

Anti-inflammatory Activity of Formononetin in a Collagen-induced Arthritis Mouse Model of Rheumatoid Arthritis

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Abstract

Background: Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation of the joints, leading to bone erosion and joint dysfunction. Although there are options for the treatment of RA, safer and more effective drugs are still being sought. Formononetin (FMN) is an isoflavonoid compound found in various plants, such as *Astragalus propinquus* Schischkin and *Spatholobus suberectus*. It has anti-tumor, anti-bacterial, anti-lipid peroxidation, and estrogen-like activities and is a noteworthy compound for screening of anti-RA drugs.

Methods: To investigate the anti-inflammatory effects of FMN in a collagen-induced arthritis (CIA) mouse model, thirty-six C57BL/6 mice were randomly divided into 6 groups: a healthy control group and 5 CIA groups. Arthritis was induced the CIA groups using chicken collagen type II. The CIA groups were divided in a control group (RA), a tripterygium glycosides (10 mg/kg body weight) treated group (TG), a low-dose (50 mg/kg) FMN group (FMN-L), a middle-dose(100mg/kg) FMN group (FMN-M), and a high-dose (200 mg/kg) FMN group (FMN-H). The control mice and CIA mice in the RA group were treated with an equal volume of 5% carboxymethylcellulose sodium. Drugs were delivered three times a week for four weeks, and the bodyweight, food-uptake, and swelling of the paws were monitored during the treatment process. Inflammatory cytokines and other biochemical indexes in the serum and joint tissues were analyzed, along with the expression levels of NF- κ B pathway-related proteins (I κ B α , p65, p-p65, TIPE2, and PCNP) in the spleen. Histopathological examinations were processed for the hind limbs.

Results: FMN-M dramatically reduced the arthritis index in the CIA mice, inhibited the inflammatory cell infiltration, and prevented damage to the synovium and cartilage. Mechanistic studies suggested that FMN might reduce inflammation by inhibiting I κ B- α degradation and by regulating the expression and release of NF- κ B p65.

Conclusions: These data suggest that FMN might be an active therapeutic agent for RA by preventing bone destruction, regulating inflammatory mediators, and suppressing NF- κ B signaling pathways.

Background

Rheumatoid arthritis (RA), one of the most common autoimmune diseases, is characterized by chronic synovial inflammation and hyperplasia, cartilage and bone destruction, and characteristic systemic lesions including cardiovascular, pulmonary, and skeletal diseases ^[1]. RA is often accompanied by a variety of complications, especially psychological aspects such as depression. The high incidence of RA is associated with a heavy burden on patients and on society ^[2]. While its etiology and pathogenesis are still unclear, previous reports indicated that pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor (TNF- α), and monocyte chemoattractant protein-1 (MCP-1), together with MCSF/RANK, provoke signals that promote osteoclast-mediated bone erosion, leading to the exacerbation of RA symptoms ^[3,4]. Notably, the induction of these inflammation cytokines is mediated by pro-inflammation

signaling pathways, including nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1), signal transduction factors, and STAT3 [5].

NF- κ B is a nuclear pro-inflammatory transcription factor that regulates the expression of specific genes that are essential for inflammation signal transduction. Positive feedback mechanisms in the NF- κ B pathway play a critical role in the production and progression of inflammation-related diseases. When the body is attacked by pathogens, activated NF- κ B is translocated from the cytoplasm to the nucleus and initiates transcription and expression of pro-inflammatory genes, such as TNF- α and IL-6 [6]. It has been previously reported that the activation of the NF- κ B pathway plays a vital role in inflammation pathogenesis upon I κ B degradation [7–9].

Tumor necrosis factor- α induced protein 8-like 2 (TIPE-2) and PEST-containing nuclear protein (PCNP) also play a crucial role in the inflammation process and body immune mechanisms. TIPE-2, a novel member of the TNF-induced protein 8 family, negatively regulates innate and adaptive immunity and plays a vital role in inflammation homeostasis [10]. Previous research reported that mice lacking TIPE-2 suffered from inflammation in multiple organs, and TIPE-2 mediated immunomodulation reduced NF- κ B signal transduction and inflammatory cell infiltration [11]. Meanwhile, TIPE-2 exists in the cytoplasm and nucleus of human RPE cells, and its expression is down regulated during inflammatory conditions, which reduces cell viability. Expression of short interference RNA (siRNA) to block TIPE-2 function improves the expression levels of TNF- α and vascular endothelial growth factor (VEGF), especially upon stimulation with lipopolysaccharides (LPS) [12]. Moreover, a decrease in TIPE-2 expression level is related to the occurrence and development of RA [13]. PCNP is a nuclear protein that mainly exists in the nucleus. This novel ring-like protein is an E3 ligase that is involved in ubiquitination-dependent degradation of many proteins. Recent studies have shown that PCNP is associated with cell cycle regulation [14].

Common anti-RA drugs include non-steroidal anti-inflammatory drugs (NSAID), disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids, and biopharmaceuticals. However, the side-effects of these anti-RA drugs, the fact that these drugs do not support a permanent cure, as well as the high cost of these medications make them unsatisfying for patients with RA. Natural medicines, belonging to the class of DMARDs, are characterized by their action on multiple targets of the disease pathway, low toxicity, and tunability, and hence play an essential role in the treatment of RA [15–16]. Among these natural drugs are the tripterygium glycosides (TG), with the main active ingredient being triptolide. TG has anti-inflammatory and immunosuppressive effects and has been used for RA treatment. *In vitro* experiments have shown that TG has anti-inflammatory effects on acute uveitis induced by bacterial LPS by downregulating inflammatory cytokines like ICAM-1, IL-1 β , and monocyte chemoattractant protein-1 (MCP-1) expression [17].

