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Wedelolactone ameliorates synovial inflammation and cardiac complications in a murine model of collagen-induced arthritis by inhibiting NF-κB/NLRP3 inflammasome activation

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Abstract

Introduction. Rheumatoid arthritis (RA) is an autoimmune disorder associated with joint damage and attendant cardiovascular complications. Wedelolactone (Wed), derived from Eclipta alba, possesses anti-inflammatory activity. Whether Wed regulates RA inflammation and related heart damage remains unknown.

Material and methods. A murine model of collagen-induced arthritis (CIA) was well-established by two subcutaneous injections of type II collagen (days 0 and 21). Wed was then administered *via* intraperitoneal injection every other day from day 28 to day 48. Joint swelling was monitored and paw thickness was calculated. Histopathological changes in synovial tissues or ankle cartilage were evaluated by hematoxylin and eosin (H&E) and Safranin O-Fast Green staining. The concentrations of inflammatory factors in serum and synovial tissues were detected by ELISA. The qRT-PCR, Western blotting, immunohistochemistry (IHC), and immunofluorescence (IF) were performed to assess receptor activator of nuclear factor kappa ligand (RANKL), matrix metalloprotease (MMP)-3, NLRP3, caspase-1 (pro- and cleaved forms), p-p65, IκBα, p-IκBα, p65, αsmooth-actin 2 (ACTA2), collagen type I and E-cadherin expression. H&E and Masson staining were used to assess the pathological alterations in the heart.

Results. Treatment with Wed ameliorated ankle joint swelling and cartilage degradation. Wed decreased the infiltration of inflammatory cells, the release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and IL-18), and the expression of RANKL and MMP-3 in serum and synovial tissues of CIA mice. Moreover, Wed increased the expression of NLRP3 and cleaved-caspase-1 in the synovium, leading to IL-1β and IL-18 secretion. Nuclear factor-kappaB (NF-κB) activation in synovial tissues was suppressed by Wed, as manifested by reduced phosphorylation of p65 and IκBα and nuclear translocation of p65. Furthermore, Wed reduced in CIA mice heart weight/body weight ratio and dampened cardiac inflammation and fibrosis that was accompanied at the mRNA level by down-regulation of ACTA2 and collagen I and up-regulation of E-cadherin.

Conclusions. These findings suggested that Wed attenuated synovial inflammation and joint damage in a mouse model of RA *via* inhibiting NF-κB/NLRP3 inflammasome activation, and ameliorated RA-induced cardiac complications. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 4, 301–310)*

Keywords: mouse; wedelolactone; collagen-induced arthritis; NLRP3; NF-κB; cardiac fibrosis

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Introduction

Rheumatoid arthritis (RA) is a chronic destructive autoimmune disorder characterized by systemic inflammation, autoantibody generation, and persistent synovitis [1]. During RA, the joints are infiltrated with a large number of inflammatory cells, accompanied by the secretion of inflammatory cytokines, especially tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [2, 3]. Nonsteroidal anti-inflammatory drugs are widely applied for the treatment of RA; however, several inevitable side effects including muscle weakness and a high risk of infection are present [4]. In addition to joint symptoms, the secretion of pro-inflammatory factors in RA also contributes to the incidence of cardiovascular complications [5, 6]. Thus, the development of effective therapeutic agents for RA is of great significance.

Wedelolactone (Wed), an Eclipta alba-derived natural bioactive compound, belongs to a class of flavonoids [7]. It exhibits a variety of biological effects, such as anti-inflammatory, anti-oxidative, anti-tumor, and anti-fibrotic [8, 9]. Wed has been reported to exert protective roles against tissue or organ damage by inhibiting nuclear factor-kappaB (NF-κB)-based inflammation [10, 11]. Wed declines the production of IL-1β, thereby relieving monosodium urate-induced inflammation [12]. In addition, Wed may have anti-toxic and cardioprotective abilities [13]. However, the function of Wed in RA and its related cardiac injury remains obscure.

The NLP family pyrin domain containing 3 (NLRP3) inflammasome, the most fully characterized inflammasome, plays a vital role in immune diseases including RA. The components of the NLRP3 inflammasome, including NLRP3 and caspase-1, are abnormally expressed in the synovium of RA patients [14–16]. Excessive activation of the NLRP3 inflammasome is tightly associated with the pathogenesis of RA [17]. Collagen type II (CII) is a major protein constituent in articular cartilage and provokes an autoimmune response resulting in joint damage. Collagen-induced arthritis (CIA) is the most widely used experimental model of RA *in vivo* [18]. A highly activated NLRP3 inflammasome was observed in CIA mice [17]. Wed has been found to efficiently suppress the activation of the NLRP3 inflammasome in both *in vitro* and *in vivo* models of inflammatory diseases [12, 19].

In the present study, we investigated whether Wed impacts the progression of RA and cardiac complications in a CIA model by modulating the NLRP3 inflammasome. Our findings help us understand the possible therapeutic role of Wed, which may provide a novel insight into future research on RA and it complications.

