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ORIGINAL ARTICLE

Effect and mechanism of AR-6 in experimental rheumatoid arthritis

Shi-xue Sun · Yun-man Li · Wei-rong Fang · Peng Cheng · Lifang Liu · Fengwen Li

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Abstract The root of *Clematis chinensis Osbeck* has been used widely in rheumatoid arthritis in Chinese traditional medicine and AR-6 is a triterpene saponin isolated from it. In this present study, we investigated in vivo effects of oral AR-6 in chronic rat adjuvant-induced arthritis (AA) and in vitro effect in macrophage and synoviocytes cells. Arthritic scores and serum inflammatory mediators were evaluated 19 days after AA induction by endermic injection of Freund's complete adjuvant in Sprague-Dawley(S-D) rats. Oral administration of AR-6 to arthritic rats resulted in a clear decrease of clinical signs compared to untreated controls. The synoviocyte and macrophage response ex vivo were then analyzed. Anti-arthritic effects of AR-6 correlated with significant decrease of NO and TNF-α produced by peritoneal macrophages, ex vivo and in vitro. AR-6 also significant decreased the proliferation of synoviocyte. These data indicate that AR-6 is a potential anti-inflammatory therapeutic and preventive agent.

Keywords Rheumatoid arthritis · *Clematis chinensis Osbeck* · Macrophages · Synoviocytes · Cytokines

S. Sun · Y. Li (✉) · W. Fang · P. Cheng
Department of Physiology, China Pharmaceutical University, 210009 Nanjing, China
e-mail: yucaoren@sina.com

L. Liu
Department of Pharmacognosy and the Key Laboratory of Modern Chinese Medicines, Ministry of Education, China Pharmaceutical University, 210038 Nanjing, China

F. Li
Department of Traditional Chinese Pharmacy, China Pharmaceutical University, 210038 Nanjing, China

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease primarily presents as a chronic symmetric polyarthritis associated with inflammation and cartilage destruction [1]. The initiating event of RA is followed by the induction of an immune response that results in inflammation in the synovial membrane, the lining of the joint, which is usually composed of macrophage and fibroblast like cells known as synoviocytes [2].

Rheumatoid arthritis is characterized by chronic inflammation of synovial tissue and the destruction of cartilage and bone in the joints [3]. Macrophages play an important role in RA, as the rheumatoid synovium is intensively infiltrated by macrophages and their numbers correlate with clinical scores [2] and articular destruction in RA [4]. Activation of the monocyte/macrophages in arthritic patients is characterized by increased expression of interleukin (IL)-1β, TNF-α, prostaglandins E_2 (PGE_2) and other pro-inflammatory mediators [3, 5]. Reports have identified NO as another pro-inflammatory mediator of arthritis in human and experimental animal studies [6, 7]. Increased concentrations of nitrites, stable metabolites of NO, have been observed in the serum and synovial fluid of patients with RA and osteoarthritis [7, 8]. Increased inducible nitric oxide synthase (iNOS) activity and NO production have also been detected in the blood mononuclear cells of RA patients and correlated with the tender and swollen joint counts [7, 9].

These cells can produce proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8), to enhance synovial inflammation [4]. In addition, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1 (ICAM-1), and reactive

oxygen species (ROS) are also known to be involved as inflammatory mediators in the induction of arthritis by augmentation of inflammation in RA [5–7].

Recently, many cytokine-targeted drugs have been applied to the treatment of RA, for example, TNF-α inhibitors (etanercept, infliximab, and adalimumab), IL-1 receptor antagonists, and nonsteroidal anti-inflammatory drugs (NSAIDs). However, NSAIDs still have undesirable side effects such as causing peptic ulcers [8]. AR-6 is a triterpene saponin isolated from the root of *Clematis chinensis Osbeck*. *Clematis chinensis Osbeck* has been frequently used to cure RA and dermatitis glandularis erythematosa in ancient China. However, the mechanism of this anti-inflammatory activity remains unclear. In this present study, the effects of AR-6 have been examined on the production of TNF-α, IL-1β, and IL-6 (as proinflammatory cytokines) both in vivo and in vitro by enzyme-linked immunosorbent assay (ELISA); we assayed the iNOS level in rats' serum and NO production in macrophage and the TNF-α-stimulated hyperplasia of FLSCs (Fig. 1).