Formononetin (FMN) is an isoflavonoid compound found in various plants, such as *Astragalus propinquus* Schischkin and *Spatholobus suberectus*. It has anti-tumor, anti-bacterial, anti-lipid peroxidation, and estrogen-like activities. Studies have demonstrated that FMN can reduce the expression

level of inflammatory cytokines such as IL-6, TNF- α , and VEGF^[18], and it is speculated that FMN could be useful in arthritis therapy. In this study, we used a collagen-induced arthritis (CIA) mouse model of human RA to investigate the mechanism of FMN in the treatment of RA by exploring the effects of FMN on the NF- κ B signaling pathway using TG as a positive control.

Methods

Reagents

FMN was provided by the Institute of Anti-aging of Binzhou Medical College; TG tablets were purchased from Zhejiang Dender Pharmaceutical Co., Ltd.; chicken type II collagen (CII) and complete Freund's adjuvant were purchased from Sigma Aldrich; acetic acid, paraformaldehyde (PFA), ethanol, formaldehyde, xylene, hematoxylin, eosin, neutral resin, and other reagents were purchased from Shanghai Yiyang Instrument Co., Ltd., China; CBA mice Th1/Th2/Th17 cytokine detection panel (detecting TNF- α , IL-6, IL-10, IL-2, IL-4, IL- γ , IL-17A) and BCA assay kit were purchased from Beijing Solar Bio-science & Technology Co., Ltd., China; goat anti-mouse IgG2a-HRP, goat anti-rabbit IgG-HRP, anti-TIPE-2 (cat# 15940), and anti-PCNP (cat# 11180) antibodies were purchased from Proteintech Group, Inc, Wuhan China; anti-I κ B- α (cat# 4812), anti-phospho-p65 (p-p65) (cat# 3033) antibodies were purchased from Cell Signaling Technology, Shanghai, China; anti-p65 (cat# ab16502) antibody was purchased from Abcam.

Animals

Thirty-six male C57BL/6 mice, weights ranging from 18–22 g, were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (experimental animal production license number: SCXK (Lu) 20140007) and raised in the specified-pathogen-free (SPF) class animal culture room in Yantai Raphael Biotechnology Co., Ltd (experimental animal license no: SCXK (lu)20170026). Mice were kept under 12/12 hours light/dark cycle, with *ad lib* feed and drinking water. The feed provides was SPF grade, and was produced by Jiangsu Province Cooperative Pharmaceutical Bioengineering Co., Ltd. Drinking water was high temperature-sterilized city tap water. The experimental study was carried out strictly in accordance with the guidelines for the rational use of animals by Yantai Animal Ethics Committee.

The animal model of collagen-induced arthritis

A CIA mouse model was used in this study, as previously described^[19]. Briefly, the CII collagen was dissolved in 0.05 M acetic acid at 2 mg/mL and kept at 4°C. The solution was fully mixed with an equal volume of complete Freund's adjuvant under sterile conditions until the liquid was milky white. The reaction was performed on ice to prevent collagen degradation.

The initial immunization was performed on day 0: 200 μ L of mixed emulsions were injected intradermally along the spinal direction at 1 cm of the mice's tail root under anesthetic conditions. The second immunization was performed on day 18: 100 μ L of the same mixed emulsions were injected into the root

of the mice tail, avoiding the initial immunization injection site as much as possible. To test the anti-RA activity of FMN, mice were randomly divided into 6 groups ($n = 6$ each): healthy control group (control, healthy mice not immunized with CIA), RA model group (RA, CIA mice), tripterygium glycosides treated group (TG, CIA mice treated with 10 mg/kg TG as positive control), low-dose FMN group (FMN-L, CIA mice treated with 50 mg/kg FMN), middle-dose FMN group (FMN-M, CIA mice treated with 100 mg/kg FMN), and high-dose FMN group (FMN-H, CIA mice treated with 200 mg/kg FMN). On day 21 to day 49, mice in the FMN groups were treated intragastrically with FMN suspension. Drugs were delivered three times a week for four consecutive weeks in a volume of 0.2 mL per mouse each time. The TG group was treated with 10 mg/kg TG suspension (0.2 mL) three times a week for four consecutive weeks. The RA group and the control group were given an equal volume of 5% sodium carboxymethyl cellulose solution three times a week for four consecutive weeks.

The measurement of body weight, swelling of the paws, and arthritis index

The body weight was measured using an electronic balance. The first measurement was just prior to the initial immunization and was subsequently performed once a week starting at day 21 until the end of the experiment to detect the changes in mice weight and the volume of the hindfeet (left, right). The thickness of the rear paws was measured with a paw volume measuring instrument (Peacock, Japan) once a week starting at day 21 until the end of the experiment. The measurement was performed three times at one-time point, and the average value was used for analysis.

The arthritis index (AI) was used to judge the outcome of the CIA mice model: 0, no redness and swelling; 1, slight swelling of the paw joint; 2, swelling of the paw joint and paw joint below the ankle joint; 3, swelling of all the paw joints including the ankle joint. The sum of the AI scores was equal to or greater than 4 indicating the successful induction of CIA (0 point for grade 0, 1 point for grade 1, 3 points for grade 2, 4 points for grade 3, with a maximum total score of 16).

The cytokine expression level in mice serum

The body weights were measured at the completion of the experiment. The blood was collected after the removal of eyeballs and left at room temperature for 30 minutes, then centrifuged at 3500 rpm for 15 minutes at 4°C. The serum was collected and stored at -80°C. The expression levels of IL-6, IL-10, and TNF- α in the serum were measured using the CBA kit.

