

# Anti-inflammatory effect of echinacoside in collagen-induced arthritis via Nrf2/Drp1 pathway

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D – writing the article; E – critical revision of the article; F – final approval of the article

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## Conflict of interest

None declared

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## Abstract

**Background.** Oxidative damage plays an important role in the progression of rheumatoid arthritis (RA). Emerging research evidence suggests that natural antioxidants may effectively ameliorate this disease.

**Objectives.** To investigate the therapeutic effect of echinacoside (ECH) in a collagen-induced arthritis (CIA) mouse model and thus elucidate the underlying molecular mechanism in RA.

**Materials and methods.** Collagen-induced arthritis mice were intraperitoneally administered 1% dimethyl sulfoxide (DMSO) (control) or 0.6 mg of ECH every other day for 1 month. Arthritis scores and the number of affected paws were assessed. On day 60, mice were euthanized, synovial tissue specimens were obtained, and serum interleukin (IL)-6 and IL-1 $\beta$  expression levels were measured. Mitochondrial morphologies, reactive oxygen species (ROS) content, expression of dynamin-related protein 1 (Drp1), IL-6, nod-like receptor protein 3 (NLRP3), kelch-like ECH-associated protein 1 (Keap1), and nuclear factor-erythroid-2-related factor 2 (Nrf2) contents in synovium were analyzed and compared between DMSO- and ECH-treated CIA mice.

**Results.** Following ECH treatment, mitochondria of CIA-induced mice were found to be elongated, while their arthritis scores, inflammation and the number of affected paws, and the expression levels of Drp1, NLRP3, IL-6, ROS, and Keap1 were all found to be significantly reduced. Conversely, the level of antioxidant factor Nrf2 was found to be elevated. Further, mitochondrial fission was found to be inhibited in synovial tissues.

**Conclusions.** Our findings validate the therapeutic efficacy of ECH in the CIA mouse model. Echinacoside may suppress oxidative stress and inhibit inflammation by regulating the Nrf2/Drp1 pathway, thus supporting its utility in the treatment of RA.

**Key words:** rheumatoid arthritis, antioxidant effect, Drp1 protein, echinacoside, mitochondrial fission

## Cite as

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## Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and bone deformities in multiple joints.<sup>1</sup> Despite significant advances in RA therapy, the functional disability rate remains high and continues to pose a significant threat to human health. Studies have shown that increased oxidative damage in synovial tissue plays a pivotal role in the progression of RA,<sup>2</sup> and increasing evidence suggests that natural antioxidant supplementation may have the potential to inhibit the progression of synovitis, and thereby serve as an effective therapeutic intervention in RA.

Oxidative damage in synovial tissues, induced by the excessive production of reactive oxygen species (ROS), plays a critical role in the progression of RA. An increased ROS content activates the release of inflammatory mediators in joints, which subsequently exacerbates the local proliferation of synovial tissues, thus eliciting an inflammatory response.<sup>3</sup> Being highly dynamic organelles, mitochondria are not only the major sources of ROS production but also the prime targets of their deleterious effects. Mitochondria constantly cycle through fusion and fission events and form net-like structures to balance their morphology and functions.<sup>4,5</sup> Studies have shown that excessive mitochondrial fission leads to the destruction of net-like structures, which in turn increases ROS production, resulting in oxidative damage. Moreover, increased mitochondrial fission has been implicated in a multitude of diseases.<sup>6,7</sup> A previous study has demonstrated increased fragmentation, an abnormal mitochondrial crest, and a damaged mitochondrial network structure in RA synovium.<sup>8,9</sup> Furthermore, our previous study established that dynamin-related protein 1 (Drp1 or DNM1L) is crucial for mitochondrial fission and plays an important role in the pathogenesis of RA. Finally, previous research also demonstrated that disease progression in RA could be significantly suppressed by inhibiting Drp1-mediated oxidative stress in synovium.<sup>9</sup>

In recent years, an increasing number of studies focused on the antioxidant role of nuclear factor erythroid 2 related factor 2 (Nrf2) in cellular defense against mitochondrial oxidative stress.<sup>10–13</sup> Studies have shown that Nrf2 activation, in response to increased oxidative stress, decreases Drp1-mediated mitochondrial fission and contributes to mitochondrial hyperfusion.<sup>14</sup> Moreover, knocking out the *Nrf2* gene resulted in reduced activity of mitochondrial respiratory chain complex I and mitochondrial fission.<sup>15–17</sup> Interestingly, mitoquinone (MitoQ), a mitochondria-targeted antioxidant, was shown to exert a protective effect by inhibiting Nrf2/Drp1-mediated mitochondrial fission.<sup>11</sup> Taken together, these reports highlight the quintessential role of Nrf2 in the maintenance of mitochondrial fission/fusion balance and the cytoprotective response against oxidative stress.

Echinacoside (ECH), a naturally occurring phenylethanoid glycoside (PhG), is the main component of the herb

cistanche (*Cistanches Herba*). Echinacoside has also been shown to possess different pharmacological activities, including neuroprotection, anti-tumor, antioxidant, anti-inflammatory, and immunomodulatory effects.<sup>18–20</sup> Furthermore, it was shown to have therapeutic effects in various cell cultures and disease models of Parkinson's disease and cancer.<sup>18,21–26</sup> A recent study demonstrated that ECH significantly inhibited Drp1 translocation into mitochondria, and regulated mitochondrial dynamics, which in turn reduced ROS production, resulting in the alleviation of neuroinflammation in rats with chronic cervical spinal cord compression.<sup>27</sup> However, the therapeutic effects of ECH in RA have not been completely explained yet.

## Objectives

In this study, we examined the effects of ECH in a collagen-induced arthritis (CIA) mouse model and elucidated the underlying molecular mechanism in the pathogenesis of RA.