Material and methods

Animals and group division. All experimental procedures followed the standards of the Animal Care and Use Committee (Hebei General Hospital). Healthy DBA/1 male mice (7 weeks old) were acclimatized for one week under a suitable laboratory environment (12 h/12 h light/dark cycle; 25 ± 1°C; 45–55% humidity; food and water freely). CIA is a common animal model for studying RA and has been performed in this study as previously described [20]. Mice were injected subcutaneously at the tail base with 100 μg of CII (2 mg/mL, YuanYe, Shanghai, China) emulsified in the same volume of complete Freund's adjuvant (SigmaAldrich, St. Louis, MO, USA) in a total volume of 0.1 mL, followed by boost immunization 21 days later with equal CII emulsified in incomplete Freund's adjuvant. Following 28 days, Wed (10 mg/kg, YuanYe) was intraperitoneally injected into CIA mice every other day until experiment termination (day 48).

Arthritis evaluation and paw thickness measurement. From day 28, the hind paw thickness of mice was measured using a caliper and the arthritis index was scored every 4 days in a blinded manner. Arthritis scores were determined as the following criteria [20]. Grade 0: Normal; Grade 1: Erythema and mild swelling limited to the tarsals or ankle; Grade 2: Erythema and mild swelling from the ankle to the tarsal bone; Grade 3: Erythema and moderate swelling from the ankle to the metatarsal bone; Grade 4: Erythema and severe swelling from the ankle to the entire limb. Score of *per* mouse ranges from 0 to 16, and a higher score represents more severe damage.

Ratios of heart weight to body weight. On day 48 of the first CII immunization, mice were euthanized and weighed. Their hearts were weighed after dissection and the heart/body weight ratio was calculated.

Histological analysis. After fixation with 4% formaldehyde, the synovial tissues, hearts, and ankle joints were embedded in paraffin and then cut into 5 μm-thick sections for hematoxylin and eosin (H&E, Solarbio, and Sangon, China) staining, Masson-trichrome staining and Safranin O-Fast Green assay. The sections were checked and photographed under a light microscope (Olympus DP73, Tokyo, Japan).

Enzyme-linked immunosorbent assay. The synovial tissues and blood were harvested from mice. The sterile saline was added to the synovial tissues at a ratio of weight (g): volume (mL) = 1:9. Tissue samples were homogenized using a glass homogenizer for 30 strokes on ice. Tissue homogenate supernatant and blood serum were obtained by centrifugation. The concentrations of IL-1β, IL-6, TNF-α, and IL-18 in serum and supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits (LianKe, Hangzhou, Zhejiang, China) as described in the manufacturer's instructions.

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Immunohistochemistry and immunofluorescence. Fixed synovial tissues were used for immunohistochemistry (IHC) and immunofluorescence (IF) analysis. After fixation, the tissues were dehydrated, paraffin-embedded, and sectioned (5 μm-thick sections). The sections were soaked in citrate buffer (9 mL citric acid and 41 mL sodium citrate added to 450 mL distilled water; pH = 6.0) and heated in the microwave oven until boiling (10 min) for antigen retrieval. They were then incubated overnight at 4°C with primary antibody. The following primary antibodies applied were: receptor activator of nuclear factor (NF) kappa B ligand (RANKL, 1:100, ABclonal, Wuhan, Hubei, China), matrix metalloprotease 3 (MMP-3, 1:100, ABclonal), phosphorylated (p)-p65 (1:100, Affinity, Changzhou, Jiangsu, China) and NLRP3 (1:200, ABclonal). The HRP-linked goat anti-rabbit antibody (1:500) for IHC or Cy3 fluorescein-labeled secondary antibody (1:200) for IF was added and incubated for 1 h at 37°C. Counterstaining for labeling cell nuclei was performed with hematoxylin for IHC or DAPI for IF. The microscope was used for the visualization of staining and for the acquisition of microphotographs.

Quantitative reverse transcription-PCR. Total RNA from synovial tissues or hearts was extracted using TRIpure Isolation Reagent (BioTeke, Beijing, China) and used for the first strand cDNA synthesis following the manufacturer's instructions (Beyotime, Shanghai, China). Quantitative reverse transcription-PCR (qRT-PCR) was carried out using Taq PCR MaterMix and SYBR Green with primers specific to RANKL, MMP-3, αsmooth-actin 2 (ACTA2), collagen type I (CI) and E-cadherin. The primers utilized for qRT-PCR were: RANKL F, 5'-ATGAAAGGAGGGAGCACG and R, 5'-GCAGGGAAGGGTTGGAC; MMP-3 F, 5'-CCTATTCCTGGTTGCTG and R, 5'-AGAGTTAGACTTGGTGGG; ACTA2 F, 5'-GAGCGTGAGATTGTCCG and R, 5'-GCTGTTATAGGTGGTTTCG; collagen I F, 5'-CGCCATCAAGGTCTACTGC and R, GAATCCATCGGTCATGCTCT; E-cadherin F, 5'-GGGACAAAGAAACAAAGGT and R, 5'-TGACACGGCATGAGAATAG. The relative expression of these mRNAs was measured using the $2^{-\Delta\Delta CT}$ method.

