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Therapeutic effects of dracocephalum heterophyllum in collagen-induced arthritis

Yalan Wang^{1,2†}, Dongming Lai^{3†}, Yunyun Geng⁴, Peng Shang^{2*} and Pu Wang^{1,2*} 

Abstract

Rheumatoid arthritis (RA) is an autoimmune and inflammatory disease characterized by synovial inflammation, joint swelling, and cartilage and bone destruction. Dracocephalum heterophyllum (DH) is a compound in traditional Chinese herbal medicine well known for its anti-inflammatory, antiviral and antioxidant activities. In the present work, the therapeutic effects of DH were investigated in collagen-induced arthritis. Arthritis severity was assessed by clinical score, X-ray, and histopathological features. Expression of inflammatory cytokines was detected by qPCR and ELISA whereas anti-type II collagen antibodies were determined by ELISA. DH treatment significantly alleviated clinical scores, synovial inflammation, joint swelling, and cartilage and bone destruction. DH also reduced the production of inflammatory cytokines, including tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and interleukin-1 β (IL-1 β), and decreased the serum levels of anti-type II collagen specific IgG antibodies in collagen-induced arthritis. The therapeutic effects of DH in collagen-induced arthritis provide evidence that DH might be a potential therapeutic drug for rheumatoid arthritis.

Keywords: Rheumatoid arthritis (RA), Dracocephalum heterophyllum (DH), Inflammatory cytokines

Introduction

RA is a chronic systemic autoimmune disease characterized by synovial inflammation (synovial hyperplasia, pannus formation), joint swelling, and cartilage and bone destruction (Alam et al. 2017; Stuart et al. 1982a). RA affects people of all ages, and it occurs more often in women than men. The causes and pathogenesis of RA are complicated and it is believed that the genes and the environment are crucial to the disease (Firestein and McInnes 2017; Yap et al. 2018). Genes and milieu insults, for example, tobacco or epigenetic factors that cause activation of autoimmune responses (Alam et al. 2017; Viatte et al. 2013).

RA is also a chronic inflammatory disease. A key inflammatory cascade includes overproduction and overexpression of TNF- α . This pathway drives an

overproduction of many cytokines such as interleukin 6 and results in sustained inflammation and joint destruction (Yap et al. 2018). Overexpression of other pro-inflammatory cytokines, interleukin 1 β , IL-1 β , is also a trigger of inflammation and joint destruction (Firestein and McInnes 2017; Ruscitti et al. 2018).

The current potent therapeutic agents for RA include conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs), biological DMARDs, and targeted synthetic DMARDs (Smolen et al. 2016). However, about 30–40% of these medicines treated patients fail to achieve the clinical target or to maintain an initial good response over time or experience adverse events leading to treatment discontinuation (Burmester and Pope 2017; Romao et al. 2014). Therefore, there is a need to develop new ideal therapeutic agents for RA.

Dracocephalum heterophyllum (DH) is a traditional Chinese herbal medicine. Several effects of DH have been reported, including anti-inflammatory, antiviral and antioxidant activity (Shi et al. 2016). It can be used as a medicine to treat various ailments such as cough,

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lymphangitis, and mouth ulcers, but the therapeutic effects of DH on rheumatoid arthritis are not clear. In this study, we used a model of collagen-induced arthritis in DBA/1 mouse to determine whether DH treatment can prevent rheumatoid arthritis.

Material and methods

Reagent

Dimethyl sulfoxide (DMSO, $\geq 99\%$, Ultra Pure Grade, AMRESCO, USA). Complete Freund's adjuvant and incomplete Freund's adjuvant were purchased from Sigma-Aldrich (USA). Bovine type II collagen (CII) was purchased from Chondrex (USA). Goat anti-mouse IgG1 and IgG2a antibodies were purchased from Sigma-Aldrich (USA). HRP-conjugated donkey anti-goat antibody was obtained from Santa Cruz Biotechnology (USA). StarSpin Animal RNA Mini Kit was purchased from GenStar (Beijing, China). PrimeScriptTM RT Reagent Kit (Perfect Real Time) was obtained from TaKaRa (Shiga, Japan). TransStart Tip Green qPCR SuperMix was purchased from TransGen Biotech (Beijing, China). Specific qPCR primers for mice tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), interleukin1 β (IL-1 β), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Genewiz (Suzhou, China). TNF- α , IL-6, IL-1 β ELISA kits were obtained from Dakewe (Shenzhen, China).

