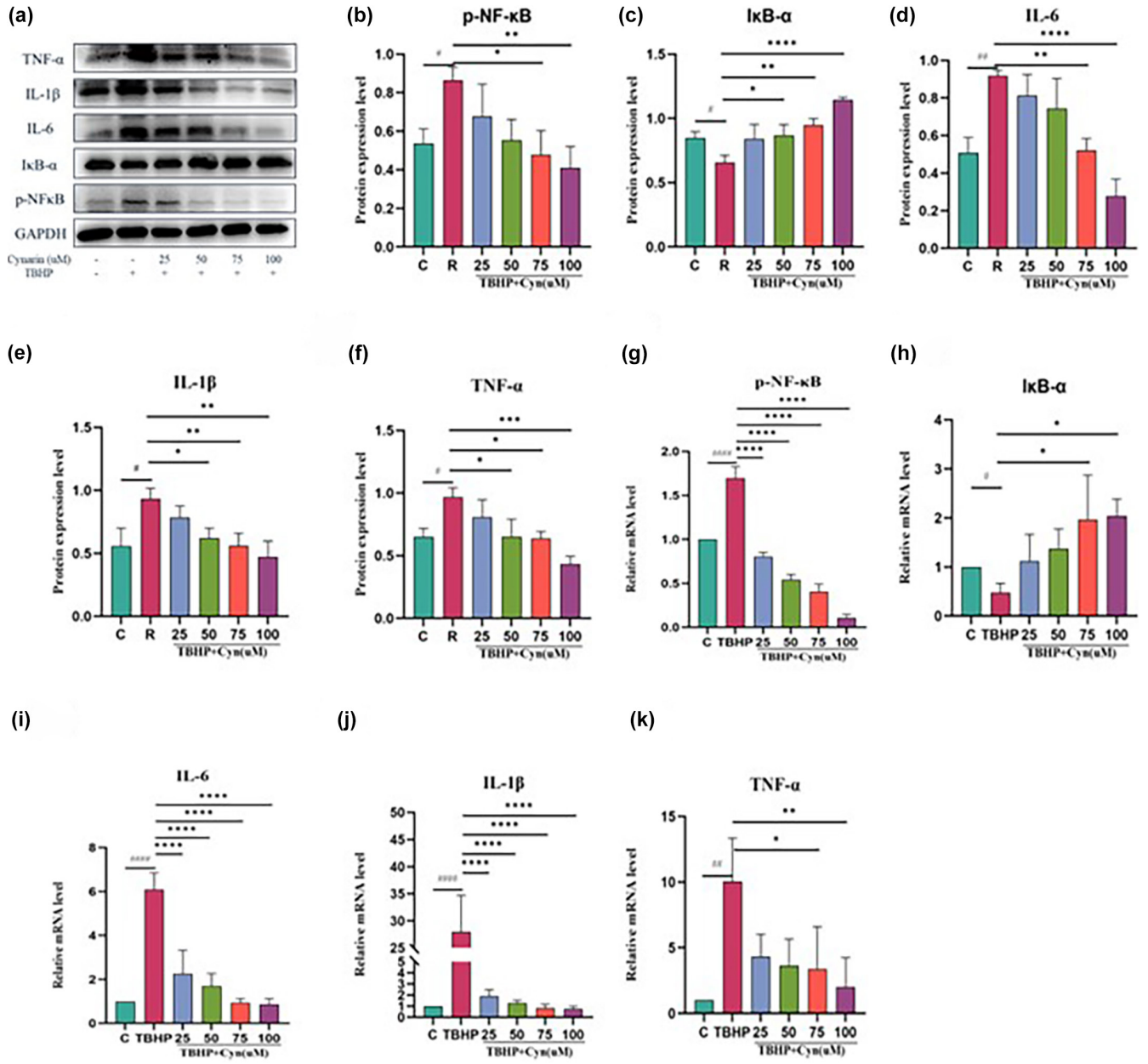

FIGURE 5 Effects of Cyn on the NF-κB pathway in AS-MSCs. The AS-MSCs were incubated with the indicated different doses of Cyn. (a) Protein levels of p-NF-κB, IκB-α, IL-6, IL-1β, and TNF-α were analyzed by Western blot. (b–f) Quantitative analysis of protein expression of p-NF-κB, IκB-α, IL-6, IL-1β, and TNF-α. (g–k) The mRNA levels of NF-κB pathway were detected by qRT-PCR. All data are presented as means ± SD and comparisons are performed by one-way analysis of variance. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ compared with control group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with model group, $n = 3$. AS, ankylosing spondylitis; Cyn, cynarin; MSC, mesenchymal stem cell; qRT-PCR, quantitative real-time polymerase chain reaction.