Western blotting. The western blotting assay was performed utilizing primary antibody against NLRP3, pro-caspase-1/ /cleaved-caspase-1, IκBα, phosphorylated (p)-IκBα, p-p65 or nuclear p65. NLRP3 antibody was purchased from ABclonal and all others were from Affinity. Proteins isolated were quantified with the BCA kit and separated by SDS-PAGE. After transfer onto PVDF membranes and blocked with 5% BSA, the samples were incubated overnight (4°C) with the diluted primary antibody (1:1000) and subsequent HRG-conjugated secondary antibody (1:10000) for 40 min (37°C). Finally, enhanced chemiluminescence (ECL, 7 Sea biotech, Shanghai, China) was used for western blotting development.

Statistical analysis. Values in the graphs were represented as means ± standard deviation (SD). One-way or two-way analysis of variance in GraphPad Prism 8.0 software was used to evaluate statistical differences. P values less than 0.05 were considered to be significant.

Results

Wed alleviates RA development in CIA-induced mice

CII is frequently used to induce RA [20]. As shown in Fig. 1A, arthritis in mice was developed by twice immunizations with CII, and then these mice were treated with Wed for exploring its role in RA. From day 28 to day 48, the ankle swelling score and paw thickness of CIA model mice were obviously greater than those in control mice, while Wed treatment lowered them (Fig. 1B, C). On day 48, as exhibited in Fig. 1D, the paws of euthanized model mice were markedly swollen, which was alleviated by Wed. Fig. 1E displayed the histopathological images of synovial tissues stained by H&E. CIA mice exhibited synovial tissue hyperplasia, joint space narrowing, and severe cartilage damage as compared to the mice in the control group. These changes were improved following Wed treatment. Moreover, Wed administration significantly declined the levels of serum IL-1β, IL-6, TNF-α, and IL-18 induced by CII (Fig. 1F), suggesting the anti-inflammatory function of Wed in CIA.

Wed improves cartilage degradation in CIA-induced synovial tissue

The pathological changes of the ankle cartilage were next evaluated. Safranin O-Fast Green staining demonstrated that CIA triggered erosion and destruction in articular cartilage. In contrast, Wed treatment resulted in a relatively intact cartilage surface and reduced injuries (Fig. 2A). Fig. 2B revealed RANKL and MMP-3 mRNA expression detected by qRT-PCR. The expression levels of RANKL and MMP-3 mRNAs were up-regulated in the synovial tissues of CIA mice and Wed significantly down-regulated their levels. Changes in the protein expression levels of RANKL and MMP-3 analyzed by IHC were consistent with the mRNA results (Fig. 2C). These data suggest that Wed prevents CIA-induced synovial tissue damage possibly through inhibiting RANKL and MMP-3 expression.

Wed suppresses CIA-induced NLRP3 inflammasome activation

NLRP3 expression in synovial tissues was assessed by IF staining. As shown in Fig. 3A, the number of NLRP3-positive cells was significantly increased in the synovium of CIA mice, which was partly reversed by Wed administration. Wed also remarkably down-regulated the protein expression levels of NLRP3 and