Animals

Seven- to 8-week-old male DBA/1 mice were provided by Beijing Vital River Laboratory Animal Technology (Beijing, China). DBA/1 mice were raised in an animal facility which was maintained at 24° C and 40–60% humidity. All mice are fed specified-pathogen-free (SPF) food and water. All procedures were preapproved by the Institutional Animal Care and Use Committee.

Preparation of DH extract

The medicinal DH was prepared following the method described previously (Dang et al. 2018; Shi et al. 2016). The whole grass of DH was harvested from North Mountain in Huzhu, Qinghai province, China. The sample was identified by Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Qinghai, China. The fresh samples were air-dried in shade, then ground into a homogeneous powder with a pulverizer (RS-FS553, Royalstar, China). Eight kilograms of air-dried DH powder was macerated in 95% ethanol (CAS No.64-17-5, Sigma-Aldrich, USA) and shook for 2 h. The samples were filtered with Whitman filter paper (Grade-1-1001-125, TISCH, USA) while the residue was further extracted under the same conditions for 3 times. The filtrate was collected, and then ethanol was completely removed by

a rotary evaporator (EYELA, Japan) at 50 °C to get the crude extract of DH. The crude ethanol extract (971 g) of DH was suspended into 500 ml water. The suspension was successively extracted 3 times by the same volume of ethyl acetate (CAS No.141-78-6, Sigma-Aldrich, USA) at room temperature to get ethyl acetate extract. The extract (5.0 mg/ml) was analyzed by HPLC (Column: Odyssil C18 (250 mm \times 4.6 mm, 5 μ m) and stored at 4 °C until used. The working DH extract (purity $\geq 95\%$) was dissolved in DMSO and further diluted with Phosphate Buffered Saline (PBS, No.ST447-1L, Beyotime Biotechnology, China). The working concentration of extract is 5 mg/mL. The final concentration of DMSO in injection solution was <1% (v/v).

Establishment of CIA model and therapeutic treatment of DH

Type II collagen (CII, 2 mg/mL) was mixed with an equal amount of complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA). The CII emulsion (CII concentration 1 mg/mL) was prepared by emulsification using a glass booster and stored in a refrigerator at 4 °C. CIA model was induced in DBA/1 mice by intradermally administering 0.1 mL of bovine type II collagen (100 μ g) emulsified with complete Freund's adjuvant in the base of the mouse tail. Twenty-one days after the initial immunization, the animals received subcutaneous booster injection of bovine type II collagen (100 μ g) emulsified with incomplete Freund's adjuvant. After the mice developed joint swelling (day 25), the mice were treated with DH by intraperitoneal administration once every other day for 50 days. CIA mice were casually divided into CIA group (CIA) and DH group (CIA+DH) with six mice per group. The animals were sacrificed at day 75 after the first immunization.

Arthritis score

The severity of arthritis in CIA was monitored once every other day after booster immunization. The arthritis severity score of the paw is used to reflect the severity of arthritis. The scores are divided into 0 (normal joints), 1 (1 toe swelling), 2 (2 or 3 toe swelling or slight swelling of the entire paw), 3 (4 toes swelling, moderate swelling of the entire paw), and 4 (whole paws severe swelling). Arthritis score (AI) = sum of limb joint scores, the highest score for each paw is 4 points, and the maximum score for each mouse is 16 points (Li et al. 2017).

X-ray examination

On day 73 of the experiment, images of the ankle and foot joints of the mice were taken by a small animal X-ray machine (XRad225Cx, Precision X-Ray Inc., North Branford, CT, USA), and then imaging examination and

analysis were conducted. The swelling degree of joint soft tissue and the destruction of joint bone were observed in CIA mice administrated with or without drugs.