Materials and methods

Reagents

AR-6: prepared by School of Traditional Chinese Pharmacy, China Pharmaceutical University. AR-6 was suspended in distilled water or PBS before administration. Freund's complete adjuvant: produced by Sigma Co, lot number: 085K8912; MTT: produced by Sigma Co., lot number: 6G086G08; LPS: produced by Sigma Co., lot number: 026D0603; Carrageenan: produced by Sigma Co., lot number: 073K0051; Concanavalin A: produced by Sigma Co, lot number: 6F306F30;ELISA kit of rat's IL-1β: produced by Jingmei Biotech Co. Ltd., lot number: 0506139; ELISA kit of rat's IL-2: produced by Jingmei Biotech Co. Ltd., lot number: 0506140; ELISA kit of rat's TNF-α: produced by Jingmei Biotech Co. Ltd., lot number: 0506141,070320.

Adjuvant arthritis (AA) induction and administration

Male Sprague-Dawley rats, weighing 150–180 g at receipt, were purchased from the experimental animal center of the Second Military Medical University, Shanghai, China.

Rats were housed in a standard laboratory under controlled temperature of 18–22°C and maintained on 12-h light/dark cycle with access to general food and water ad libitum.

Adjuvant arthritis was induced by intradermal injection of Freund's complete adjuvant (0.1 ml/per rat) into the right hind paw.

In primary AA rats' model, AR-6 (32, 16, 8 mg/kg) was administered once daily, for 5 consecutive days before adjuvant injection; in secondary AA model, AR-6 (32, 16, 8 mg/kg) was administered once daily, from day 21 to day 25.

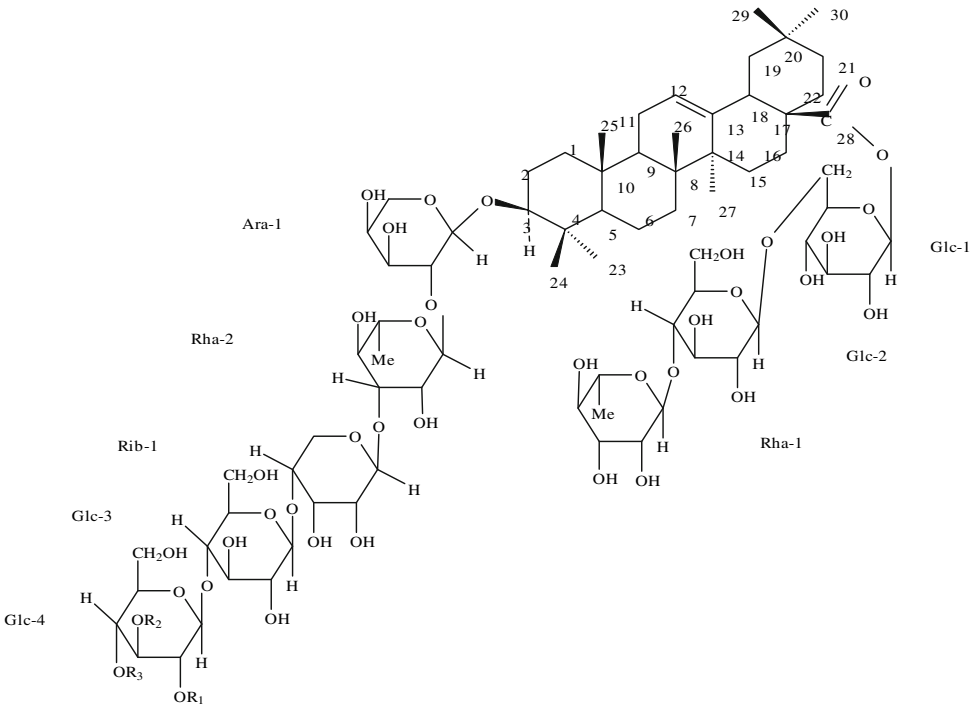

Fig. 1 Chemical structure of AR-6

Primary and secondary arthritis indexes

Rats' primary arthritis indexes were essayed on the 7th day after AA was induced. Volumes of the right hind paws were measured as an index of primary symptom, body weights and organ indexes were essayed as well.

Rats' secondary arthritis indexes ware essayed on the 25th day AA was induced. Paw swelling (ml) was calculated by subtracting the paw volume at day 0 from the related one at day 15–21. Swollen ratio was calculated by dividing the paw volume at day 0 from the paw swelling. The mean swollen ratio was used to compare the data among the control and model groups. Body weights and organ indexes were essayed as well.

Evaluation of AA severity was performed by independent observers with no knowledge of the treatment protocol. The results were analyzed and compared using the Student *t*-test for paired data.

Inflammatory mediators in secondary AA rats

NOS in rats' blood serum and PGE_2 in rats' right hind paws were assayed. NOS kit was used to assay iNOS in blood serum. To detect other rat mediators (IL-1β, IL-6, TNF-α), specific ELISA kits were used.