high levels of chronic inflammation.[25–28] Although Nrf-2 is the most important modulator of HO-1, other factors also have been reported to regulate the expression of HO-1, such as hypoxia-inducible factor 1α (HIF-1α).[29–31] Besides, within the HMOX1 promoter, biding sites for NF-κB have been proved in immune cells particularly.[32] As a pro-inflammatory transcription factor, NF-κB plays a key role in innate immunity and inflammation regulation via modulating the expression of cell surface receptors, chemokines, and cytokines.[33] In the background of inflammation, with the proteasomal degradation and ubiquitylation of IκB-α, and then p65 migrated into nucleus binding to DNA sequences to promote transcription of the pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α.[34] In AS-MSCs, we found that the expression levels of p-NF-κB, IL-6, IL-1β, and TNF-α were higher and IκB-α was lower compared with HC-MSCs, which indicated that NF-κB pathway was activated. Considering these findings,

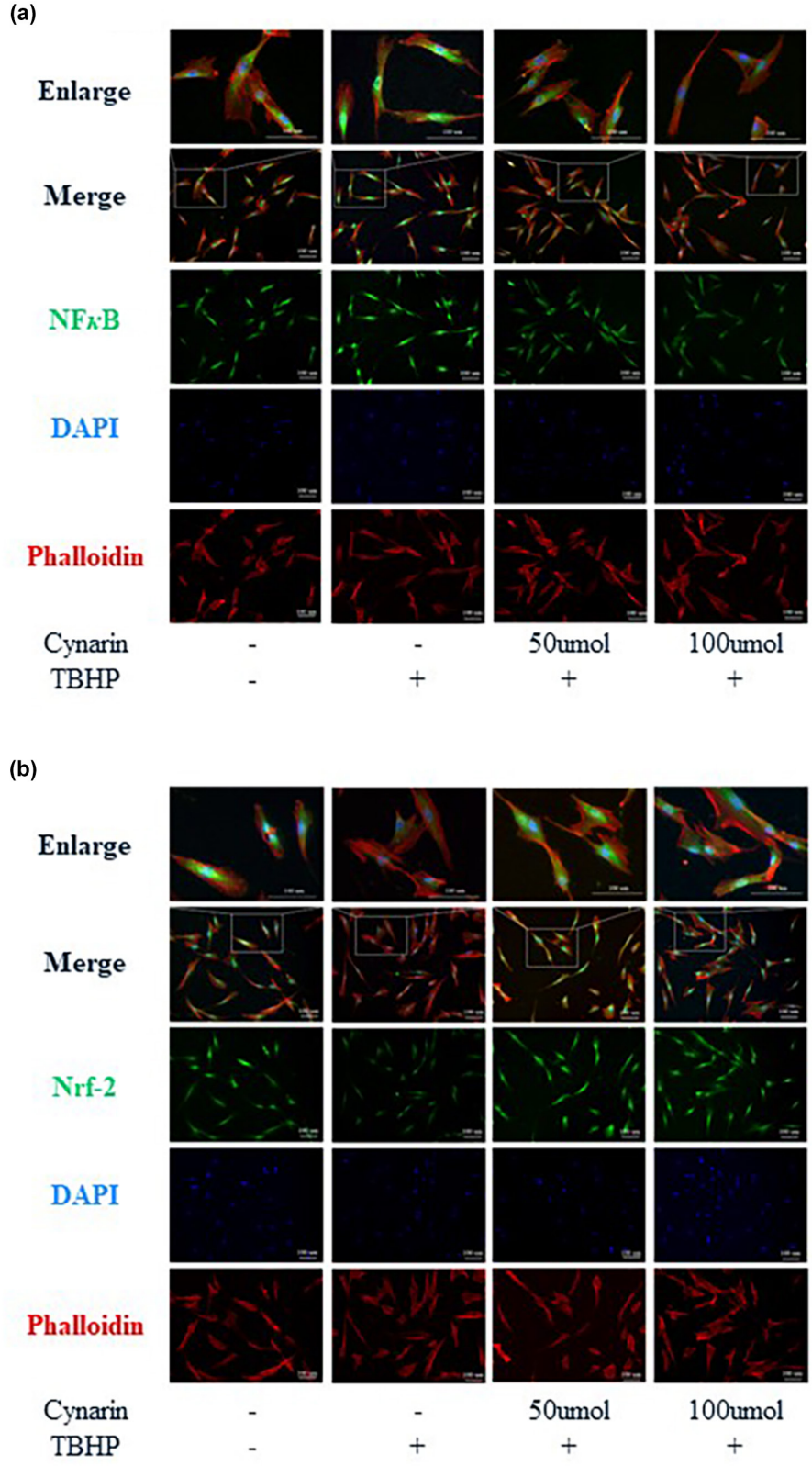

FIGURE 6 Effects of Cyn on nuclear translocation of NF-κB and Nrf-2 in TBHP-stimulated AS-MSCs. (a) The nuclear translocation of NF-κB detected by immunofluorescence (scale bar: 100 μm). (b) Immunofluorescence to detect the effect of Cyn on nuclear translocation of Nrf-2 in TBHP-stimulated AS-MSCs. (scale bar: 100 μm). AS, ankylosing spondylitis; Cyn, cynarin; MSC, mesenchymal stem cell.

it is fair enough to believe that promoting Nrf-2 activation or suppressing the NF-κB activation are effective for treatment of AS.

The ROS often defined as partial metabolites of oxygen characterized by possessing strong oxidizing abilities which is important to the progression of many inflammatory diseases.[35] In mitochondrial matrix, ROS can react with manganese SOD to product H_2O_2 which can enter into cytoplasm leading to activation of NF-κB and inflammasomes.[36–40] Studies have reported that the level of ROS was increased in

leukocytes in patients with AS and it was deemed as a mediator of AS pathogenesis.[41,42] Moreover, studies have also shown that AS serum promoted ROS production in MSCs and inhibited the generation of ROS can rescue AS-MSCs from senescence.[4] In this study, the ROS production was increased compared with HC-MSCs and all the findings indicated that targeting ROS is essential in the treatment of AS.