HE staining

On day 75, the mice were sacrificed, and the right hind knee joints and ankle joints were fixed with 4% paraformaldehyde for 48 h. The joint tissues were decalcified and dehydrated at gradient concentrations of ethanol. Paraffin-embedded tissues were sectioned and stained by H&E staining. The pathological changes of the ankle of the mice were observed under a microscope (Leica DM750, Germany) and images were obtained with a 20X objective.

Detection of inflammatory cytokines

Also on day 75, the mice were sacrificed, spleen and paw tissues were ground in liquid nitrogen using a porcelain mortar (60 mL) until they became a fine powder. The extraction buffer corresponding to each method was added to samples of the obtained tissue powder. Total RNA was extracted using TRIzol (Invitrogen) and reverse-transcribed into cDNA according to the manufacturer's instructions. (Kim et al. 2017). Primer sequences used for real-time PCR were as follows: TNF- α forward 5'-TAGCTCCCAGAAAAGCAAGC-3' and reverse 5'-TTTTCTGGAGGGAGATGTGG-3'; IL-6 forward 5'-CCACTTCACAAGTCGGAGGCTTA-3' and reverse 5'-GCAAGTGCATCATCGTGTTCATAC-3'; IL-1 β forward 5'-TTGACGGACCCCAAAAGATG-3' and reverse 5'-AGAAGGTGCTCATGTCCTCA-3'; GAPDH forward 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'. The program procedure of DNA amplifications was a pre-denaturation at 95 °C for 5 min, 40 cycles of 95 °C for

10 s, and 60 °C for 30 s. All samples were tested in triplicate, and cytokine mRNA expression was normalized to GAPDH mRNA. Relative differences in gene expression among study groups were determined using the comparative Ct (Δ Ct) method. Fold expression was calculated using the formula Δ Ct.

Blood was obtained from the orbital venous sinus. Serum levels of TNF- α , IL-6, and IL-1 β were determined using a specific enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Detection of anti-type II collagen antibodies

To detect anti-type II collagen antibodies in the serum, bovine type II collagen (2 mg/mL) embed on ELISA plates overnight at 4 °C. Serum from mice was added in 1:3 dilution and incubated for 90 min at 37 °C. Goat anti-mouse IgG1 or IgG2a were used at 1:700 dilution as the first detection reagent. HRP-conjugated anti-goat antibody was used at 1:1500 dilution as the secondary detection reagent. The concentration of anti-type II collagen antibodies was shown as OD values.