Primary cells culture of macrophages and macrophage-derived inflammatory mediators

Macrophages were cultured in 24-well plates with 500 μl RPMI 1640 medium with 10% FBS, penicillin (200 U/ml) and streptomycin (0.2 mg/ml), as described by Mamani-Matsuda [10].

Macrophages were stimulated by LPS and inflammatory mediators in cell supernatant were assayed. Griess reaction was used to assay NO in cell supernatant; ELISA kit was used to detect concentration of TNF-α. The results were analyzed and compared using the Student *t*-test for paired data.

Primary cells culture of synoviocyte

Rats' knee joint fibroblast synoviocyte cells (FLSCs) were isolated from Sprague-Dawley rats as described by Youn [11], with some minor modifications. Synoviums were separated from joints and placed in a Petri dish containing 10 ml Hanks' balanced salt solution with penicillin (100 U/ml) and streptomycin (100 Ag/ml). The synoviums were triturated and were incubated in 0.1% collagenase-II (diluted with Hanks' balanced salt solution) at 37°C for 120 min. Isolated cells were filtered through a 100 Am nylon mesh. The cells were collected by centrifugation and resuspended in DMEM with 10% heat-inactivated newborn calf serum. The cells were plated in a 75 cm^2 tissue culture flask and incubated at 37°C.

Cytotoxicity study

The cytotoxicity effect of AR-6 was investigated by MTT reduction assay [12]. Briefly, 1×10^5 to 2×10^5 FLSCs were incubated in 96-well plates in DMEM with AR-6 (0.1, 1, 10 μg/ml) in a 5% CO_2 incubator. After 72-h incubation, 20 μl of MTT reagent (5 mg/ml) was added to each well and the plate was then incubated for 4 h. The amount of soluble formazone produced by cellular reduction of MTT was then determined by measuring the absorbance at 490 nm with an ELISA reader.

Proliferation assay

The effect of AR-6 was investigated on the proliferation of FLSCs as judged by MTT reduction assay [12]. 1×10^4 to 2×10^4 FLSCs were incubated in 96-well plates in DMEM and stimulated by TNF-α with or without AR-6 (0.1, 1, 10 μg/ml) in a 5% CO_2 incubator. After 72-h incubation, 20 μl of MTT reagent (5 mg/ml) was added to each well and the plate was incubated for 4 h. The amount of soluble formazone produced by cellular reduction of MTT was then determined by measuring the absorbance at 490 nm on an ELISA reader.

Histological study on secondary arthritis mice

Male mice (weighing 18–22 g at receipt) were purchased from experiment animal center of Nantong University. Mice were housed in a standard laboratory under controlled temperature of 18–22°C and maintained on 12-h light/dark cycle with access to general food and water ad libitum.

Adjuvant arthritis was induced by intradermal injection of Freund's complete adjuvant (0.1 ml/per rat) into the right hind paw. To observe the effect on secondary AA mice, AR-6 (32, 16, 8 mg/kg) was administered once daily from day 21 to day 25.

On 25th day, mice were executed and the hind paws, with skin on the upper side removed, were fixed in 4% formaldehyde for 24 h and decalcified in an EDTA solution (pH 7.4). The paws were then dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin/eosin.

Statistical analysis

Data were analyzed by ANOVA followed by post hoc test to compare the treatment groups with model group. A difference was considered distinct difference at $P < 0.05$, significant distinct difference at $P < 0.01$. All values shown as mean ± SD.

Results

Effect on Freund's complete adjuvant-induced rats

Results of Fig. 2 indicate that AA severity significantly decreased after oral ministration of AR-6, compared with groups treated with 0.5% carboxymethylcellulose sodium alone ($P < 0.01$). There were no particular behaviors, clinical or physiological signs observed in animals treated with all dose levels of AR-6, suggesting that these AR-6 doses probably have no toxic effects in vivo. Furthermore, weight progression and organ indexes in AR-6-treated rats (Fig. 2) were very similar to those of control animals, while AA animals treated with dexamethasone had a significant growth delay.

Effects on histopathology of secondary AA mice

In normal group, the articular cavities were very clean, each of which has one to four layers of synovial cells. The articular surfaces were smooth, and there were loose connective tissues and fatty tissues under the synovial membranes. In the model control group, there was obviously hyperplasia in the synovial membranes of articular capsule. Synovial cells became proliferated, enlarged in size and disorderly arranged. The loose connective tissues under the synovial membranes became hyperaemia, edema and infiltrated with inflammatory cells. In AR-6-treated groups (high and middle dose levels), there were no obviously hyperplasia in the synovial membranes, with synovial cells arranged in orders and weak hyperemia in the tissue under