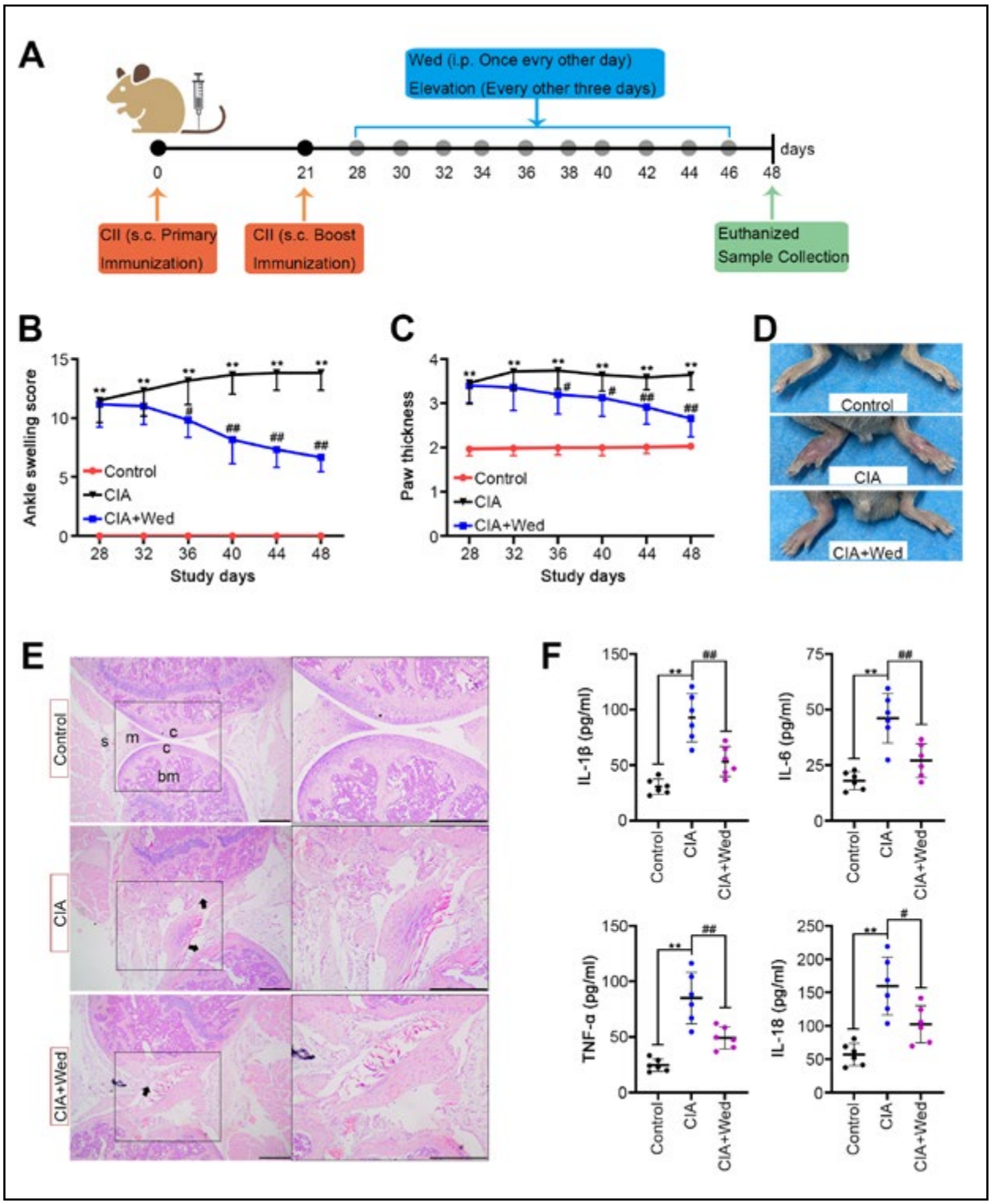

Figure 1. Wedelolactone ameliorates collagen-induced arthritis in mice. **A.** Experimental scheme was depicted. Arthritis in mice was induced *via* immunization twice, followed by Wedelolactone (Wed) administration every other day from day 28 to day 48; **B**, **C.** On days 28, 32, 36, 40, 44, 48, arthritis scores of mice were performed and paw thickness was recorded in different treatment groups; **D.** Representative pictures of hind paws on day 48; **E.** On day 48, mice were euthanized and synovial tissues were harvested for histological study (H&E staining; size bar, 500 μm). Abbreviations: bm — bone marrow; m — meniscus; s — synovial; c — cartilage. Black arrow — cartilage destruction; **F.** Collagen-induced changes in serum inflammatory cytokines (IL-1β, IL-6, TNF-α and IL-18) were determined by ELISA. The results were shown as mean and standard deviation of at least 6 mice each group. $^{**}P < 0.01$ for CIA *vs.* Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for CIA + Wed *vs.* CIA.

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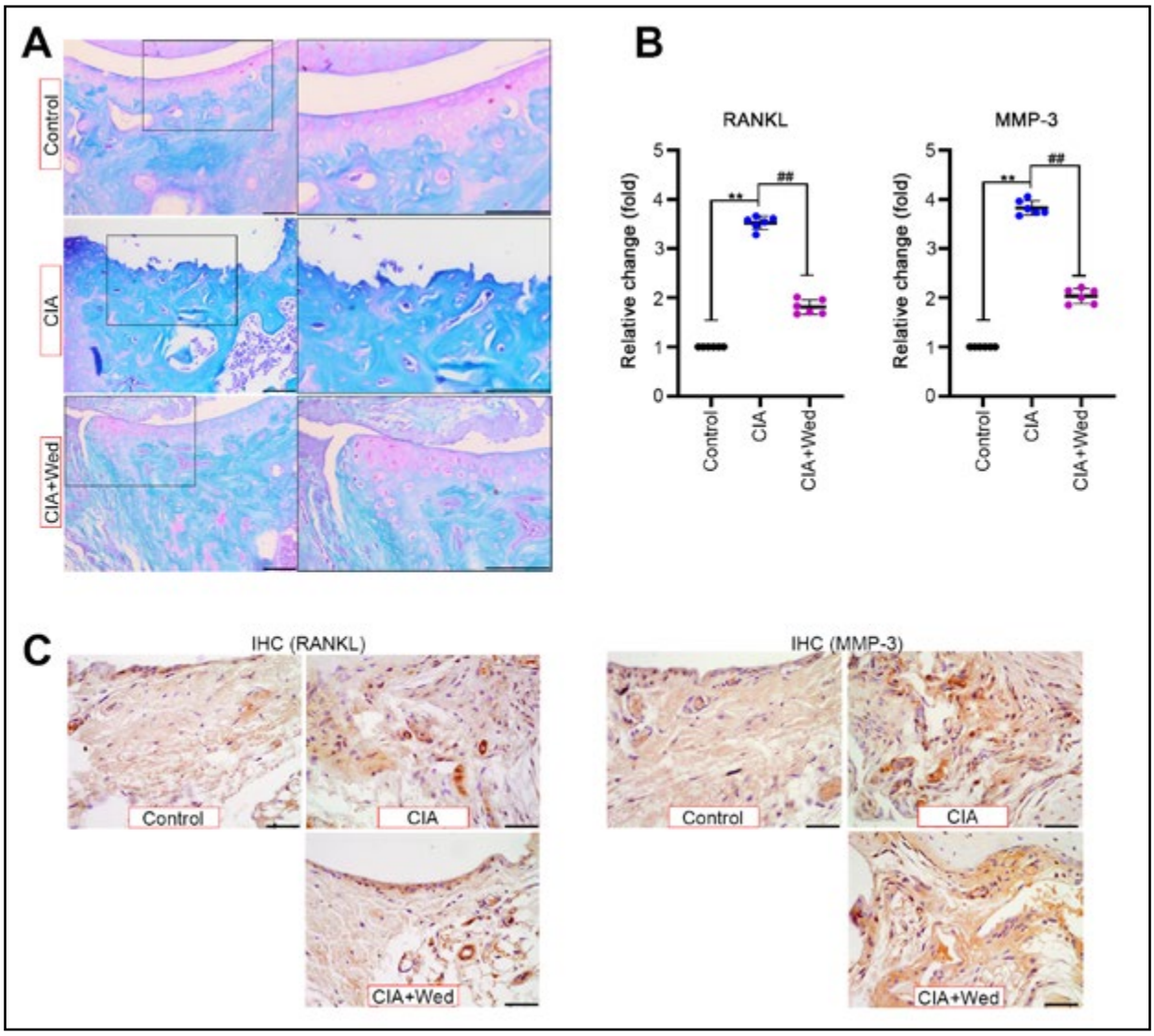