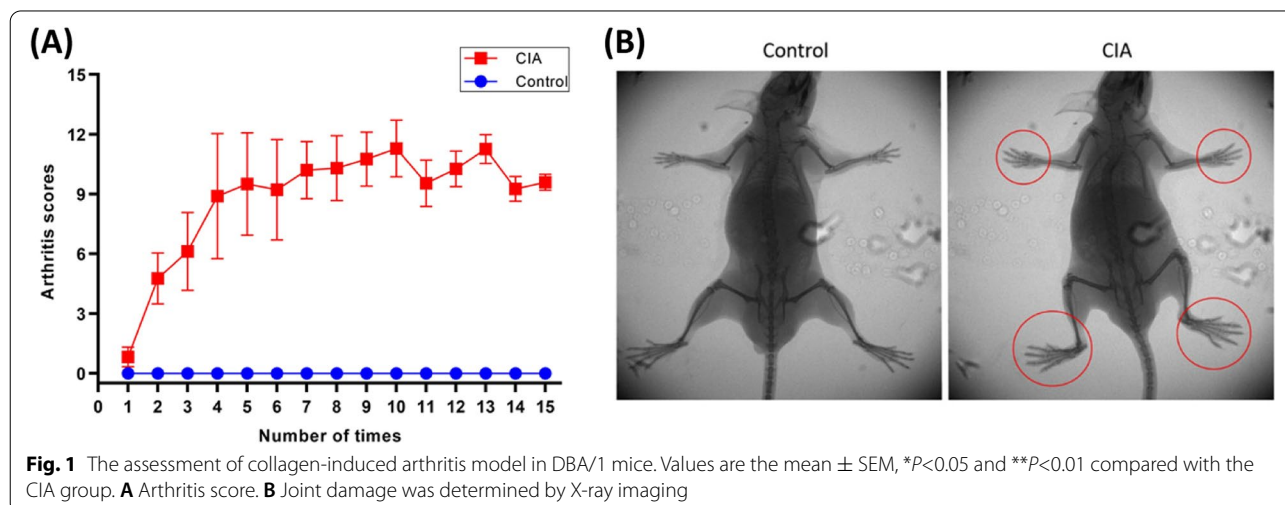
Statistical analysis

The results were analyzed by one-way analysis of variance. All data are represented as mean \pm SEM. All statistical analyses were performed with GraphPad Prism 8.0 and P value < 0.05 was considered to be statistically significant.

Results

DH ameliorated disease severity in CIA mice

To assess the extent to which DH inhibits the development of autoimmune arthritis, a collagen-induced arthritis mouse model was established. The result in Fig. 1 showed that the control group did not show signs of swell



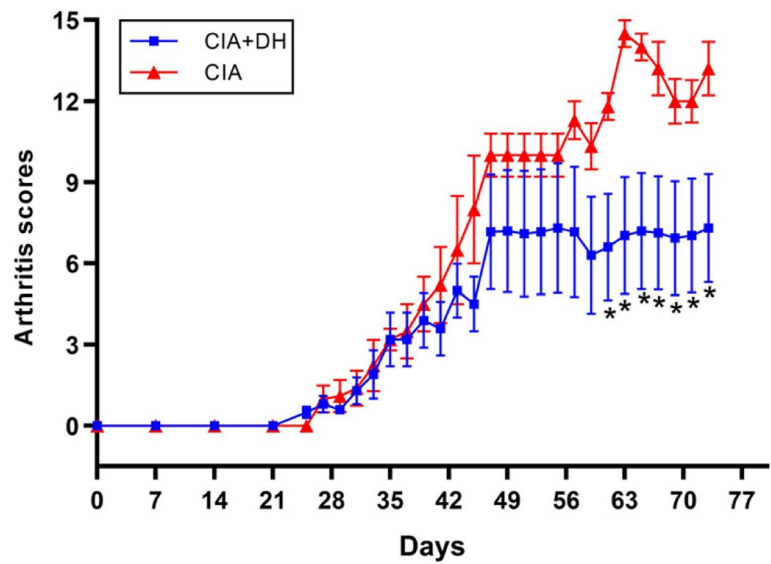


Fig. 2 Effects of DH on disease severity in CIA mice. Values are the mean ± SEM, * $P < 0.05$ and ** $P < 0.01$ compared with the CIA group