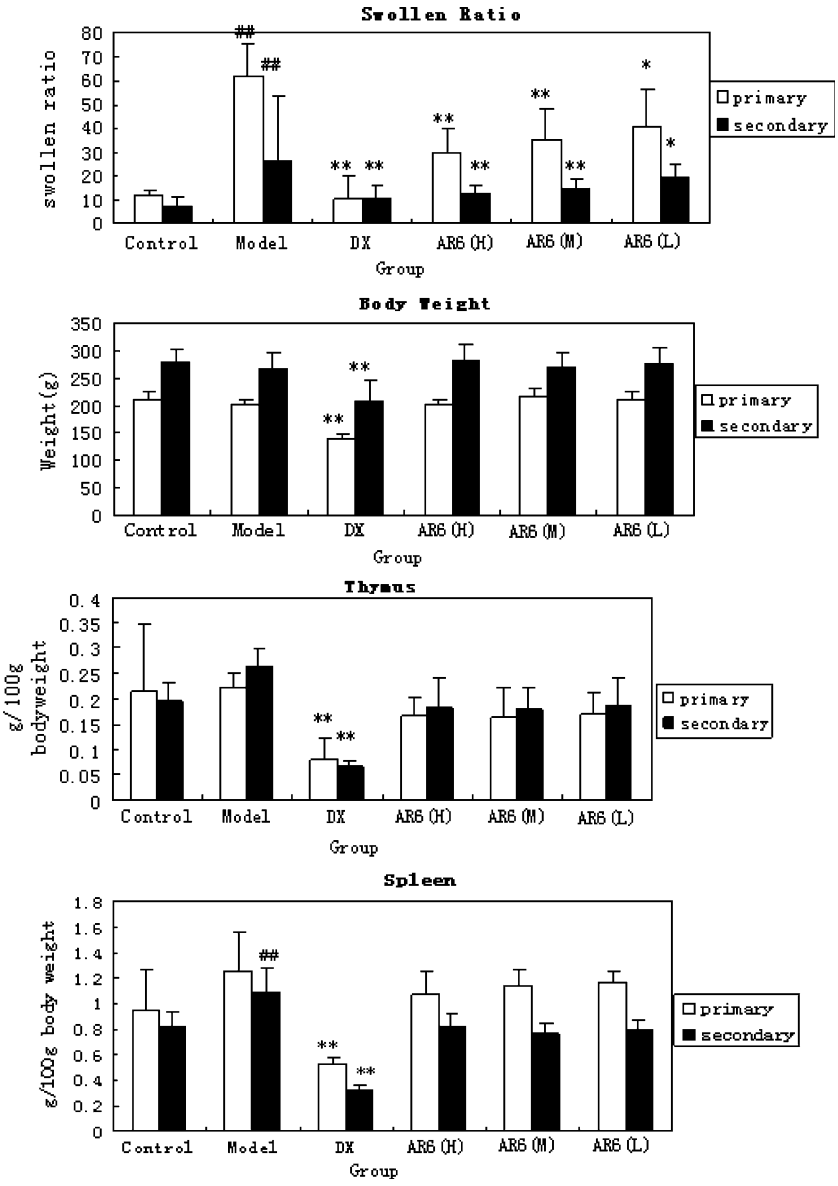

Fig. 2 The effect of AR-6 on swollen ratio, body weight, thymus, and spleen of primary and secondary adjuvant arthritis rat paw edema ($\bar{x} \pm S$, $n = 8$). $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$; $^{**}P < 0.05$ versus model. *AR-6(H)* AR-6 high dose group(32 mg/kg), *AR-6(M)* AR-6 middle dose group(16 mg/kg), *AR-6(L)* AR-6 low dose group(8 mg/kg)

the synovial membrane, and there were few inflammatory cells infiltrated into the synovial membranes. By comparison, the effect of high and middle dose levels of AR-6-treated group was better than that of the low dose-treated group, and its result was also slightly better than the DX and TGP groups (Fig. 3).

Inflammatory mediators in secondary AA rats

Results of Fig. 4 indicate that cytokines levels in serum and PGE_2 in right hind paw of AA rats significantly decreased in animals treated by oral AR-6, compared to those treated with carboxymethylcellulose sodium alone.

Inhibition of macrophage-derived inflammatory mediators by AR-6

As presented in Fig. 5, AR-6 have no significant effect on the secretion of TNF-α and NO in freshly isolated peritoneal macrophages from normal mice ($P > 0.05$).

AR-6 can significantly inhibit the over secretion of TNF-α and NO induced by LPS. These ex vivo data suggest the role of macrophage inflammatory response as a possible target for AR-6 in vivo.