Histopathological examination

On the last day of the experiment, all mice were sacrificed after anesthesia and blood collection. The chest and abdominal cavity of experimental mice were opened, and thymus and spleen were removed, washed with normal saline, and dried on filter paper. The weight of thymus and spleen was recorded to calculate for the thymus and spleen index (organ index = organ wet weight/mice body weight *100%).

The malleolus joints were fixed in 10% neutral formalin solution for 48 hours, followed by decalcification in EDTA at 4°C for 30 days. The sections were stained with hematoxylin-eosin (H&E) and toluidine blue to monitor the osteoclasts. Histopathological changes were observed under the microscope.

Western blot analysis

The protein expression levels of I κ B α , p65, p-p65, TIPE-2, and PCNP in the spleen were detected using Western blot, as follows: the tissue samples (100 mg) were cut into small pieces of about 3 mm \times 3 mm with surgical scissors. Each block was immersed in 0.5 mL of ice-cold RIPA lysis buffer (containing protease inhibitor and phosphatase inhibitor, purchased from Beyotime Biototechnology Co., Ltd., Shanghai, China), shaken gently until the sample material was completely homogeneous (glass beads were used for grinding to help achieve better homogenization). The lysates were transferred into a chilled centrifuge tube and centrifuged at 12,000 rpm for 15 min at 4 °C. Next, the supernatant was transferred to another pre-cooled centrifuge tube and the protein concentration was determined using the BCA method. The protein samples were mixed with 5x loading buffer and boiled for 10 minutes, then cooled to room temperature and mixed. The samples (25 μ g total protein) were separated on 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF). The membranes were blocked with 5% (w/v) BSA in Tris-buffered saline containing 0.1% Tween-20 for two hours at room temperature and subjected to antibody incubation. The dilution ratio of the primary antibodies used was as follows: anti-p65 (1:1000), anti-p-p65 (1:1000), anti-I κ B α (1:1000), anti-TIPE-2 (1:1000), anti-PCNP (1:1000), and anti-GAPDH (1:4000). GAPDH served as the internal loading control. HRP-conjugated secondary antibody labeling of protein bands was detected by enhanced chemiluminescence (ECL) detection system (Tanon Science & Technology Co., Ltd, Shanghai, China). The gray values measured with Image J software were normalized against GAPDH .

Immunohistochemistry analysis

The expression level of NF- κ B p65 in ankle tissues was detected by immunohistochemistry (IHC). The paraffin-embedded ankle tissue sections were heated at 62 °C for 1 h for dewaxing, then refilled with an ethanol gradient and treated with Tris-EDTA/citrate buffer for antigen repair. The slides were then washed with 3% H₂O₂/carbinol and blocked with 5% BSA for 30 minutes. The slides were incubated with an anti-p65 antibody overnight, washed, and then incubated with horseradish peroxidase (HRP) secondary antibody for 30 minutes at 37 °C. The signal was developed with DAB kit, and slides were re-stained with haematite, destained in 1% hydrochloric acid/ethanol, and washed with pure water for 20 minutes. The signal was detected under the microscope, and the integral optical density (IOD) was analyzed with Image J software.

Statistical analysis

The data were processed with Graph Pad Prism 7.0. The error bars represent the mean \pm SD. The single factor ANOVA was used to evaluate the difference between the groups. $p < 0.05$ was considered statistically significant.

Results

The behavior of the experimental mice

Before the second immunization, there was no obvious difference in appearance of the mice of each group compared with the control group. In contrast, after the second immunization, compared with the control group, the fur of mice in each CIA group gradually lost its luster, and the hind limbs appeared red and swollen, with reduced activity and weight loss. The health condition of mice in each drug-treated group improved after the intervention with anti-AR drugs, among which the health improvement in the FMN-M (100 mg/kg)-treated group was the most obvious. There was no significant difference between the FMN-M group and the TG-treated group.

Body weight change and arthritis index of CIA mice

The timeline of C II-induced arthritis and drug treatments is shown in Fig. 1. The activity of FMN in the disease progression of the CIA mice was determined by assessing inflammation development. The most obvious symptoms of RA are weight loss and increased paw swelling. As shown in Fig. 2 (A- C), the bodyweight of mice in the RA group decreased significantly ($p < 0.01$) compared with the control group, while the paw volume increased dramatically ($p < 0.01$) on the 21st day following CII injection. Over time, the mice paw volume in the RA group showed a continuous increase and was significantly different from the control group ($p < 0.01$). After drug treatment, there was an obvious improvement compared with that in the RA group, and the effect of TG treatment was most significant. The body weight increased quickly, and the paw swelling decreased significantly ($p < 0.05$) compared with the RA group. Consistently, the bodyweight of CIA mice also increased in the different dose of FMN group, along with the decrease of paw volume. The body weight had significantly increased on day 21, day 35 and day 42 following the first immunization in all the three FMN treated groups compared with the RA group ($p < 0.05$ or $p < 0.01$). The paw volume had significantly decreased in the FMN-L and FMN-M groups compared with the RA group ($p < 0.05$) among which the FMN-M treated group displayed the optimal effect (Fig. 2, panel B). The arthritis score clearly demonstrated that the clinical score of CIA mice paws in the RA group reached the highest level on day 35, while the clinical score of mice was significantly reduced at day 35, day 42 and day 49 in the drug-treated groups of TG, FMN-L, and FMN-M but not in the FMN-H group.