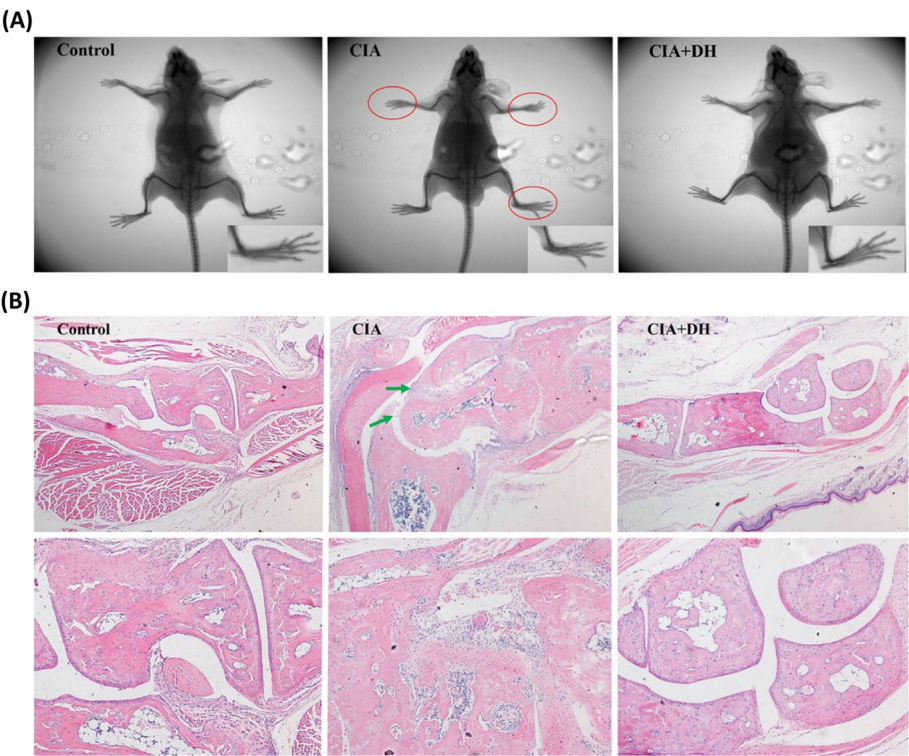


Fig. 3 Effects of DH on the pathology of ankle tissue in CIA mice. **A** Joint damage was determined by X-ray imaging. **B** Soft tissue and structure of the ankle of CIA mice were determined by H&E staining

and redness in four paws, and the CIA group showed swelling in four paws.

Arthritis score directly shows the severity scale of arthritis. Each mouse was scored every other day after the second immunization (day 25). The results showed that on day 47, compared with the CIA group, the score of arthritis in the DH group was relieved and exhibited significant differences after day 61 ($*p<0.05$) (Fig. 2). In the light of this result, DH displayed the therapeutic effect in RA, particularly in the late phase of collagen-induced arthritis.

The effect of DH on the pathology of the joints in CIA mice

The effect of DH treatment on joint swelling and destruction in CIA mice is observed by X-ray imaging. Our data showed that joint soft tissue was not swollen and the joint was undamaged in the control group. In the CIA group, joint soft tissue was obviously swollen. In comparison, joint soft tissue swelling was significantly relieved in the DH treatment group (Fig. 3 A).

H&E staining was performed for histopathological evaluation of mouse joints to examine the extent of arthritic damage, including synovial hyperplasia, pannus formation, inflammatory cell infiltration, and cartilage and bone damage. In the control

group, the synovial membrane did not proliferate, no inflammatory cells infiltrated, and the articular cartilage surface was smooth and structurally intact. In the CIA group, the synovial membrane proliferated and with a large number of inflammatory cells infiltrated, and a small amount of inflammatory exudate was seen in the joint cavity. The cartilage and the synovial tissue structure from the DH-treated mice showed much less damage when compared with the CIA group (Fig. 3 B).

Decreased inflammatory cytokine expression in the DH-treated mice

Multiple pro-inflammatory cytokines have been involved in the pathological processes of CIA. Among them, cytokines such as TNF- α , IL-6, and IL-1 β are highly expressed in CIA, thereby contributing to bone damage in inflamed joints (Joosten et al. 2008; Tao et al. 2017). To investigate whether DH treatment inhibited cytokine expression, quantitative RT-PCR and ELISA analysis were used to detect the level of cytokines produced. The results displayed that the mRNA expressions of TNF- α , IL-6, and IL-1 β were significantly reduced in the spleen and paw of the DH treatment group (Fig. 4).