Cytotoxicity assay and effect on the proliferation of FLSCs stimulated by TNF-α

The cytotoxicity assay was conducted to determine whether AR-6 have a cytotoxicity effect on normal FLSCs. Results were illustrated in Fig. 7. Dexamethasone exhibit high cytotoxicity on FLSCs ($P < 0.05$); there was no significant difference in cell viability between control and AR-6-treated cells ($n = 6$ in each case).

To investigate whether AR-6 can inhibit the proliferation of FLSCs stimulated by TNF-α, a proliferation assay was carried out. TNF-α significantly increased the proliferation of untreated FLSCs ($P < 0.01$). In contrast, AR-6 significantly inhibited the proliferation of TNF-α stimulated FLSCs, at concentrations of 10, 1 μg/ml ($P < 0.05$), and 0.1 μg/ml ($P < 0.01$). Lower concentrations had a better effect ($P < 0.01$) (Fig. 7).

Discussion

This present study evaluated anti-arthritic potential of AR-6 in both in vivo and ex vivo rat models, which were very close to its human counterpart. AR-6 has a wide range of biological activities and a potential therapeutic interest in

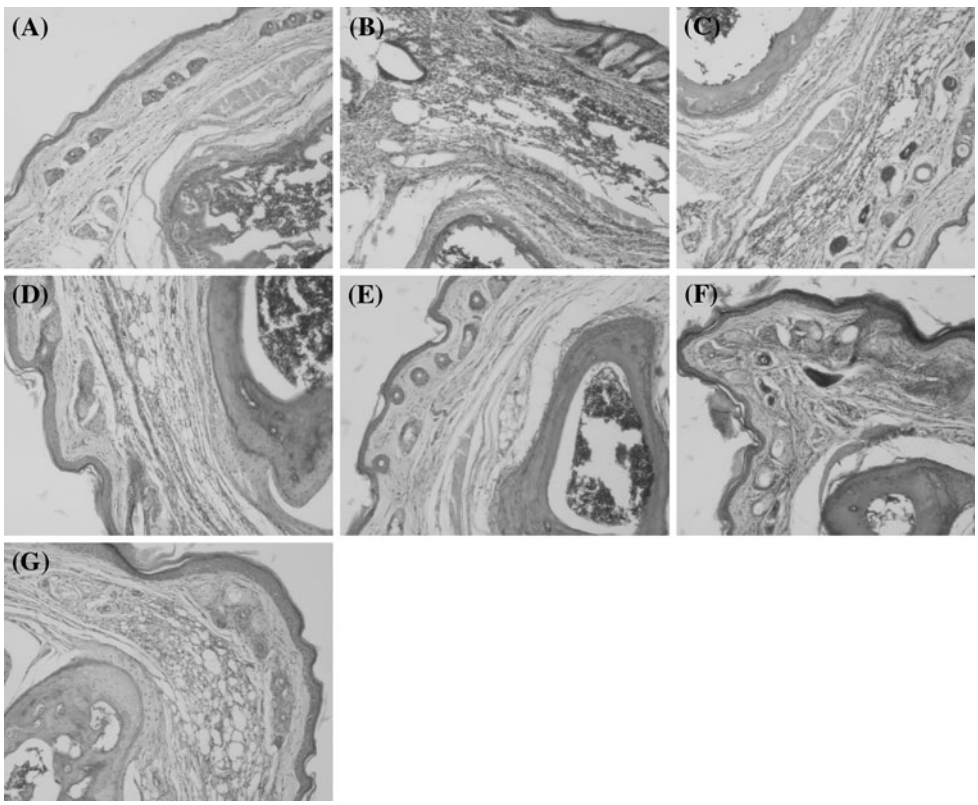

Fig. 3 Photographs of articulation slices of mouse. **a** Normal hind paw histology of rats, **b** histopathological finding from the right hind paw in CIA rats, **c** histopathological finding from the right hind paw in CIA rats treated with CPA (cyclophosphamide, 7 mg/kg), **d** histopathological finding from the right hind paw in CIA rats treated with TGP (180 mg/kg), **e** histopathological finding from the right hind paw in CIA rats treated with AR-6 (32 mg/kg), **f** histopathological finding from the right hind paw in CIA rats treated with AR-6 (16 mg/kg), **g** histopathological finding from the right hind paw in CIA rats treated with AR-6 (8 mg/kg). All the magnification is 200×