Figure 2. Wedelolactone alleviates collagen-induced damage in synovial tissues. **A.** Safranin O-fast green staining showed cartilage erosion in synovial tissues. Size bar, 100 μm; **B.** QRT-PCR assay was performed for RANKL and MMP-3 mRNA levels in synovial tissues; **C.** Immunohistochemical (IHC) studies for RANKL and MMP-3. Size bar, 50 μm. Data were shown as mean and standard deviation. $^{**}P < 0.01$ for CIA *vs.* Control, $^{\#\#}P < 0.01$ for CIA + Wed *vs.* CIA.

cleaved-caspase-1 induced by CIA in the synovium. There were no changes in pro-caspase-1 expression in these groups (Fig. 3B). ELISA results showed that Wed declined CIA-induced elevation in IL-1β and IL-18 concentrations in synovial tissues (Fig. 3C). These findings suggest that Wed is a possible suppressor of NLRP3 inflammasome activation during RA development.

Wed inhibits the NF-κB signaling pathway

Abnormal NF-κB activation may lead to the development of inflammatory diseases such as RA [21]. IHC staining for p-p65 showed that the p-p65-positive cells in synovial tissues of CIA mice were greatly enhanced compared with control mice. Treatment with Wed abolished the CIA-induced increase in p-p65 (Fig. 4A). Western blotting assay was performed to detect the expression of NF-κB-related proteins. The results revealed that significant increases in p-IκBα, p-p65, and nuclear translocation of p65 protein were observed in the CIA group, which was partially abolished by Wed (Fig. 4B). Taken together, Wed suppresses the NF-κB signaling pathway in CIA.

Wed ameliorates CIA-caused cardiac injury

The potential effect of Wed on the hearts of CIA mice was further investigated. Compared with the control group, the ratio of heart/body weight was elevated in