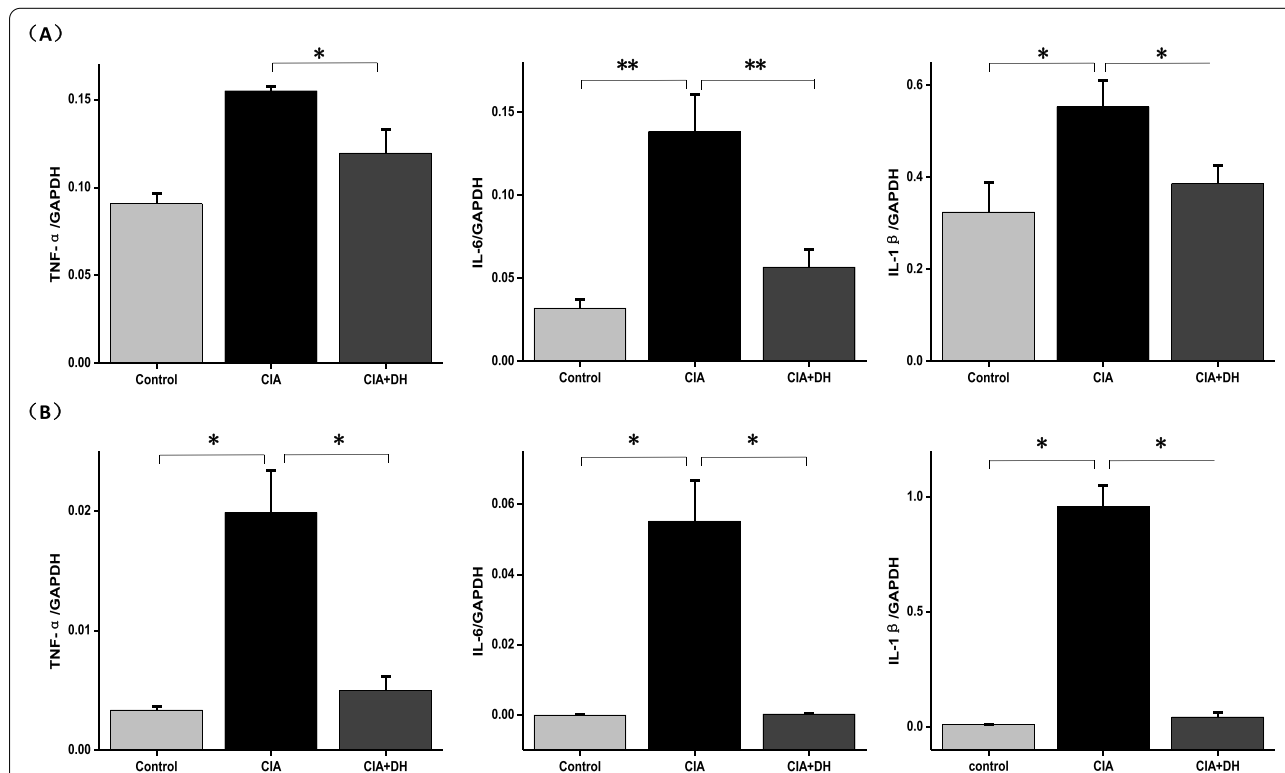
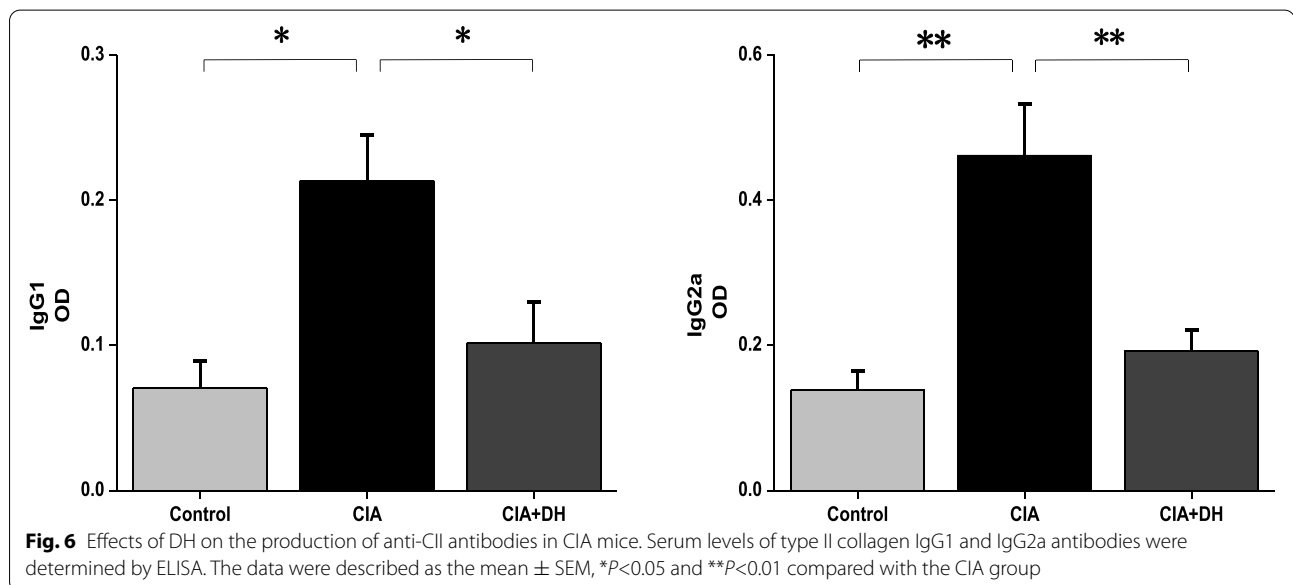