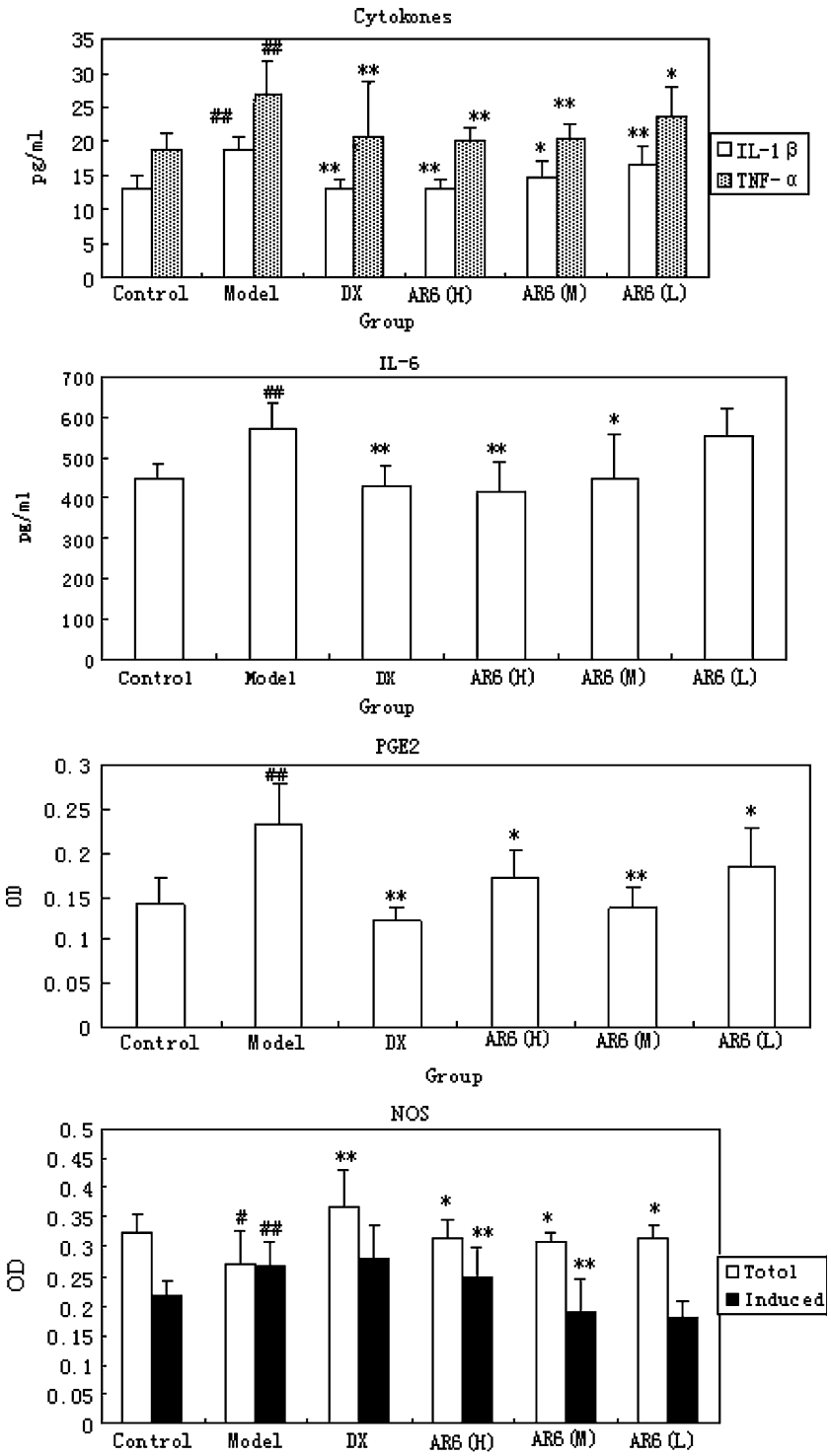

Fig. 4 The effect AR-6 on cytokines levels in serum in and PGE_2 in right hind paw secondary adjuvant arthritis rats ($\bar{x} \pm S$, $n = 8$). $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$; $^{**}P < 0.05$ versus model. *AR-6(H)* AR-6 high dose group (32 mg/kg), *AR-6(M)* AR-6 middle dose group (16 mg/kg), *AR-6(L)* AR-6 low dose group (8 mg/kg)

human. AR-6 significantly reversed growth delay and prevented development of severe AA in rats (resumed in Fig. 2). In further studies, AR-6 showed preventive effects in vivo in its purified form. This property correlated with AR-6-mediated inhibition of murine macrophage activation and inflammatory mediator release ex vivo and in vitro (Figs. 3, 4, 5, 6, and 7).