## Materials and methods

### Reagents

Bovine type II collagen (cat. No. 20022) and incomplete Freund's adjuvant (IFA; cat. No. 7002) were procured from Chondrex, Inc. (Woodinville, USA). Freund's complete adjuvant (FCA) (cat. No. F5881) and dimethyl sulfoxide (DMSO) (cat. No. D2650) were purchased from Sigma-Aldrich (St. Louis, USA). Echinacoside (cat. No. B115837) was purchased from Aladdin (Shanghai, China). The specific antibodies anti-kelch-like ECH-associated protein 1 (Keap1) monoclonal antibody (cat. No. 60027-1-Ig) and anti-Nrf2 polyclonal antibody (cat. No. 16396-1-AP) were procured from Proteintech Group, Inc. (Rosemont, USA). Anti-Drp1 rabbit polyclonal antibody (cat. No. GB11855), anti-NLRP3 (nod-like receptor protein 3) rabbit polyclonal antibody (cat. No. GB11300), anti-interleukin (IL)-6 rabbit polyclonal antibody (cat. No. GB11117) were purchased from Servicebio Technology Co., Ltd. (Wuhan, China). IL-6 Mouse Uncoated enzyme-linked immunosorbent assay (ELISA) Kit (cat. No. 88-7064) and IL-1 $\beta$  ELISA kit (cat. No. 88-7013) were procured from Invitrogen (Waltham, USA). ROS Fluorescent Probe-Dihydroethidium (DHE) was purchased from Vigorous Co., Ltd. (Beijing, China). The BCA protein assay kit (cat. No. P0010S) was purchased from Beyotime Biotechnology (Shanghai, China).

### Study design and sample size

Based on experience, the CIA mouse model was established and then randomly divided into control (n = 8) and ECH treatment (n = 6) groups. The sample size was

determined based on our previous experience. Arthritis scores, histological changes, changes in blood and local joint inflammatory factors, and changes in local joint mitochondria morphology and expression of antioxidant factors were observed in both groups.

## Inclusion and exclusion criteria

Seven-week-old male DBA/1 mice, purchased from Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China), were housed in a specific pathogen-free facility with free access to food and water, as described previously.<sup>9</sup> Initially, the mice were immunized with an intradermal injection of 200 µg/mouse bovine type II collagen (emulsified with an equal volume of FCA) at the tail base. On day 21, mouse immunization was boosted at a different location with an intradermal injection of 200 µg/mouse bovine type II collagen (emulsified with an equal volume of IFA). From day 22, the mice began to develop symptoms, which presented as swollen joints and slow movement, and by day 27, mice with successful model induction were included in the experiment, and mice with failed induction were excluded.

## Randomization, blinding and outcome measures

On day 28, the mice were randomly assigned to intraperitoneal injections of DMSO (vehicle,  $n = 8$ ) or ECH (0.6 mg/mouse,  $n = 6$ ) once every other day for 1 month. The clinical score was assessed by 2 independent, blinded observers. On day 60, the mice were sacrificed, and whole knee joints and blood samples were collected. The animal study was approved by the Ethics Committee of Shaanxi University of Chinese Medicine (Xianyang, China; approval No. SUCMDL20210308017).

## Clinical and histological assessment of arthritis

The clinical scoring system, “arthritis score” (each paw was ranging from 0 to 4), was employed to assess the severity of arthritis as described previously, with a maximum score for each mouse of 16.<sup>9</sup> On day 60, the mice were euthanized, and their whole knee joint tissue sample was collected. Subsequently, the tissue specimens were fixed in 10% paraformaldehyde overnight and embedded in paraffin. Then, they were stained using hematoxylin and eosin (H&E) and toluidine blue. Histopathological analyses were performed to assess cartilage damage and bone erosion.

## Transmission electron microscopy analysis

Synovial tissue specimens were fixed in glutaraldehyde at 4°C for 2 h. Then, the specimens were post-fixed with 1% citric acid-0.1M phosphate buffer (PB) for 2 h at room

temperature. Before embedding, the tissue specimens were dehydrated and cleared. Ultra-thin sections (70 nm) double stained with uranylacetate and lead citrate were directly examined using transmission electron microscopy (TEM) (TEM HT7700; Hitachi High Technologies America, Inc., Schaumburg, USA).

## Immunohistochemical (IHC) analysis

Synovial tissue sections were fixed in 10% paraformaldehyde overnight, embedded in paraffin and sectioned. Then, samples were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min and heated with antigen retrieval solution. After blocking non-specific binding sites with 5% bovine serum albumin (BSA) (cat. No. AR0004; Boster Biological Engineering Co., Ltd., Wuhan, China) at 37°C for 10 min, the sections were probed using anti-Drp1 antibody (1:200), anti-IL-6 antibody (1:200) and anti-NLRP3 antibody (1:100) at 4°C overnight. Subsequently, the sections were incubated with the secondary antibody, horseradish peroxidase (HRP) goat anti-rabbit IgG antibody (1:2000) (cat. No. AS014; ABclonal Inc., Woburn, USA) for 1 h at room temperature, and visualized using diaminobenzidine. Images were obtained under a light microscope (Olympus Corp., Tokyo, Japan).

## Enzyme-linked immunosorbent assay

The blood was obtained when the mice were sacrificed on day 60 and clotted at room temperature for 2 h to obtain serum. IL-6 and IL-1 $\beta$  in the serum were measured according to the manufacturer’s protocol (cat. No. 88-7013, 88-7064; Invitrogen). Finally, the absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, USA).

## Reactive oxygen species analysis

Another portion of synovial tissue was placed in liquid nitrogen and then cut into frozen sections. The cellular ROS content in crystalline synovial membrane sections was determined with probe-DHE according to the manufacturer’s protocol. In mitochondria, when DHE gets oxidized by superoxide, it emits bright red fluorescence. Briefly, DHE was diluted to a final concentration of 20 µM with a serum-free medium. Then, the cell suspension was incubated with DHE at 37°C in the dark for 30 min, and nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI). The images were acquired using an inverted fluorescence microscope (model IX-53; Olympus Corp., Tokyo, Japan).

## Immunofluorescence staining

Nuclear factor-erythroid-2-related factor 2 accumulation and Keap1 expression were analyzed with immunofluorescence. Briefly, synovial sections were fixed

with 10% paraformaldehyde overnight and embedded in paraffin before sectioning. Then, samples were incubated in a blocking solution (containing Triton X-100 and 5% BSA) for 1 h at room temperature. After washing, the cells were incubated with anti-Nrf2 antibody (1:50) and anti-Keap1 antibody (1:50) at 4°C overnight. They were then incubated with Cy3-conjugated secondary antibody (1:500) for 2 h and counter-stained with DAPI. The fluorescence signals were evaluated using an standing fluorescence microscope (Nikon Eclipse C1; Nikon Corp., Tokyo, Japan).