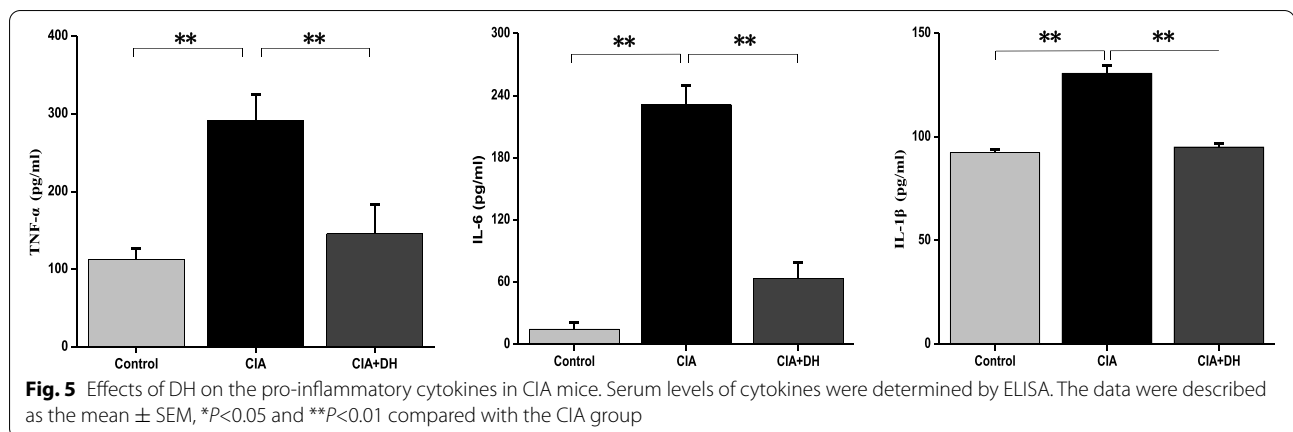