To elucidate the effects of AR-6 on proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, ELISA assays were performed. TNF-α and IL-1β have been reported to be involved in early joint swelling as well as in chronic joint inflammation [13], and are considered key mediators in the joint inflammation and in the destruction of cartilage and bone in patients with RA [14–16]. TNF-α is a pivotal

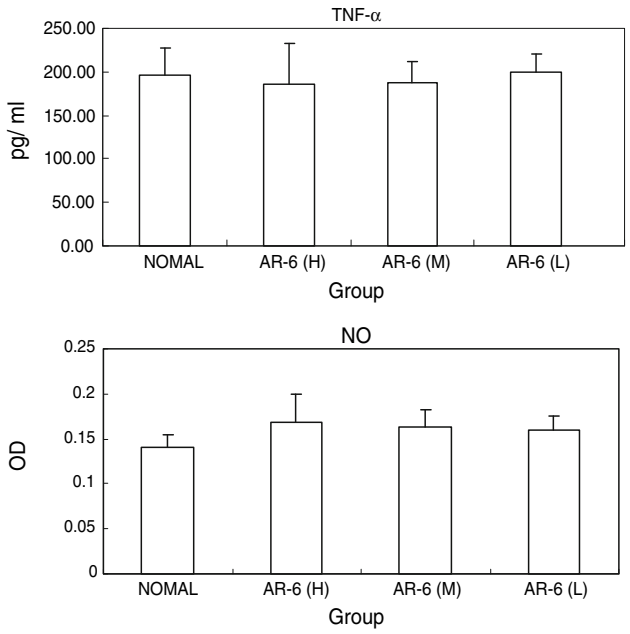

Fig. 5 The effect of AR-6 on TNF-α and NO excretion of normal mice's macrophages ($\bar{x} \pm S$, $n = 6$). *AR-6(H)* AR-6 high dose group (10 μg/ml), *AR-6(M)* AR-6 middle dose group (1 μg/ml), *AR-6(L)* AR-6 low dose group (0.1 μg/ml)

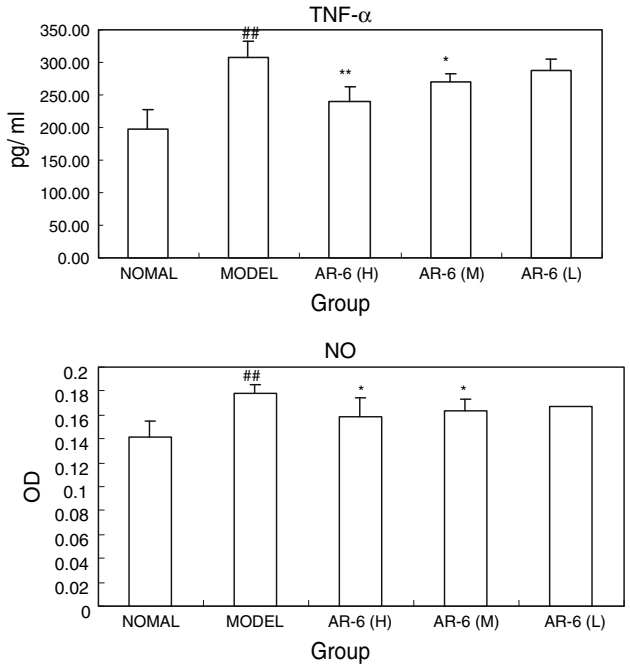

Fig. 6 The effect of AR-6 on LPS induced TNF-α and NO excretion of mice's macrophages ($\bar{x} \pm S$, $n = 6$). $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$; $^{**}P < 0.05$ versus model. *AR-6(H)* AR-6 high dose group (10 μg/ml), *AR-6(M)* AR-6 middle dose group (1 μg/ml), *AR-6(L)* AR-6 low dose group (0.1 μg/ml)

mediator in inflammatory arthritis including RA [17]. TNF-α is an autocrine stimulator as well as a potent paracrine inducer of other inflammatory cytokines such as IL-1β and