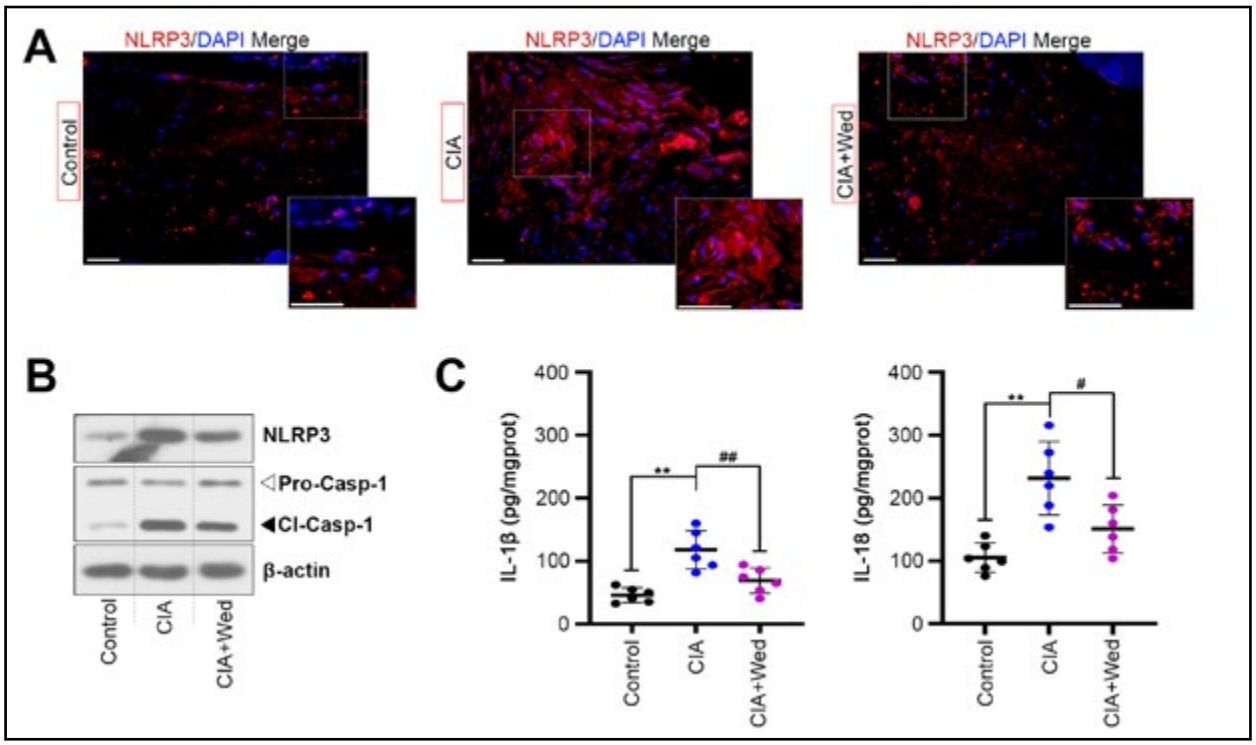

Figure 3. Wedelolactone inhibits collagen-induced NLRP3 inflammasome activation. **A.** NLRP3 expression in synovial tissues was displayed by immunofluorescence. Size bar, 50 μm; **B.** Representative immunoblots of NLRP3, pro-caspase-1 (pro-Casp-1) and cleaved-caspase-1 (Cl-Casp-1), with β-actin as a control; **C.** ELISA showed the levels of IL-1β and IL-18 in synovial tissues. Values were indicated as mean and standard deviation. $^{**}P < 0.01$ for CIA *vs.* Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for CIA + Wed *vs.* CIA.

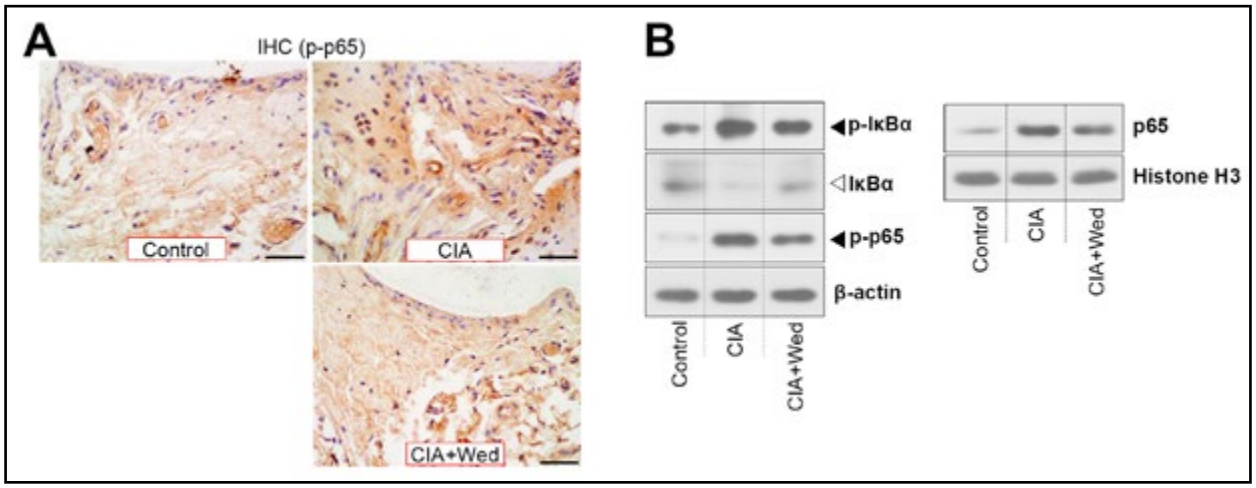

Figure 4. Wedelolactone hinders the activation of NF-κB signaling pathway. **A.** P-p65 expression in synovial tissues (IHC staining, 400×); **B.** Western blotting analysis of IκBα, p-IκBα, p-p65 and nuclear p65.

the CIA group. However, treatment with Wed diminished it (Fig. 5A). H&E staining was used to reveal the pathological alterations in heart tissues. The myocardial cells in the control mice were morphologically intact with centrally-located nuclei. CIA resulted in myocardial structural damage and inflammatory cell infiltration into the heart, but Wed treatment partially ameliorated the CIA-induced cardiac morphological alterations (Fig. 5B). Masson staining was applied for fibrosis evaluation. No significant fibrosis was observed in the hearts of control mice. A strongly blue-stained area was present in the myocardium of CIA mice, while Wed reduced the intensity of myocardial fibrosis (Fig. 5C).