The organ index of CIA mice

The relative weight of spleen and thymus is reflected by the spleen and thymus index. According to previous reports, the spleen and thymus indexes had increased in autoimmune arthritis. In this study, we found that the spleen index in the RA group increased dramatically compared with the healthy control group ($p < 0.01$, as shown in Table 1), indicating the successful induction of RA in the CIA model. Compared with the RA group, the spleen index in the FMN-treated groups was reduced, with $p < 0.01$ in FMN-L and FML-M groups, and $p < 0.05$ in the FMN-H treated group. Thus, the FMN-M treatment showed the optimal effect in attenuating CIA-induced increase of the the spleen index, and was comparable to that of the TG treatment.

On the other hand, while the thymus index in the RA group had increased compared with the control group ($p < 0.05$), drug treatment had no significant effect ($p > 0.05$) on this increase (Table 1).

Table 1
Effect of anti-RA drugs on the spleen index and the thymic index in a mouse model of rheumatoid arthritis

Group	spleen index/%	thymic index/%
Control	0.29 ± 0.04	0.12 ± 0.02
RA	0.64 ± 0.17 ^{##}	0.14 ± 0.02 [#]
TG	0.29 ± 0.03 ^{**}	0.13 ± 0.02
FMN-L (50 mg/kg)	0.33 ± 0.06 ^{**}	0.14 ± 0.02
FMN-M (100 mg/kg)	0.31 ± 0.04 ^{**}	0.13 ± 0.02
FMN-H (200 mg/kg)	0.40 ± 0.11 [*]	0.16 ± 0.02

$p < 0.05$, the RA group compared with the control group. * $p < 0.05$, ** $p < 0.01$, the drug-treated groups compared with the RA group. Data are expressed as the mean ± SD ($n = 6$). RA, rheumatoid arthritis; TG, tripterygium glycosides ; FMN, formononetin.

Effects of FMN on the serum levels of IL-10, IL-6, and TNF- α of CIA mice

To evaluate the role of FMN in the regulation of cytokine production in CIA mice, we measured the serum levels of pro-inflammatory cytokines IL-6 and TNF- α , and of the anti-inflammatory factor IL-10 in each group.

As shown in Figure-3 (panels A-C), the serum levels of IL-6 ($p < 0.05$) and of TNF- α ($p < 0.01$) were higher in the RA group compared with the healthy control group, while the expression level of IL-10 decreased ($p < 0.05$) in the RA group. At the end of the experiment, we found that treatment with TG, FMN-L (50 mg/kg), and FMN-M (100 mg/kg) significantly reduced the release of TNF- α ($p < 0.01$) and of IL-6 ($p < 0.05$). At the same time, FMN-M (100 mg/kg) treatment increased the expression level of IL-10 ($p < 0.05$) compared with the RA group, while this effect of FMN on IL-10 expression in CIA mice disappeared when the dose was increased to 200 mg/kg (Fig. 3, panel A).

Control: healthy control mice without CIA induction. RA: CIA mice with rheumatoid arthritis (RA) induced by C II treatment without anti-RA drug treatment. TG: CIA mice treated with tripterygium glycosides (TG). FMN: CIA mice treated with formononetin (FMN) at a dose of 50 mg/kg, 100 mg/kg, or 200 mg/kg, as indicated. The serum levels of IL-10 (A), TNF- α (B), and IL-6 (C) were determined using the CBA kit. Data are expressed as the mean ± SD ($n = 6$). * $p < 0.05$, ** $p < 0.01$, the drug-treated groups compared with the RA group. # $p < 0.05$, ## $p < 0.01$ the RA group compared with the control group.

Effect of FMN in the ankle histological parameters of CIA mice

The histological staining (H&E) of mice in different groups is shown in Fig. 4. In the RA group (CIA mice without drug treatment), the articular tissues showed signs of cartilage destruction, damage to extraarticular tissues, and joint space disorders. Compared with the RA group, the TG group and the FMN treatment groups displayed significant improvement, with less immune cell infiltration and slight inflammation of synovium during arthritis formation of CIA mice (Fig. 4a, panels C-D-E-F). We performed qualitative detection of cartilage proteoglycan by toluidine blue staining. The reduction of proteoglycans and incomplete staining of joints in the mice of the RA group indicated the severe damage in cartilages, while the presence of relatively normal proteoglycans in the CIA mice after TG and FMN treatment showed that cartilage had remained relatively intact. These results demonstrated that FMN inhibited the progression of arthritis in CIA mice by protecting proteoglycans (Figure-4b-C-D-E).

Effect of FMN treatment on the NF- κ B signaling pathway in CIA mice

There may be a potential link between NF- κ B p65 expression and inflammation, but the mechanism is still unclear. Many studies have shown that NF- κ B signaling pathway can modulate a variety of mediating molecules in the early stages of the immune response and inflammation. The NF- κ B signaling pathway is one of the most classical pathways for various biological responses to inflammation [20]. To investigate whether the NF- κ B signaling pathway was involved in the effect of FMN treatment of rheumatoid arthritis, we used Western blotting and IHC staining to explore the NF- κ B pathway activation in CIA mice. The results of Western blotting indicated that, compared with the RA group, the NF- κ B p65 expression level was reduced in all samples after the different dose of FMN treatment, and that the expression levels of pp65 and PCNP were also reduced, while the expression of I κ B α and TIPE2 was upregulated (Fig. 5a, panels A-E). These findings were further confirmed with IHC staining. As shown in Fig. 5b (panel B), where brown indicates the signal of NF- κ B p65 expression, in the sections of the RA group, the p65 expression was overwhelming, while the expression was dramatically reduced in the TG and FMN-treated groups, as shown in Fig. 4b (panels C-F). These results were statistically analyzed in Fig. 4c. The relative ratio of NF- κ B p65 positive staining area in the RA group was higher than that in the control group, and this difference was reversed after FMN treatment. These results suggest that FMN might play a protective role in CIA mice by blocking NF- κ B pathway activation.