## Statistical analyses

All statistical analyses were conducted using R software (v. 4.3.2; R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS v. 29.0 (IBM Corp., Armonk, USA). The normality of data was assessed using the Shapiro–Wilk test. In this study, all data exhibited non-normal distribution and were presented as median (1<sup>st</sup> quartile (Q1), 3<sup>rd</sup> quartile (Q3)). Mixed analysis of variance (ANOVA) was employed for the analysis of repeated measurement data with independent treatments. This analysis was conducted using the ‘ezANOVA’ function from the ‘ez’ package in R. We performed Mauchly’s test for sphericity and  $p > 0.05$ , which indicates variants are independent. Mixed ANOVA allows for more accurate modeling of the data by accounting for both within-subject (time) and between-subject (group) effects, which is essential for the robustness and validity of the results. The nonparametric 2-way ANOVA was used for repeated measures between independent treatments. The test for repeated measures is used when the assumptions of a standard ANOVA (such as normality of residuals) are not met. This analysis was conducted using the ‘Anova’ function from the ‘car’ package in R. We performed multiple comparisons using the glht() function in the R package ‘multcomp’. ‘glht’ stands for general linear hypothesis tests and is used to perform multiple pairwise-comparisons for parametric models. Differences between 2 independent treatment groups were compared using the Wilcoxon rank sum test. A significance level of  $p < 0.05$  was considered statistically significant.

## Results

### ECH ameliorates disease progression of CIA in mice

Echinacoside has been shown to have therapeutic effects in various cell cultures and disease models of Parkinson’s disease and cancer. To investigate whether ECH treatment has a therapeutic effect on RA, we used the CIA mouse model. Collagen-induced arthritis mice were randomized and treated with ECH (0.6 mg/mouse) or DMSO (control) every other day for 1 month. Notably, we observed that administration of ECH in CIA mice significantly decreased the arthritis scores from day 32 (Fig. 1A, Table 1,2 and

**Table 1.** Comparison of arthritis scores at different time between the 2 groups

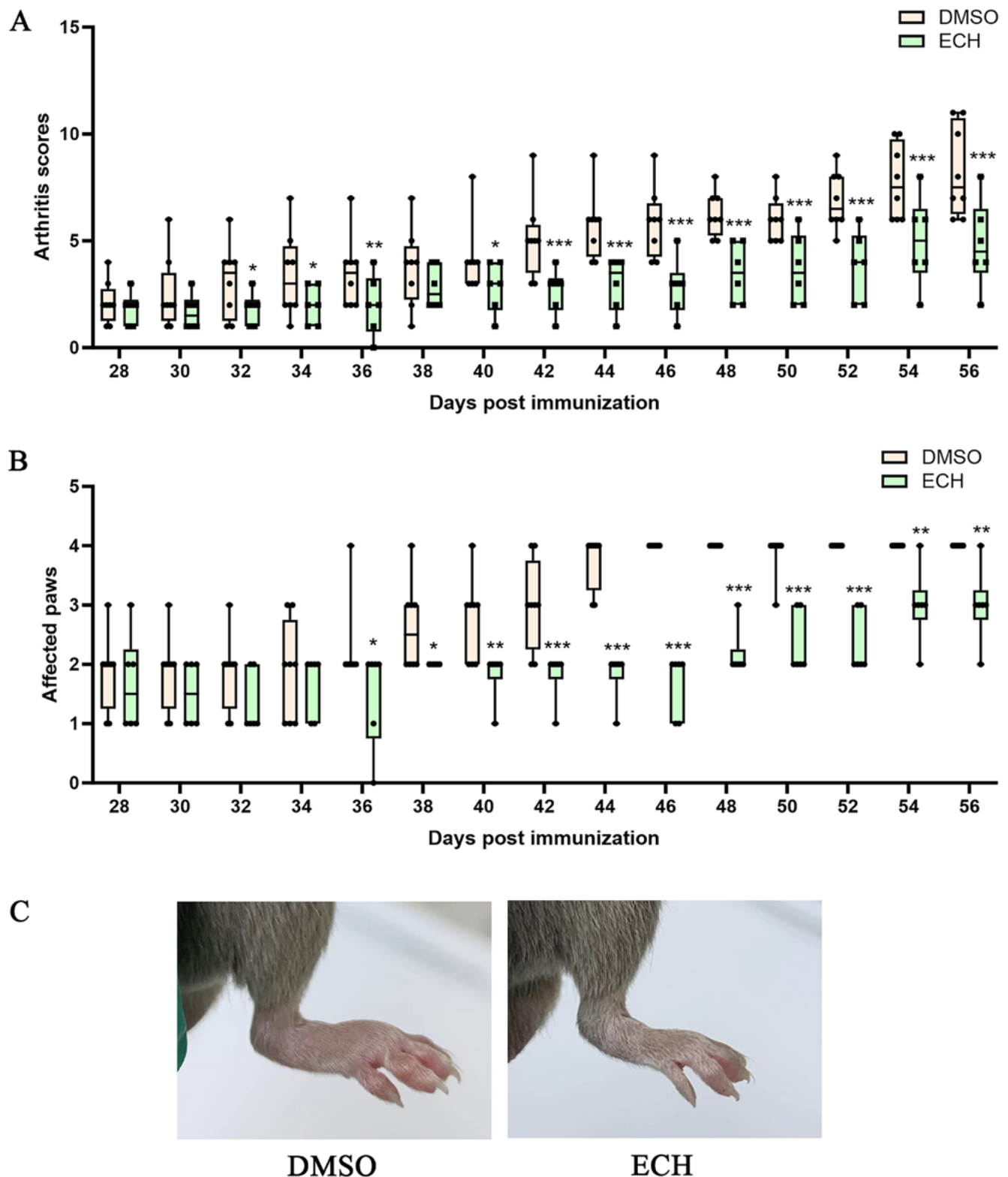
Days post immunization	Clinical parameters (arthritis scores)	
	DMSO-treated mice (n = 8)	ECH-treated mice (n = 6)
28	2.00 (1.25, 2.75)	2.00 (1.00, 2.25)
30	2.00 (1.25, 3.50)	1.50 (1.00, 2.25)
32	3.50 (1.25, 4.00)	2.00 (1.00, 2.25)*
34	3.00 (2.00, 4.75)	2.00 (1.00, 3.00)*
36	3.50 (2.00, 4.00)	2.00 (0.75, 3.25)**
38	4.00 (2.25, 4.75)	2.50 (2.00, 4.00)
40	4.00 (3.00, 4.00)	3.00 (1.75, 4.00)*
42	5.00 (3.50, 5.75)	3.00 (1.75, 3.25)***
44	6.00 (4.25, 6.00)	3.50 (1.75, 4.00)***
46	6.00 (4.25, 6.75)	3.00 (1.75, 3.50)***
48	6.00 (5.25, 7.00)	3.50 (2.00, 5.00)***
50	6.00 (6.00, 6.75)	3.50 (2.00, 5.25)***
52	6.50 (6.00, 8.00)	4.00 (2.00, 5.25)***
54	7.50 (6.00, 9.75)	5.00 (3.50, 6.50)***
56	7.50 (6.25, 10.75)	4.50 (3.50, 6.50)***