ACTA2, collagen I, and E-cadherin expression in the hearts was also measured at the mRNA level by qRT-PCR assay. It was found that CIA increased the expression of ACTA2 and collagen I, but reduced

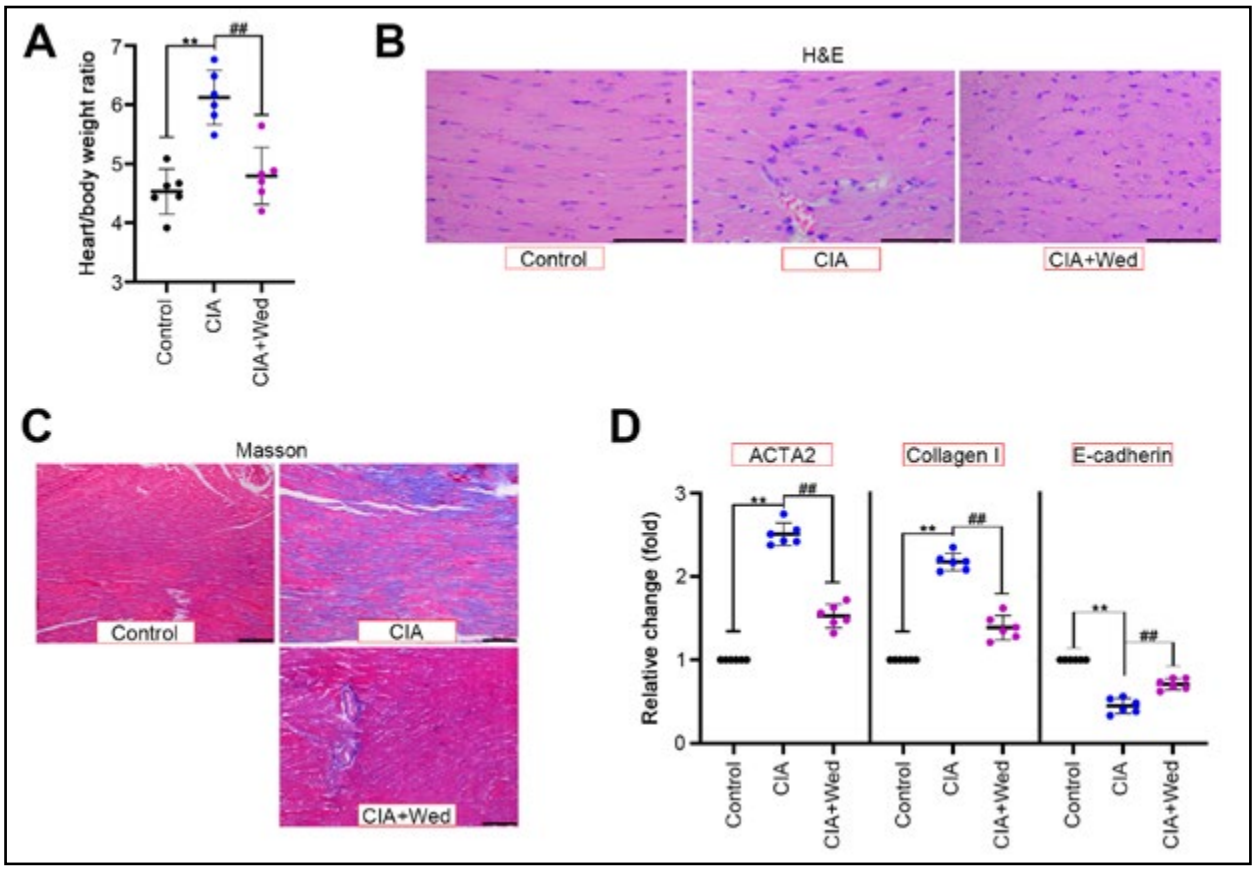

Figure 5. Wedelolactone improves CIA-caused cardiac injury. **A.** The ratio of heart weight to body weight was calculated; **B.** Pathological alteration in cardiac tissues was shown by H&E staining. Size bar, 100 μm; **C.** Masson staining was performed to evaluate cardiac fibrosis. Size bar, 100 μm; **D.** QRT-PCR results of ACTA2, Collagen I and E-cadherin in cardiac tissues. Results were expressed as mean and standard deviation. $^{**}P < 0.01$ for CIA *vs.* Control, $^{\#\#}P < 0.01$ for CIA + Wed *vs.* CIA.

the expression of E-cadherin mRNA. These alterations were significantly reversed after Wed administration (Fig. 5D).

Discussion

Rheumatoid arthritis is a progressive autoimmune disorder affecting synovial tissue and articular cartilage [22]. CIA is a widely accepted animal model for RA, which is of great significance for the exploration of RA pathogenesis and the development of therapeutic strategies. In the present study, wedelolactone was found to ameliorate synovial and articular cartilage damage caused by CIA. The findings also indicate that Wed may be a suppressor of NLRP3 inflammasome activation and NF-κB signaling pathway. Our work presents strong evidence supporting the protective effect of Wed in RA development.