Discussion

Natural drugs are potential resources for anti-RA treatment [20, 21]. A growing number of studies have demonstrated that natural drugs play an important role in anti-inflammation therapies. For example, TG, represented by triptolide, has been widely used in anti-RA treatment as one of the DMARDs. Our study found that FMN showed similar activities of anti-inflammation effects. The results showed that the

therapeutic effects of FMN on the arthritis of CIA mice were achieved by inhibiting the NF- κ B signaling pathway, by down-regulating IL-6, and TNF- α expression and release, as well as by upregulating IL-10.

The CIA model is one of the most classical and widely used animal models in anti-arthritis drug studies and is used in the study of disease pathogenesis and validation of potential therapeutic targets [22, 23]. Hence, in this study, we adopted the CIA model by inducing inflammation in C54 background mice with chicken collagen type II. By scoring the paw swelling and the arthritis index during the experiment, we monitored the disease progression [24] and evaluated the anti-arthritis activity of FMN. The results showed that 100 mg/kg FMN reduced the paw volume of CIA mice and significantly decreased the arthritis index, which was functionally equivalent to treatment with 10 mg/kg TG. On day 49 following disease induction, the mice paw swelling and arthritis index scores in the FMN-M group were significantly lower than those on day 21, indicating that FMN could significantly delay the disease progression of CIA. Additionally, animals in the RA group (without anti-RA drug treatment) suffered from weight reduction due to loss of appetite, disturbance of the immune system and persistent inflammation, which can also be used as an acute clinical signal to reflect the severity of the disease [25, 26]. Thus, increased feeding is usually taken to counteract or prevent weight loss in RA animals [26]. However, the FMN treatment did, at least in part, prevent the weight loss of CIA mice in our experiment, with FMN-M displaying the strongest effect. Furthermore, the disruption of joint and synovial tissue usually reflects RA progression and thus becomes one of the most important targets of RA therapy [27, 28]. Our data showed that FMN alleviated the inflammation and destruction of joint and synovial tissues of CIA mice at an optimal dose of 100 mg/kg body weight. Histopathological analysis demonstrated that no synovial inflammation and hyperplasia, or cartilage destruction in the mice of the healthy control group, while in the RA group mice had obvious inflammation, immune cell infiltration, synovial hyperplasia, and cartilage destruction. In the FMN groups and the TG group, drug treatment significantly alleviated synovial inflammation, synovial hyperplasia, and articular cartilage destruction.

At present, synovial inflammation and joint destruction are the two main problems faced by RA patients. TNF- α overexpression leads to excessive production of many cytokines, such as IL-6 [29, 30]. TNF- α and IL-6 are also the two key inflammatory cytokines media in the process of RA sustainable development [31]. IL-6 promotes the aggregation of osteoclasts and inhibits the synthesis of proteoglycan [32]. Osteoclasts are the key factor causing joint erosion [33], while proteoglycan is the main component of articular cartilage, and the reduction of proteoglycan leads to the degradation of articular cartilage. Moreover, IL-10 is an important multifunctional cytokine with anti-inflammatory activity, capable of inhibiting the cytokine producing Th1 cells [34]. Therefore, in this study, we analyzed the expression levels of pro-inflammatory cytokines TNF- α and IL-6, as well as of anti-inflammatory factor IL-10 in serum of CIA mice. The content of TNF- α and IL-6 significantly decreased in the FMN-treated group, while the content of IL-10 increased dramatically.

Although our data indicated that FMN had a significant therapeutic effect on the CIA mice, one phenomenon has attracted our attention, that is, mice treated with the high dose (200 mg/kg) of FMN

were not better off than the mice treated with a middle or low dose of FMN. Importantly, only the middle dose (100 mg/kg) of FMN optimally increased the level of the anti-inflammatory factor IL-10 in the serum and reduced the swelling degree of paws, while these effects were lost at a high dose of FMN. Treatment with the middle dose of FMN also led to a lower content of pro-inflammatory cytokines of IL-6 and TNF- α in the serum compared with mice treated with the high or low dose of FMN. The results also indicated that with an increase in FMN dose, the content of TNF- α in serum showed a dose-dependent increase, although the reason for this is still unclear.

NF- κ B is the most classical pathway involved in the activation and regulation of inflammatory responses [35]. Recent studies have shown that the activated NF- κ B pathway promoted the release of many inflammatory cytokines such as TNF- α and IL-6 in stimulated macrophages and synovial cells, thus playing an essential role in the pathogenesis and progression of RA [36, 37]. Research data have shown that TIPE-2 was preferentially expressed by immune cells, and could inhibit the NF- κ B pathway activation in T cells and macrophages [32]. The downregulation of TIPE-2 expression activated the NF- κ B pathway, a process that might be related to the occurrence and development mechanisms of RA [13]. Therefore, we considered the NF- κ B pathway as a potential therapeutic target in RA given the anti-inflammatory effects of inhibiting I κ B- α , TIPE-2 degradation, and blocking the activation of the NF- κ B pathway. Our study investigated the inhibitory effects on the progression of RA of the members of the NF- κ B pathway, including p65, p-p65, I κ B- α , TIPE-2, and PCNP in both the RA group and the drug-treated groups. The results showed that FMN could down-regulate the expression of p-p65 and PCNP, and inhibit the degradation of I κ B- α and TIPE-2. Again, the data revealed that the middle dose of FMN appeared to yield optimal effects and there was no benefit to treating with high dose FMN. In summary, FMN plays an anti-RA role mainly by inhibiting the activation of the NF- κ B pathway.