DMSO – dimethyl sulfoxide; ECH – echinacoside. The normality of measurement data was assessed using the Shapiro–Wilk test. In this study, the data exhibited non-normal distribution and were presented as mean (1<sup>st</sup> quartile (Q1), 3<sup>rd</sup> quartile (Q3)). The results were analyzed with mixed analysis of variance (ANOVA) test; \* $p < 0.05$  vs the control; \*\* $p < 0.01$  vs the control, \*\*\* $p < 0.001$  vs the control. The Shapiro–Wilk test was shown in Supplementary Table 1.

**Table 2.** Comparison of arthritis scores at different time between the 2 groups (mixed ANOVA test)

Effect	Dfn	Dfd	SSn	SSd	F-value	p-value
Intercept	1	12	3,538.305	240.756	176.36	<0.001
Group	1	12	208.006	240.756	10.368	0.007
Time	14	168	451.695	170.578	31.776	<0.001
Group:time	14	168	44.66	170.578	3.142	<0.001

Dfn – degrees of freedom in the numerator; Dfd – degrees of freedom in the denominator; SSn – sum of squares in the numerator; SSd – sum of squares in the denominator. The results were analyzed with mixed analysis of variance (ANOVA) test. The difference of arthritis score between the 2 groups was statistically significant ( $F = 10.368$ ,  $p = 0.007$ ). The difference of arthritis scores at different time points was statistically significant ( $F = 31.776$ ,  $p < 0.001$ ). The interaction of group and time point was statistically significant ( $F = 3.142$ ,  $p < 0.001$ ). The post hoc between-group analysis was shown in Supplementary Table 2.



**Fig. 1.** Echinacoside (ECH) ameliorates disease progression of collagen-induced arthritis (CIA) in mice. Following the induction of CIA, mice were randomized and treated intraperitoneally with dimethyl sulfoxide (DMSO) or 0.6 mg of echinacoside (ECH) every other day for 1 month. Arthritis scores and number of affected paws in CIA mice were compared between the DMSO and ECH groups (A, B). Mixed analysis of variance (ANOVA) was employed for the analysis of repeated measurement data with independent treatments. The non-parametric two-way ANOVA was used for repeatedly measured counted data between independent treatments. Data are expressed as mean (1<sup>st</sup> quartile (Q1), 3<sup>rd</sup> quartile (Q3)). In a box plot, the top whiskers contain 25% high-value data, the box contains 50% intermediate data, the middle line of the box indicates the median, and the bottom whiskers contain 25% low-value data. Data points represent each piece of data. Representative paw images of CIA mice following DMSO and ECH administrations (C)

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Table 3.** Comparison of affected paws at different time between the 2 groups

Days post immunization	Clinical parameters (affected paws)	
	DMSO-treated mice (n = 8)	ECH-treated mice (n = 6)
28	2.00 (1.25, 2.00)	1.50 (1.00, 2.25)
30	2.00 (1.25, 2.00)	1.50 (1.00, 2.00)
32	2.00 (1.25, 2.00)	1.00 (1.00, 2.00)
34	2.00 (1.00, 2.75)	2.00 (1.00, 2.00)
36	2.00 (2.00, 2.00)	2.00 (0.75, 2.00)*
38	2.50 (2.00, 3.00)	2.00 (2.00, 2.00)*
40	3.00 (2.00, 3.00)	2.00 (1.75, 2.00)**
42	3.00 (2.25, 3.75)	2.00 (1.75, 2.00)***
44	4.00 (4.00, 4.00)	2.00 (1.75, 2.00)***
46	4.00 (4.00, 4.00)	2.00 (1.00, 2.00)***
48	4.00 (4.00, 4.00)	2.00 (2.00, 2.25)***
50	4.00 (4.00, 4.00)	2.00 (2.00, 3.00)***
52	4.00 (4.00, 4.00)	2.00 (2.00, 3.00)***
54	4.00 (4.00, 4.00)	3.00 (2.75, 3.25)**
56	4.00 (4.00, 4.00)	3.00 (2.75, 3.25)**

DMSO – dimethyl sulfoxide; ECH – echinacoside. The normality of measurement data was assessed using the Shapiro–Wilk test. In this study, the data exhibited non-normal distribution and were presented as mean (1<sup>st</sup> quartile (Q1), 3<sup>rd</sup> quartile (Q3)). The results were analyzed with non-parametric two-way analysis of variance (ANOVA) analysis. \*p < 0.05 vs the control; \*\*p < 0.01 vs the control; \*\*\*p < 0.001 vs the control. The Shapiro–Wilk test was shown in Supplementary Table 3.

Supplementary Table 1,2) and number of affected paws from day 36 (Fig. 1B, Table 3,4 and Supplementary Table 3,4), thus ameliorating arthritis symptoms compared with the control group (Fig. 1C). Therefore, our findings suggest that ECH could have a potential therapeutic effect in CIA mice.