Synovial inflammation is a hallmark of chronic inflammatory joint diseases including RA [23]. IL-1β, IL-6, TNF-α and IL-18 are important pro-inflammatory cytokines that have been implicated in the accumulation of inflammatory cells and synovial tissue destruction in RA [24]. Wed has been reported to function as an anti-inflammatory agent in models of some inflammatory disorders, such as arthritis [12], fungal keratitis [25], and colitis [26]. In our study, serum IL-1β, IL-6, TNF-α and IL-18 levels elevated in CIA mice were significantly decreased after successive Wed administration, indicating that Wed has anti-inflammatory activity in this RA animal model. Moreover, inflammatory cytokines can stimulate the production of RANKL in cartilage [27, 28]. It has been shown that RANKL acts not only as a mediator of osteoclastogenesis and bone resorption but also as a critical regulator of the inflammatory reaction in the genetic model of RA in which overexpressed RANKL aggravated the course of the disease [29–32]. Specific MMPs, including MMP-1, MMP-3, and MMP-13, have been reported to degenerate articular cartilage matrix components and play predominant roles in joint destruction in RA [33–35]. MMP-3, a member of the MMP family that is produced primarily by synoviocytes [36, 37], is responsible for the cleavage

of extracellular matrix proteins, resulting in severe inflammation and joint destruction [38]. Our results obtained by IHC and qRT-PCR analysis showed that Wed reduced synovial RANKL and MMP-3 expression in the CIA murine model, further indicating the protective function of Wed against synovial tissue and cartilage damage.

Accumulating studies have confirmed that NLRP3 inflammasome activation is a critical mechanism in the pathogenesis of RA [17, 39, 40]. Collagen type II is known to induce the activation of NLRP3, as demonstrated by up-regulated NLRP3 and cleaved-caspase-1 expression in the joints of CIA mice as well as increased serum pro-inflammatory IL-1β and IL-6 levels [41]. Activated NLRP3 inflammasome has been observed in synovial tissues of RA patients and CIA mice [17, 42]. Negative regulation of NLRP3 leads to the reduction of downstream pro-inflammatory cytokine release, which promotes cartilage degradation and synovial inflammation [43, 44]. It has been also reported that joint inflammation in CIA mice can be alleviated by inhibition of the NLRP3 inflammasome [44]. Wed has been considered to be an inhibitor of NLRP3 inflammasome formation in macrophages [12] and animal colitis model [19]. In line with previous findings in different experimental models of inflammation, our study demonstrated that Wed blocked the activation of NLRP3 inflammasome and synovial inflammation in CIA mice. Stimulation of IL-1β production by NLRP3 is thought to induce the production of RANKL, matrix metalloproteinase 3 (MMP-3), and downstream inflammatory cytokines such as IL-6 [45]. Our findings collectively imply that Wed may prevent RA development *via* inhibiting the NLRP3 inflammasome activation.

Wed has been reported to exert anti-inflammatory effects by negatively modulating the activation of IκBα/NF-κB [7, 46]. The previous investigation showed that NF-κB activation contributes to the secretion of inflammatory cytokines TNF-α, IL-6, bone erosion, and joint injury [47–50]. Blockage of NF-κB appears to play a crucial role in the modulation of an inflammatory cascade of RA [48]. Here, we found that the NF-κB signaling pathway was activated by CIA in synovial tissues. Wed treatment hindered CIA-induced phosphorylation of IκBα and p65 as well as p65 nuclear translocation, suggesting the inhibitory role of Wed in NF-κB activation in RA. Additionally, activated NF-κB increases the expression of downstream RANKL and MMP-3 in the joints of mice with CIA [51]. NF-κB acts as an upstream regulatory factor of NLRP3, and activation of NF-κB can induce NLRP3 inflammasome activation [52, 53]. Our study indicates that the anti-inflammatory effect of Wed in RA is associated with the suppression of NF-κB/ /NLRP3 inflammasome activation.

Emerging evidence suggests that patients with RA are at significant risk for cardiovascular diseases [54]. Wed has previously been shown to be beneficial in alleviating tissue injury including the kidney and liver [11, 46]. Wed appears to be useful in preventing heart diseases [13]. Whether Wed affects RA-associated cardiac complications and the underlying molecular mechanism are yet not explored. In our study, Wed decreased CIA-induced cardiac inflammation and fibrosis, along with down-regulation of fibrotic markers ACTA2 and collagen I and up-regulation of E-cadherin. Our work demonstrated for the first time that Wed might prevent the development and progression of RA-related cardiac damage. Additional experiments will be required to determine the action of Wed in the heart. In addition, the protective effects of Wed in tissue injuries have been linked with the suppression of the NF-κB signaling pathway [11, 46]. Further investigations are required to elucidate whether NF-κB is involved in Wed-mediated protection against RA-associated complications.

In conclusion, our study suggested that Wed, a natural bioactive compound, alleviated RA development in the CIA mouse model. Wed might improve CIA-induced joint inflammation and cartilage destruction *via* the suppression of NF-κB/NLRP3 inflammasome activation. Wed also attenuated RA-induced cardiac fibrosis. Together, these results indicate that Wed may be a novel promising agent for the treatment of RA and related cardiovascular complications.

Authors' contribution

JJC designed the study, performed the experiments and reviewed the draft of the manuscript. XRN and HXZ analyzed the results of the experiments. YHN performed the experiments and drafted the manuscript. All authors have read and approved the manuscript.

Data availability statement

All data supporting our findings are included in this manuscript.

Experimental ethics

All experimental procedures followed the standards of the Animal Care and Use Committee of Hebei General Hospital.

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Conflict of interest

The authors claim that they have no competing interest.

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