Conclusions

In this study, we found that FMN treatment inhibited the release of TNF- α and IL-6, while increasing the release of IL-10, suggesting that FMN had a good therapeutic effect on collagen α -induced arthritis in mice. The mechanism might be related to its inhibition of I κ B- α degradation, thus blocking the activation of the NF- κ B signaling pathway. Therefore, we believe that FMN is a potential candidate for RA therapy, which is worthy of further studies, including elucidating the appropriate dose for the anti-RA drugs and carrying out additional mechanistic and safety studies.

Abbreviations

RA: Rheumatoid arthritis; IL: Interleukin; TNF- α : Tumor necrosis factor; MCP-1: Monocyte chemotactic protein-1; NF- κ B: Nuclear factor- κ B; AP-1: Activator protein-1; TIPE-2: Tumor necrosis factor- α induced protein 8-like 2; PCNP: PEST-containing nuclear protein; siRNA: Short interference RNA; VEGF: Vascular endothelial growth factor; LPS: Lipopolysaccharides; NSAID: Non-steroidal anti-inflammatory drugs; DMARDs: Disease-modifying anti-rheumatic drugs; TG: Tripterygium glycosides; FMN: Formononetin;

CIA: Collagen-induced arthritis; CII: Type II collagen; PFA: Paraformaldehyde; SPF: Specified-pathogen-free; FMN-L: Low-dose formononetin group; FMN-M: Middle-dose formononetin group; FMN-H: High-dose formononetin group; AI: Arthritis index; PVDF: Polyvinylidene fluoride membranes; HRP: Horseradish peroxidase; IOD: integral optical density; ANOVA: Analysis of variance; Th: T helper; H&E: Hematoxylin and eosin; IHC: Immunohistochemical.

Declarations

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of Laboratory Animal Management and Animal Welfare of Binzhou Medical University.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Data curation, Ying Zhao, Wenxue Lu, Wenxing Shi, and Qing Lv; Formal analysis, Guiwu Qu, Shuyang Xie, Defang Li, Qizhi Cao, and Fengmei Zhou; Writing—original draft, Ying Zhao; Writing—review & editing, Guiwu Qu.

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Figures

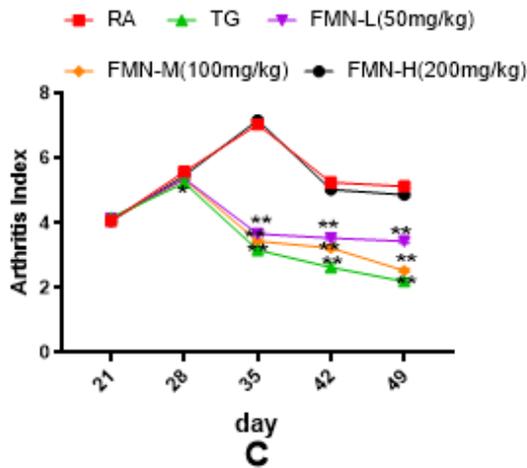
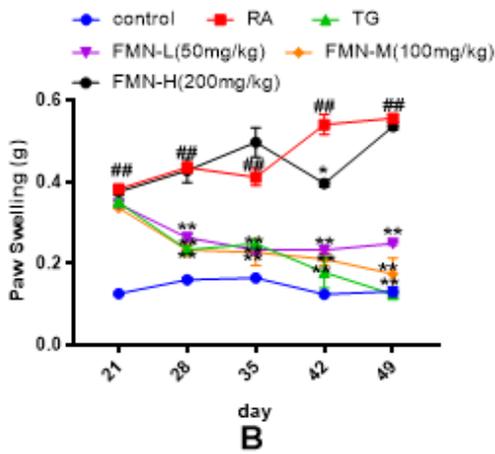
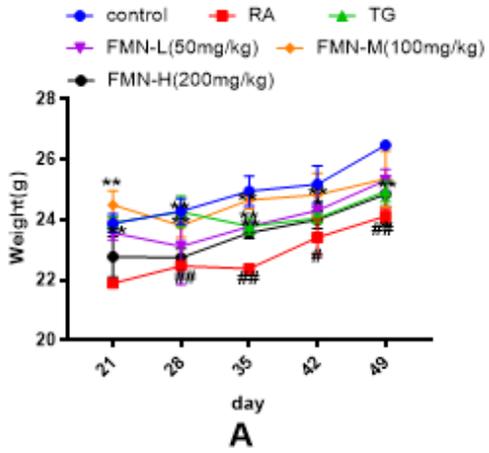


Figure 2

Effect of drug treatments on inflammation development in a mouse model of rheumatoid arthritis. Effects of FMN treatment on body weight (A), paw swelling (B), and arthritis index (C). Control: healthy control mice without collagen-induced arthritis (CIA). RA: CIA mice with rheumatoid arthritis (RA) induced by CII treatment but without anti-RA drug treatment. TG: CIA mice treated with TG. FMN-L (50 mg/kg): CIA mice treated with a low dose of FMN. FMN-M (100 mg/kg): CIA mice treated with the middle dose of

FMN. FMN-H (200 mg/kg): CIA mice treated with a high dose of FMN. Data are expressed as the mean \pm SD (n = 6). \square p < 0.05, $\square\square$ p < 0.01: the drug-treated groups compared with the RA group. # p < 0.05, ## p < 0.01: the RA group compared with the control group.