### ECH inhibits mitochondrial fission in the synovium of CIA mice

Next, we examined the effect of ECH administration on the synovial membrane in CIA mice. Histological analysis revealed that ECH-treated CIA mice exhibited intact joint architecture and showed a significant reduction in synovial inflammation (Fig. 2D,F) compared to controls (Fig. 2C,E). As suggested by our previous study, disease

progression in RA is correlated with abnormal mitochondrial fission.<sup>9</sup> Therefore, we compared the mitochondrial morphologies of synovium between the DMSO- and ECH-treated CIA mice by TEM. Interestingly, the mitochondria of synovial tissue specimens from ECH-treated CIA mice were found to be elongated (Fig. 2B) compared with that of control (Fig. 2A). Therefore, our results propose that ECH may ameliorate RA symptoms by inhibiting mitochondrial fission in the synovium of CIA mice.

### ECH suppresses inflammation in synovium by attenuating Drp1-mediated mitochondrial fission

Our previous studies found that by inducing abnormal mitochondrial division, Drp1 mediates oxidative stress in synovium and the release of inflammatory factors, aggravating synovial inflammation in RA.<sup>9</sup> To further examine the role of ECH on inflammation, we analyzed the expressions of Drp1, NLRP3 and IL-6 in synovium of CIA mice by performing IHC analysis. Notably, in synovial tissue specimens of CIA mice, the expression levels of Drp1, NLRP3 and IL-6 were found to be significantly downregulated compared to controls (Fig. 3A–C). Furthermore, we performed ELISA to analyze the expressions of IL-6 and IL-1 $\beta$  in peripheral serum samples of CIA mice. Our analysis revealed that, in ECH-treated CIA mice, the expression levels of IL-6 were significantly lower, in contrast to IL-1 $\beta$  expression levels and compared with that of controls (Fig. 3D, Table 5,6 and Supplementary Table 5). Our findings indicate that ECH may, therefore, suppress inflammation in synovium by attenuating Drp1-mediated mitochondrial fission in CIA mice.

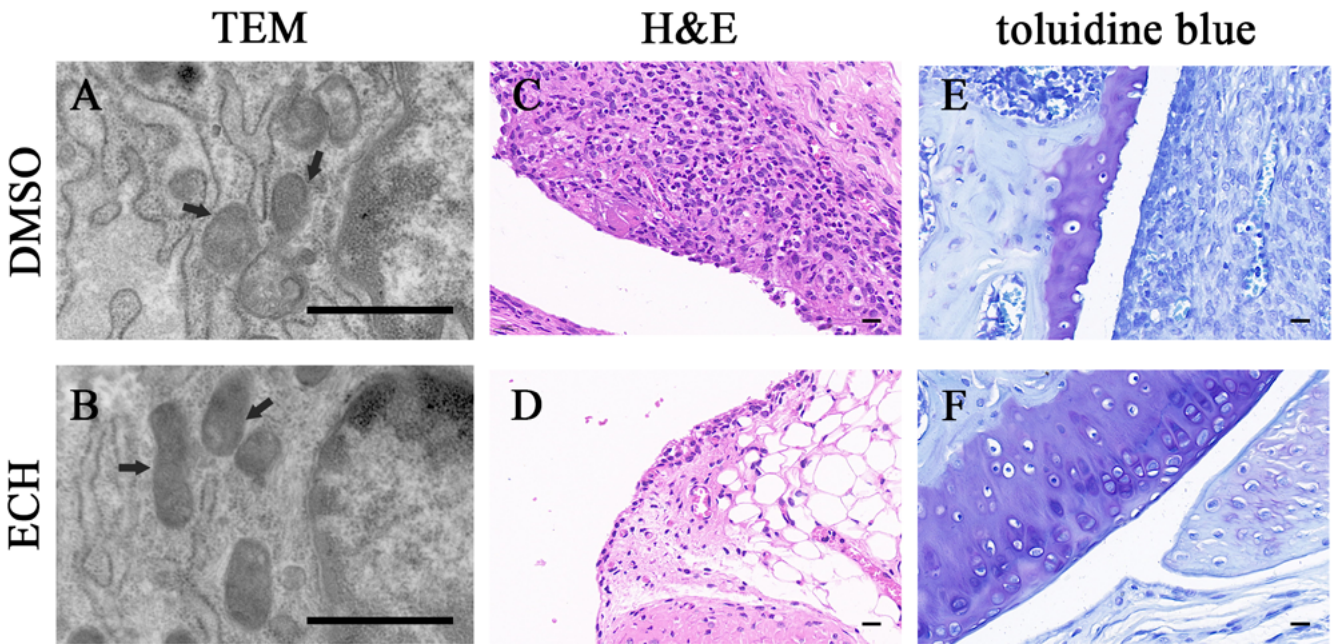
### ECH inhibits oxidative stress-mediated inflammation via Keap1-Nrf2 signaling pathway

Oxidative damage in synovial tissues, induced by excessive production of ROS, plays a critical role in the progression of RA. Therefore, we examined the impact of ECH treatment on oxidative stress response in the synovial tissues of CIA mice. To achieve this, we first determined the intracellular ROS levels by DHE staining. Expectedly, treatment with ECH slightly reduced the levels of ROS in synovial tissue

**Table 4.** Comparison of affected paws at different time between the 2 groups (nonparametric two-way ANOVA analysis)

Effect	Dfn	Sum Sq	F	p-value
Intercept	1	1291.43	4126.478	<0.001
Group	1	57.3	183.0938	<0.001
Time	14	86.09	19.6484	<0.001
Group:time	14	19.08	4.3545	<0.001
Residuals	180	56.33		

Dfn – degrees of freedom in the numerator; Sum Sq – sum of squares. The results were analyzed with nonparametric analysis of variance (ANOVA) analysis. The difference of affected paws between the 2 groups was statistically significant (F = 183.0938, p < 0.001). The difference of affected paws at different time points was statistically significant (F = 19.6484, p < 0.001). The interaction of group and time point was statistically significant (F = 4.3545, p < 0.001). The post hoc between-group analysis was shown in Supplementary Table 4.