The artichoke is a plant cultivated in the Americas as a healthy food which has antioxidant capacity due to its rich phenolic compound content, including 1,3-, 3,4-, and 4,5-di-O-caffeoylquinic acid.[43,44] Cyn 1,3-di-O-caffeoylquinic acid was considered as the one possessing the highest capacity to suppress LDL oxidation and cholesterol biosynthesis.[45,46] In addition, the scavenging capacity of Cyn against ROS, such as hydroxyl radical, superoxide anion radical, and peroxyl radicals, was already reported in previous literatures.[47,48] In endothelial cells, the extracts from the artichoke alleviated oxidative stress induced by low-density lipoprotein and promoted the expression of endothelial nitric oxide synthase.[49] Moreover, Cyn can inhibit the activity of NF-κB in LPS-stimulated THP-1 human leukemic cells and acute alcohol-induced liver injury.[19,50] However, until this moment, the potential therapeutic value of Cyn against AS was not reported. In this study, the docking affinities of Cyn-p65-IκB-α and Cyn-Keap1-Nrf-2 were predicted by molecular docking analysis, and these indirect reaction mechanisms were verified in biological experiments. In AS-MSCs, Cyn stimulation suppressed the degradation of IκB-α, phosphorylation of p65, and the nuclear translocation of p65, which activated the expression of IL-1β and IL-6 and TNF-α in the nucleus, demonstrating that NF-κB is a downstream target of Cyn in anti-inflammatory function. To determine the effect of Cyn on the Nrf2/HO-1 pathway, the Nrf2 and HO-1 expression levels were detected. Our results suggested that Cyn increased the nuclear translocation of Nrf-2 and upregulated the expression of HO-1, resulting in the reduction of ROS. The trend and level of upregulation of protein and gene expression was consistent within the same group. To summarize, Cyn alleviates the inflammation via the Nrf-2/ROS/NF-κB axis in AS-MSCs. The potential mechanism is illustrated in Figure 7.

To be honest, one potential drawback should be pointed out; the safe concentrations of Cyn were selected by cell proliferation assay using cell counting kit-8 in vitro, which were not the actual physiological ranges in the in vivo experiments. In other words, the safe concentrations may not be easily reproduced in the in vivo experiments. However, the conclusion that Cyn worked safely in vivo might be inferred from the results of the current study and the previous studies. On one hand, in this study, the concentrations were proven to be safe in vitro, which was demonstrated in Figure 3b. On the other hand, Cyn played an antioxidant role in mice at a safe concentration and had few side effects in the human body as an immunosuppressive agent, which was also demonstrated in other previous studies.[43,50] Hence, it might be speculated that the levels of Cyn were likely to be achieved in animal models and human studies.