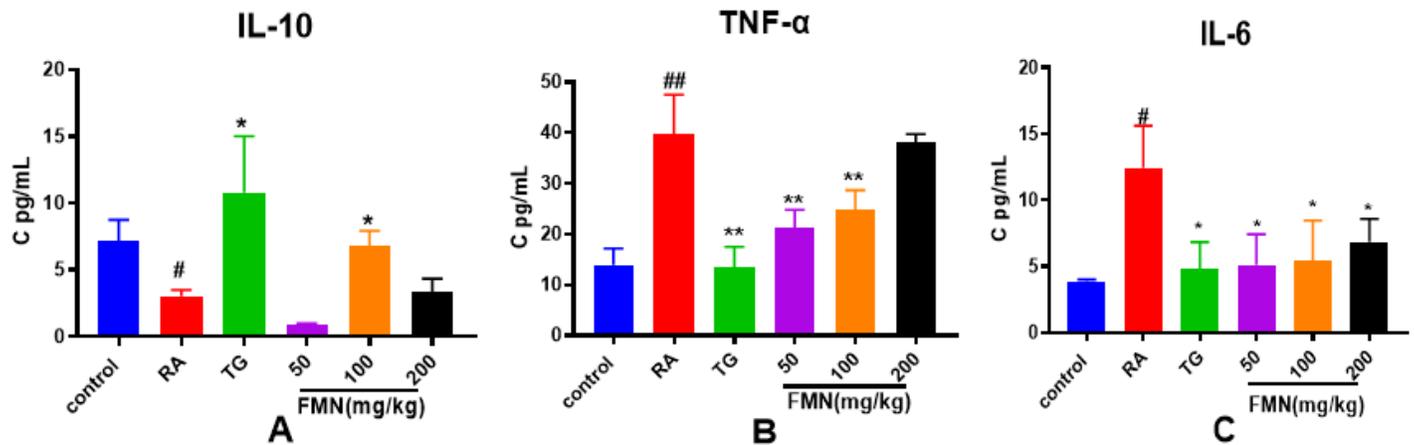
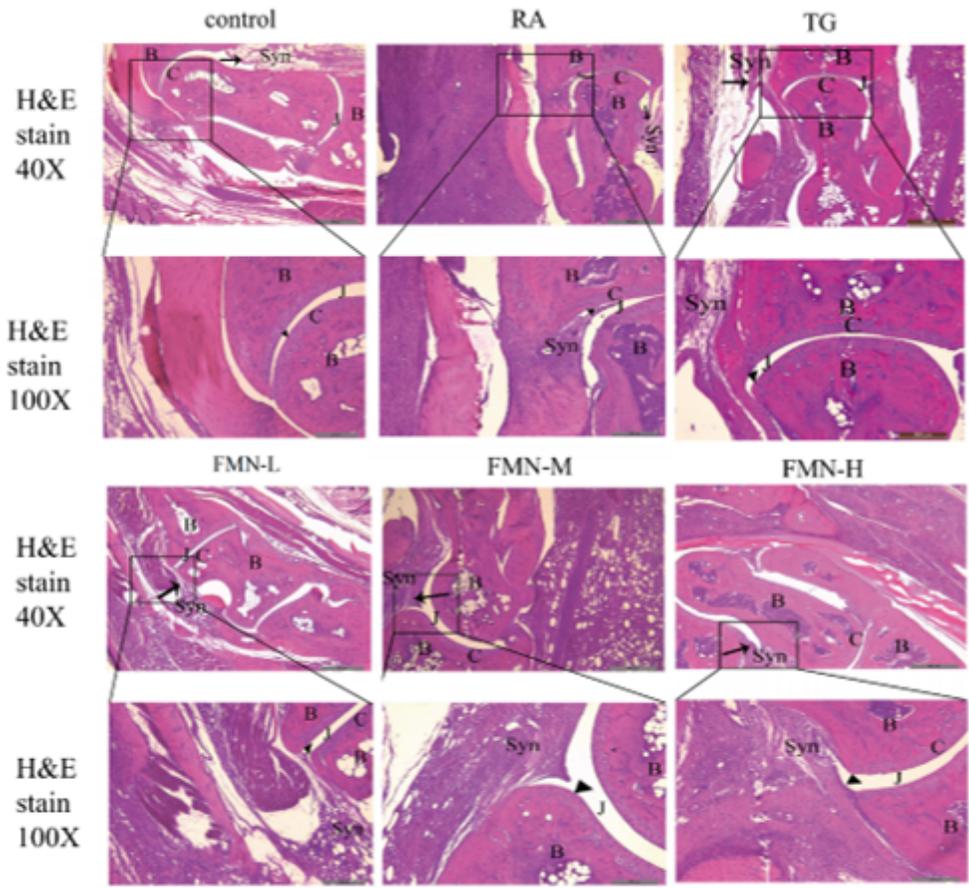
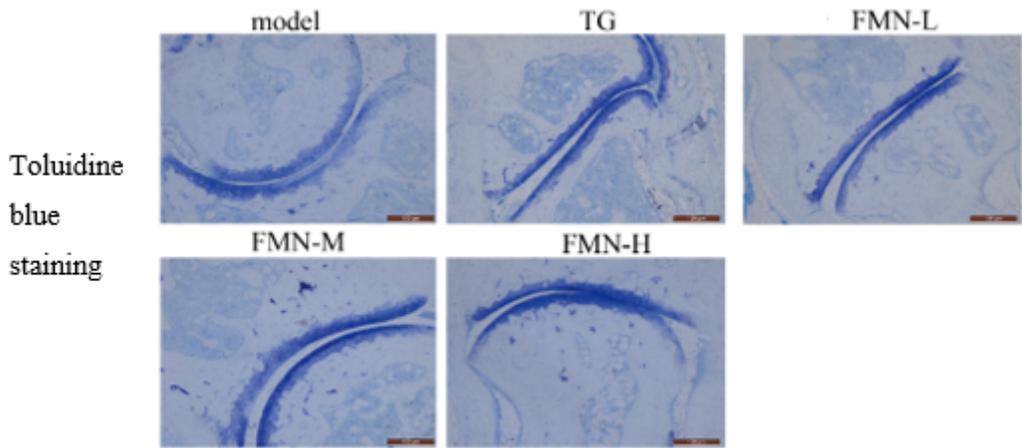