**Fig. 2.** Echinacoside (ECH) inhibits mitochondrial fission in the synovium of collagen-induced arthritis (CIA) mice. Representative transmission electron microscopy (TEM) images of mitochondrial morphology, scale bar = 1  $\mu$ m (A, B). Representative images of hematoxylin & eosin (H&E) staining, magnification =  $\times$  40, scale bar = 20  $\mu$ m (C, D). Representative images of toluidine blue staining, magnification =  $\times$ 40, scale bar = 20  $\mu$ m (E, F)

**Table 5.** Comparison of cytokines in blood serum between the 2 groups

Cytokines	Cytokines in blood serum	
	DMSO-treated mice (n = 8)	ECH-treated mice (n = 6)
IL-6 [pg/mL]	41.55 (19.30, 71.13)	10.41 (7.86, 31.70)*
IL-1 $\beta$ [pg/mL]	32.39 (26.77, 34.29)	35.64 (29.01, 38.93)

IL – interleukin. The normality of measurement data was assessed using the Shapiro–Wilk test. In this study, the data exhibited non-normal distribution and were presented as mean (1<sup>st</sup> quartile (Q1), 3<sup>rd</sup> quartile (Q3)). The results were analyzed with Wilcoxon rank sum test; \* $p < 0.05$  vs the control. The Shapiro–Wilk test was shown in Supplementary Table 5.

specimens of CIA mice compared with that of controls (Fig. 4A). Moreover, Nrf2 has an important role in the maintenance of mitochondrial fission/fusion balance and the cytoprotective response against oxidative stress. Finally, to investigate the effect of ECH on the Keap1-Nrf2 signaling pathway, we detected the expression levels of Keap1 and Nrf2 using immunofluorescent assay. Notably, we observed that ECH treatment significantly increased Nrf2 nuclear translocation and decreased Keap1 levels in the synovial cytoplasm of CIA mice (Fig. 4B,C). Our findings suggest that ECH might attenuate oxidative stress response in synovium via the Keap1-Nrf2 signaling pathway.

## Discussion

In the present study, we found that ECH has a protective antioxidant role in CIA mice. Such findings support the anti-oxidative effect of ECH in different diseases, including osteoarthritis, ethanol-induced liver injury and inflammatory disease.<sup>27–30</sup> To the best of our knowledge,

**Table 6.** Comparison of cytokines in blood serum between the 2 groups (Wilcoxon rank sum test statistics)

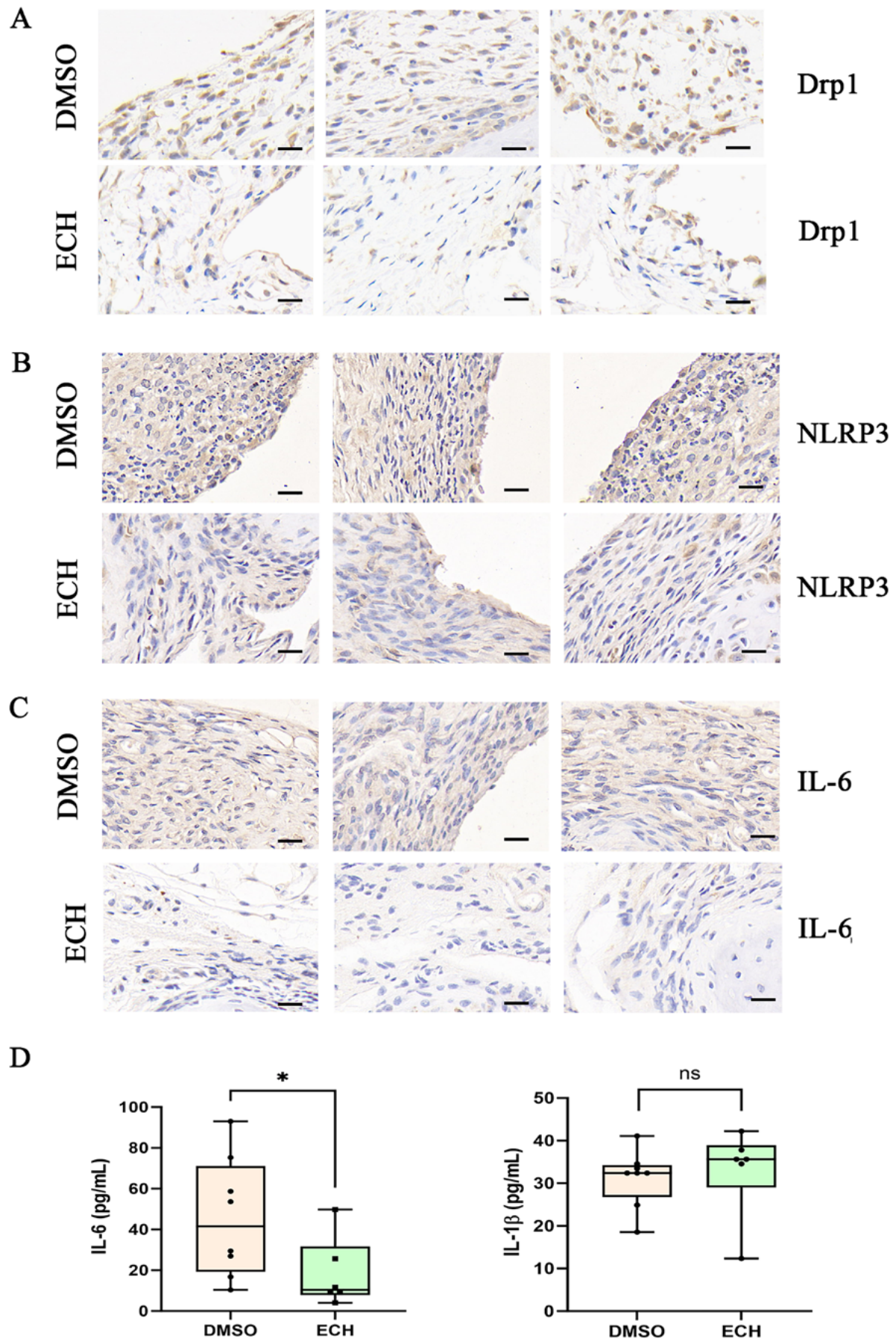
Test statistics	IL-6 expression	IL-1 $\beta$ expression
Mann–Whitney U	7.000	12.500
Wilcoxon W	28.000	48.500
Z	–2.197	–1.495
Asymp. Sig. (2-tailed)	0.028	0.135
Exact Sig. [2*(1-tailed Sig.)]	0.029 <sup>b</sup>	0.142 <sup>b</sup>

IL – interleukin. <sup>a</sup> grouping variable: group. <sup>b</sup> Not corrected for ties. The difference of IL-6 expression between the 2 groups was statistically significant ( $Z = -2.197$ ,  $p = 0.028$ ). The difference of IL-1 $\beta$  expression between the 2 groups was not statistically significant ( $Z = -1.495$ ,  $p = 0.135$ ).

this study is the first of its kind to validate the therapeutic efficacy of ECH in the CIA mouse model. A key finding of this study is that ECH might suppress oxidative stress-mediated inflammation via the Nrf2/Drp1 pathway.