There were also other some limitations which should be mentioned in the current study. First, the molecular mechanism has not been validated in vivo. The artichoke was an edible herbal medicine widely studied due to its hepatoprotective and antioxidative effects.[50] Since Roman times, the leaves of the artichoke were used for hepatoprotection in traditional European medicine and the artichoke was used as a choleretic for its hepatoprotective and lipid-lowering actions in today's Germany.[44] Cyn was one of the most important antioxidant components in the artichoke. In animal models, Cyn could play an antioxidant role in mice at a safe concentration.[50] Besides, Cyn binds strongly to CD28 and has no significant binding with other T-cell receptors indicating that Cyn may have few side effects.[43] Therefore, there is a theoretical basis for applying Cyn to clinical treatment

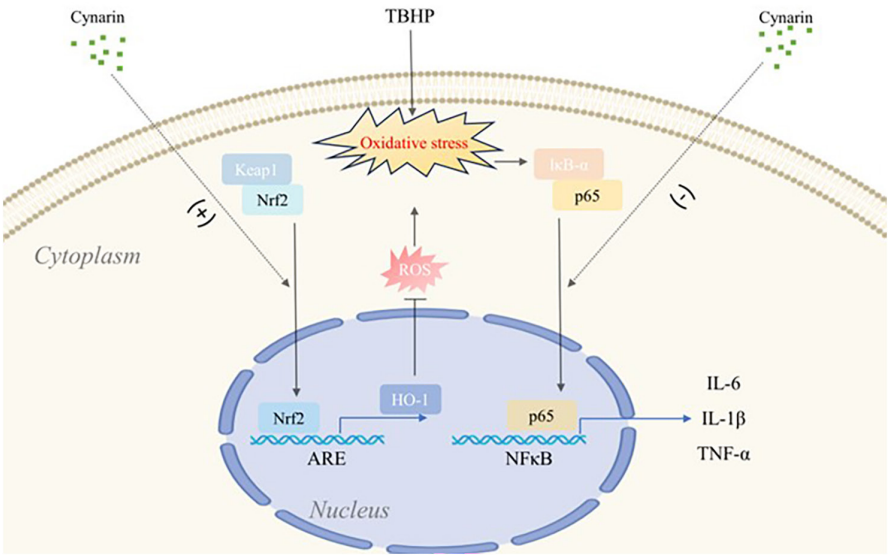

FIGURE 7 Schematic of Cyn regulation in the inflammatory response. Cyn might induce nucleus translocation of Nrf2 to enhance antioxidant capacity with reduction of intracellular ROS. Meanwhile, the NF-κB activation was impaired by Cyn to ameliorate the MSC-mediated inflammatory response. Cyn, cynarin; ROS, reactive oxygen species.

as a mild anti-inflammatory drug in the future. Second, this study did not investigate the effect of Cyn on new bone formation in AS. To the best of our knowledge, this study was the first time to investigate the molecular mechanism of Cyn in the treatment of AS. We can also predict potential targets and possible binding patterns of Cyn. Our findings indicated that Cyn significantly suppressed excessive inflammatory responses via the Nrf-2/ROS/NF-κB signaling axis in AS-MSCs. Using the AS-MSCs, we revealed the role of Cyn in anti-inflammation, anti-oxidative stress, Nrf-2/ROS/NF-κB regulation, and inhibiting gene and protein expression of proinflammatory factors, such as IL-1β and IL-6 and TNF-α, as well as explored its molecular docking using Autodock tools. These findings provide powerful evidence for Cyn as a promising natural immunosuppressive agent in the treatment of AS.

AUTHOR CONTRIBUTIONS

C.S., K.W., and B.Q. wrote the manuscript. C.S., K.W., and B.Q. designed the research. C.S., K.W., J.L., and M.Q. performed the research. C.S., K.W., Y.Q., B.W., and Y.Y. analyzed the data.

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CONFLICT OF INTEREST STATEMENT

The authors declared no competing interests for this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INFORMED CONSENT

All participating subjects or their legal guardians signed the written informed consents.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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