Figure 3

Effect of FMN on the IL-10, TNF- α , and IL-6 expression of CIA mice. Control: healthy control mice without CIA induction. RA: CIA mice with rheumatoid arthritis (RA) induced by C II treatment without anti-RA drug treatment. TG: CIA mice treated with tripterygium glycosides (TG). FMN: CIA mice treated with formononetin (FMN) at a dose of 50 mg/kg, 100 mg/kg, or 200 mg/kg, as indicated. The serum levels of IL-10 (A), TNF- α (B), and IL-6 (C) were determined using the CBA kit. Data are expressed as the mean \pm SD (n = 6). \square p < 0.05, $\square\square$ p < 0.01, the drug-treated groups compared with the RA group. # p < 0.05, ## p < 0.01 the RA group compared with the control group.



(a)

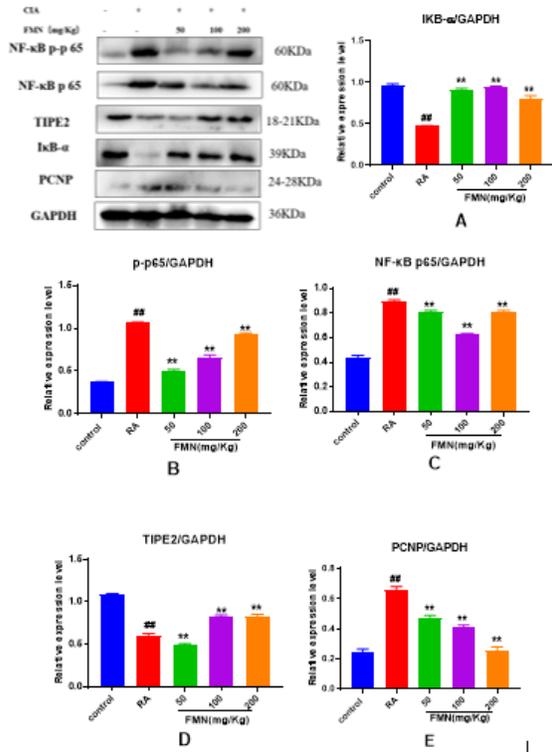


(b)

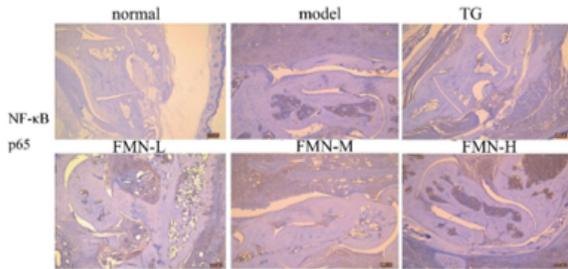
Figure 4

The effects of FMN treatment on the histopathological changes of CIA mice (40x, 100x). (a) H&E staining. control, healthy control without CIA induction. No abnormality of cartilage plate (arrow), and no damage in the thick cartilage (arrowhead). RA: CIA mice. Inflammation and hyperplasia of the synovium (arrow), damaged cartilage (arrowhead), and the decreased thickness of the cartilage plate. TG: CIA mice treated with TG. Slight inflammation and hyperplasia of the synovium (arrow), minor decreased thickness

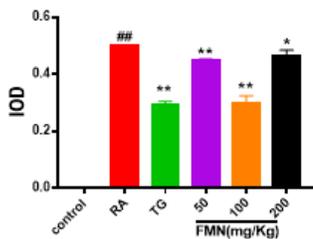
of cartilage plate (arrowhead). FMN-L: CIA mice treated with a low dose of FMN. Inflammation and hyperplasia of the synovium (arrow), decreased thickness of the cartilage plate (arrowhead). FMN-M: CIA mice treated with the middle dose of FMN. Minor inflammation (arrow) and minimally damaged cartilage (arrowhead). FMN-H: CIA mice treated with a high dose of CIA. Inflammation (arrow) and damaged cartilage (arrowhead). (b) Toluidine blue staining. Toluidine blue staining indicated the reduction of proteoglycans and incomplete staining of joints in the mice of the RA group. In contrast, the proteoglycan levels were normal, and the articular cartilage was relatively complete, in the FMN-treated groups. C: articular cartilage; J: joint space; Syn: synovial membrane; B: bone.



(a)



(b)



(c)

Figure 5

The effects of FMN treatment on the NF-κB signaling pathway activation in CIA mice. (a) Western blot analysis was used to detect the expressions of p65, p-p65, IκBα, TIPE-2, and PCNP. the relative expression level was determined by measuring the grey level of each band and normalize to the GAPDH expression level using Image J software. (b) and (c): Immunohistochemical (IHC) staining of NF-κB p65 protein. Magnification: x40. Scale bars: 100 μm. The protein expression level was determined by measuring the

integrated optical density (IOD) in IHC. Data are expressed as the mean \pm SD. n = 3 in each group. * p < 0.05, ** p < 0.01, the FMN-treated groups compared with the RA group. ## p < 0.01, the RA group compared with the control group.