Oxidative stress plays an important role in the progression of RA, and treatment with antioxidant drugs can effectively ameliorate the clinical symptoms of the disease.<sup>2,3,13</sup> Previous studies have shown that ECH exerts several pharmacological effects, including antioxidant and anti-inflammatory ones.<sup>18,19</sup> According to a report from 2021, ECH alleviates ethanol-induced oxidative stress and liver steatosis by reducing ROS levels and increasing antioxidant oxidase levels.<sup>29</sup> Further strengthening the results, we found that ROS levels declined sharply following ECH treatment. Consistent with previous observations,<sup>29–32</sup> we revealed that ECH administration significantly decreased arthritis scores and the number of affected paws in CIA mice. Meanwhile, it was also visible that in mice treated with ECH, malondialdehyde (MDA) expression levels were moderately downregulated in contrast

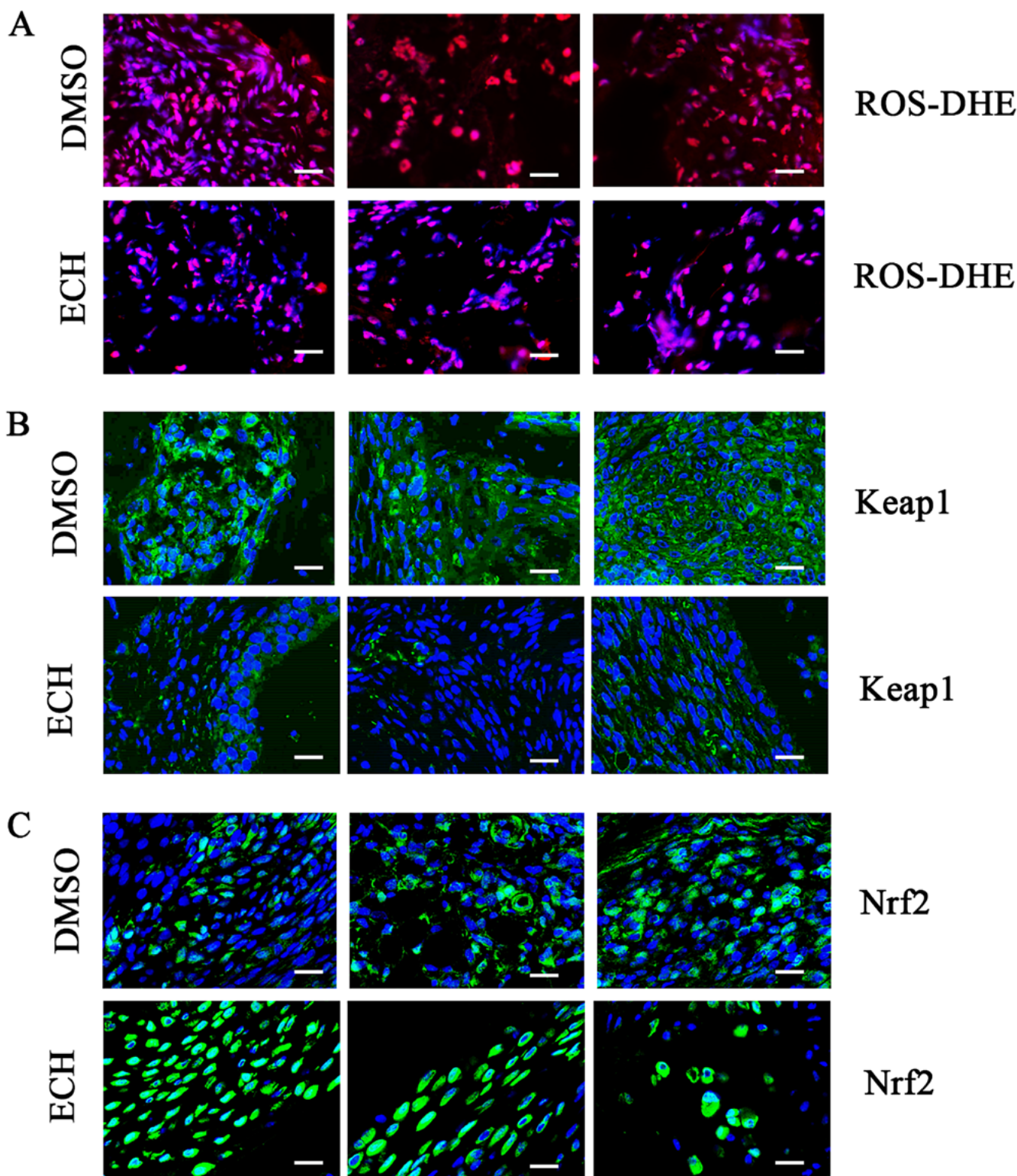




**Fig. 3.** Echinacoside (ECH) suppresses inflammation in synovium by attenuating dynamin-related protein 1 (Drp1)-mediated mitochondrial fission. Representative immunohistochemical (IHC) images showing the expressions of Drp1, NLRP3 and interleukin (IL)-6 in the synovium of collagen-induced arthritis (CIA) mice (A). Relative expression levels of IL-6 and IL-1 $\beta$  in the peripheral serum of dimethyl sulfoxide (DMSO)- and ECH-treated CIA mice, as determined using enzyme-linked immunosorbent assay (ELISA) (B). The results were analyzed using the Wilcoxon rank sum test. Data are expressed as mean (1<sup>st</sup> quartile (Q1), 3<sup>rd</sup> quartile (Q3)). In a box plot, the top whiskers contain 25% high-value data, the box contains 50% intermediate data, the middle line of the box indicates the median, and the bottom whiskers contain 25% low-value data. Data points represent each piece of data

\* $p < 0.05$ ; ns  $p > 0.05$ .