Fig. 4 Effects of DH on the production of pro-inflammatory cytokines in CIA mice. The data were described as the mean \pm SEM, $*P<0.05$ and $**P<0.01$ compared with the CIA group. **A** Cytokines of spleen tissues were determined by RT-PCR. **B** Cytokines of paw in CIA mice were determined by RT-PCR



We also found that DH treatment decreased the serum levels of TNF- α , IL-6, and IL-1 β (Fig. 5). The down-regulation of cytokine levels in the DH administration group was related with decreased severity of CIA.

DH treatment down-regulated production of anti-CII antibodies

Accumulating evidences indicated that anti-type II collagen IgG antibodies were critical to the development of collagen-induced arthritis (Stuart et al. 1982a). It has been shown that arthritis in DBA/1 mice is associated with high levels of type II collagen-specific IgG antibodies (Stuart et al. 1982b). To explore the effect of DH treatment on IgG antibodies for type II collagen in arthritic mice, we tested serum levels of type II collagen IgG

antibodies. The results showed that DH-treated mice had significantly lower levels of anti-type II collagen IgG1 and IgG2a antibodies in CIA mice (Fig. 6).

Discussion

Dracocephalum heterophyllum (DH), a perennial plant of the family Labiatae, possesses various pharmacological effects involved in anti-inflammatory and antibacterial activities (Shi et al. 2016; Zheng et al. 2016). In this study, we examined the therapeutic effect of DH in Collagen-induced arthritis (Joosten et al. 1997). The present results showed that DH treatment could significantly decrease the arthritis scores (Fig. 2), alleviate joints swelling (Fig. 3 A), synovial proliferation, inflammatory cell infiltration, inflammatory extract in the joint cavity, cartilage,

and bone damage (Fig. 3 B) in CIA mice. It means that DH can relieve the development and progression of arthritis.

The pathogenesis of rheumatoid arthritis is complicated. There has been considerable progress in pro-inflammation cytokines that contribute to the etiology of RA. Inhibition of cytokine (including TNF- α , IL-6, and IL-1 β) production and expression can alleviate rheumatoid arthritis (Haleagrahara et al. 2017; Koopman et al. 2016). Consistent with these studies, the CIA group had higher levels of TNF- α , IL-6, and IL-1 β . By contrast, the DH treatment group reduced TNF- α , IL-6, and IL-1 β levels and was significantly different from the CIA group (Figs. 4 and 5). Our findings showed that DH administration relieved collagen-induced arthritis by inhibiting the production and expression of pro-inflammatory cytokines.

Anti-type II collagen antibodies play an important role in the collagen-induced arthritis. Autoantibody production precedes the first clinical symptoms of arthritis and significantly hasten the onset of disease (Dayer et al. 1990; Mikecz et al. 1990). The studies have demonstrated that the antibodies to type II collagen were detected in rheumatoid arthritis patients (Clague and Moore 1984; Rowley et al. 2008). The levels of anti-type II collagen antibodies as an evaluation index of rheumatoid arthritis are of great significance in practical applications. Study on collagen-induced arthritis in rats also supported the hypothesis that a correlation between autoantibody levels and arthritis severity, and others have found that joint damage has never been seen without high levels of anti-type II collagen antibodies (Clague et al. 1980; Stuart et al. 1982a). DH treated mice significantly inhibited the production of anti-type II collagen IgG1 and IgG2a antibodies (Fig. 6), which alleviated disease severity.

In the present study, mice with collagen-induced arthritis (CIA) were treated with DH after the first signs of arthritis. DH at a later stage of CIA slowed the progression of the disease. A clear reduction of the levels of anti-type II collagen antibodies and expression of synovial TNF- α , IL-6, and IL-1 β after DH treatment in the late stage of arthritis. DH seems to reduce the late stage of the arthritis process and may be considered to be potential agents for the treatment of destructive arthritis.

Conclusion

Together, administration with DH alleviated collagen-induced arthritis by the remission of synovial inflammation (synovial hyperplasia, inflammatory cell infiltration, inflammatory extract in the joint cavity), joints swelling, and cartilage and bone destruction. It also

down-regulated pro-inflammatory cytokines, including TNF- α , IL-6, and IL-1 β as well as anti-type II collagen IgG1 and IgG2a antibodies. These results indicated that DH can protect against arthritis via inhibiting inflammatory responses, and the study provides a new possibility for the treatment of rheumatoid arthritis with DH.

Abbreviations

RA: Rheumatoid arthritis; DH: *Dracocephalum heterophyllum*; HE: Hematoxylin eosin staining; qPCR: Quantitative real-time PCR; ELISA: Enzyme-linked immunosorbent assay; TNF- α : Tumor necrosis factor alpha; IL-6: Interleukin 6; IL-1 β : Interleukin-1 β ; DMARDs: Disease-modifying anti-rheumatic drugs; DMSO: Dimethyl sulfoxide; CII: Bovine type II collagen; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PBS: Phosphate buffered saline; CFA: Complete Freund's adjuvant; IFA: Incomplete Freund's adjuvant.

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Authors' contributions

YLW, DML, and YYG participated in the experimental design and manuscript draft and performed the experiments. PS and PW participated in the design, coordination, and drafts of the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

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

Declarations

Competing interests

The authors declare that they have no competing interests.

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