**Fig. 4.** Echinacoside (ECH) inhibits oxidative stress-mediated inflammation via the Keap1–Nrf2 signaling pathway. Representative immunofluorescence images (magnification = ×40, scale bar = 20 μm) illustrating the expression levels of reactive oxygen species (ROS), intracellular Keap1 and Nrf2 (A, B, C) in the synovium of dimethyl sulfoxide (DMSO)- and ECH-treated collagen-induced arthritis (CIA) mice

Keap1 – kelch-like ECH-associated protein 1; Nrf2 – nuclear factor-erythroid-2-related factor 2.

to glutathione (GSH) levels when compared to controls (data not shown). Our findings demonstrated that ECH significantly ameliorates inflammation in CIA mice, thus supporting its therapeutic utility in RA.

It is well known that increased mitochondrial fission directly promotes ROS production<sup>33</sup> and our previous report found that increased mitochondrial fission plays a crucial role in the progression of the disease.<sup>9</sup> In this

study, we observed that mitochondria of synovial tissue specimens from ECH-treated CIA mice were found to be elongated, thus indicating a significant reduction in mitochondrial fragmentation compared to DMSO-treated mice. In addition, the expression of ROS mediators was found to be downregulated after ECH administration. Supporting these results, our histological analysis revealed that ECH-treated CIA mice exhibited intact joint architecture and showed a significant reduction in synovial inflammation and bone erosion compared to controls. Collectively, these findings suggest that ECH can inhibit mitochondrial fission in the synovium via downregulation of ROS, which ultimately leads to the amelioration of clinical RA symptoms. Given that mitochondrial fusion and fission are essential for regulating mitochondrial morphology and governing its functions,<sup>6</sup> and several studies have shown that ECH may affect mitochondrial functions,<sup>31,32</sup> it is possible that ECH may modulate mitochondrial autophagy and apoptosis to affect CIA-mediated inflammation.

Dynamin-related protein 1 is one of the key regulators of mitochondrial fission.<sup>5</sup> According to a report from 2020, ECH was shown to attenuate neuroinflammation by regulating Drp1-dependent mitochondrial fission in rats subjected to chronic cervical cord compression.<sup>27</sup> Moreover, our previous study demonstrated elevated expression of Drp1 in RA, and that Drp1-targeted therapy may yield clinical benefits and improve prognosis in RA.<sup>9</sup> In line with this finding, we found that ECH treatment downregulated Drp1 expression and reduced synovial inflammation in CIA mice. Further strengthening these results, the serum expression level of the proinflammatory cytokines IL-6 were found to be significantly lower in ECH-treated CIA mice compared to the control mice. Collectively, these findings suggest that ECH might suppress inflammation in synovium by attenuating Drp1-mediated mitochondrial fission, and thus Drp1-targeted ECH therapy may improve the prognosis for patients with RA.

It has been well established that Nrf2 is closely linked to the regulation of mitochondrial biogenesis, dynamics and mitophagy, and is involved in antioxidant defense.<sup>15,34,35</sup> Studies have shown that Nrf2 activation could decrease mitochondrial fission through the degradation of Drp1, thus unraveling a novel mechanism by which Nrf2 mitigates oxidative stress.<sup>14</sup> According to a report from 2022, ECH alleviates osteoarthritis in rats by activating the Nrf2-HO-1 signaling pathway.<sup>28</sup> Another report from 2021 indicated that ECH targets Nrf2 to regulate oxidative stress and improve alcohol-induced liver injury.<sup>29</sup> In the current study, we found that ECH treatment significantly increased Nrf2 nuclear translocation and decreased Keap1 levels in the synovial cytoplasm of CIA mice. Interestingly, we also observed that ECH treatment significantly reduced NLRP3 expression. Hennig et al. have shown that ROS regulates NLRP3 inflammasome activation, and further demonstrated a correlation between Nrf2 activation and NLRP3 inflammasome inhibition in many different disease models

related to inflammation.<sup>36</sup> Therefore, in line with previous reports, the results of this study indicate that ECH might activate the Nrf2 transcription factor and increase its nuclear translocation, in turn leading to the inhibition of NLRP3 and the alleviation of synovial inflammation associated with RA. Collectively, our findings suggest that ECH attenuates oxidative stress response in synovium via the Nrf2/Drp1 pathway.

## Limitations

The primary limitation of this study is that the results may not be clinically relevant to humans with RA. Further, mice were given tap water and solid food ad libitum. Weight loss may have resulted in bone erosion. In addition, tests involving gene knockout mice are needed to further study the interactions of ECH with Drp1 and Nrf2. Finally, we did not perform safety tests and did not examine whether there are any gender differences. Hence, further investigations are warranted.

## Conclusions

The results of this study highlight that ECH treatment led to a decline in Drp1 expression levels, which altered mitochondrial morphology and reduced ROS production. Therefore, our findings validate the therapeutic efficacy of ECH in the CIA mouse model. In conclusion, ECH may suppress oxidative stress and inhibit inflammation by regulating the Nrf2/Drp1 pathway, thus supporting its utility in the treatment of RA.

## Supplementary data

The Supplementary materials are available at <https://doi.org/10.5281/zenodo.10665399>. The package includes the following files:

Supplementary Table 1. Comparison of arthritis scores at different time between the 2 groups (Shapiro–Wilk test).

Supplementary Table 2. Comparison of arthritis scores at different time between the 2 groups (post hoc between-group analysis).

Supplementary Table 3. Comparison of affected paws at different time between the 2 groups (Shapiro–Wilk test).

Supplementary Table 4. Comparison of affected paws at different time between the 2 groups (post hoc between-group analysis).

Supplementary Table 5. Comparison of cytokines in blood serum between the 2 groups (Shapiro–Wilk test).

## Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Consent for publication

Not applicable.

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