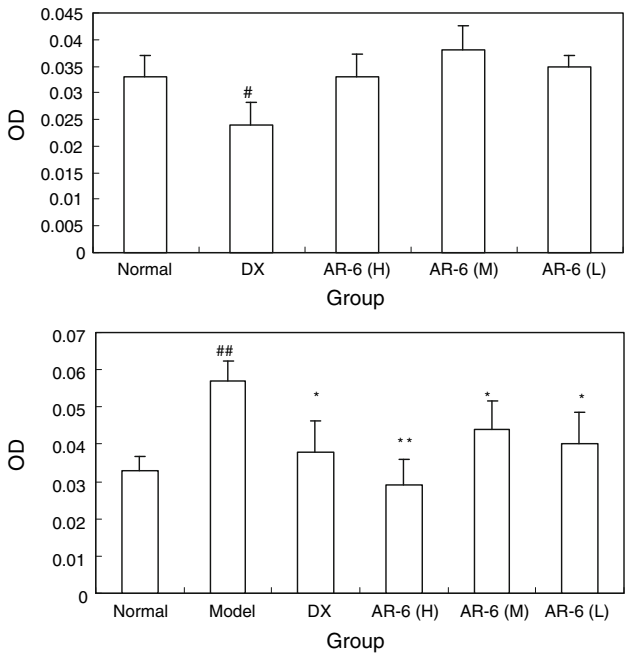

Fig. 7 The effect of AR-6 on TNF-α induced proliferation of rats synovial fibroblasts cells ($\bar{x} \pm S$, $n = 4$). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus control, $^{*}P < 0.05$, $^{**}P < 0.05$ versus model. *AR-6(H)* AR-6 high dose group (10 μg/ml), *AR-6(M)* AR-6 middle dose group (1 μg/ml), *AR-6(L)* AR-6 low dose group (0.1 μg/ml)

IL-6. The blockade and inhibition of TNF-α reduces the production of other inflammatory cytokines in cultured synovial cells from RA patients [18]. IL-6 is a proinflammatory cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis [17]. IL-6 is known to be responsible for the increase of serum g-globulin and the emergence of rheumatoid factors [19]. High levels of IL-6 have been observed in both sera and synovial fluids from the affected joints of patients with RA [20]. In present studies, AR-6 significantly reduced the production of IL-1β, IL-6, and TNF-α. These data indicated that AR-6 may have the potential to regulate proinflammatory cytokines in synoviocytes.

NO is a product of the enzymatic conversion of arginine catalyzed by a family of three distinct synthases (NOS). Expression of the inducible isoform of NOS (iNOS) and a raised concentration of NO may play a key role as a mediator of apoptosis in the pathogenesis of RA [21]. The transcription of iNOS may be involved in the induction of RA by augmentation of inflammation [22]. Our studies have demonstrated that AR-6 significantly inhibits the expression of iNOS in blood serum.

The synthesis of prostaglandins is initiated by the cyclooxygenase-catalyzed oxidation of arachidonic acid into prostaglandin H_2. The main product of the cyclooxygenase path way, namely PGE_2, plays a key role in the

erosion of cartilage and juxtaarticular bone. Joints from RA patients exhibited abundant PGE_2 expression, to higher levels than those found in synovial tissues from osteoarthritis patients or nonarthritic synovial tissues; it also showed that PGE_2 may induce over expression of cytokines, such as TNF-α, IL-1β. Therefore, PGE_2-targeted therapy is under consideration for the treatment of RA by inhibiting inflammation and angiogenesis [23–25]. During this study, AR-6 effectively involved in the regulation of the inflammatory process as a potential PGE_2 inhibitor.

Macrophages, once activated in vivo by auto antibody or by antigen-specific T-cell-derived lymphokines, are the major source of TNF-α, IL-1β, and iNOS-mediated NO during immune response [9, 26]. Although essential for the elimination of invasive antigens, chronic expression of NO resulted in a variety of inflammatory disorders including RA and many other autoimmune diseases [27]. In vitro, it was showed that AR-6 inhibited the production of TNF-α and NO from activated human macrophages [28]. In vivo, present work confirmed the anti-inflammatory potential of AR-6 as it clearly showed the anti-arthritic property of this molecule corroborated decreasing NO and TNF-α production capacity of macrophages ex vivo. We did not observe any apparent toxicity in AR-6-treated macrophage. Finally, ex vivo analysis of freshly isolated rat macrophages pointed to AR-6 mediated lowering of their activation markers.

The exact mechanism of anti-inflammatory effects of AR-6 in arthritis remains to be clarified. In addition to ex vivo data, we clearly show that AR-6 decreased the production of TNF-α, NO, major inflammatory and pro-arthritic mediators of macrophages.

RA is now known to share many pathogenetic features with osteoarthritis including synovial activation with the release of proinflammatory cytokines into the synovial fluid [29, 30].

The synovial reaction in RA patients is characterized by abundance of many cytokines, chemokines, and growth factors [31]. In particular, some proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and mediators, including iNOS and PGE_2, are known to play key roles in the pathogenesis of RA [32]. Thus, in the present study, the anti-inflammatory activity of AR-6, which has been used for the Oriental treatment of arthritis, on proinflammatory cytokines and mediators in FLSCs stimulated by TNF-α (proinflammatory cytokines) was investigated.

Recently, plant extracts have attracted more and more interests with few side effects and low cytotoxicity [33]. In order to determine the cytotoxicity of AR-6, a cytotoxicity assay was carried out. We used adrenal cortex hormone dexamethasone as a positive control in this study. As expected, AR-6 did not show any cytotoxic effect on normal FLSCs even at high concentration (10 μg/ml).

Synovial hyperplasia appears to be associated with proinflammatory cytokines, especially TNF-α, which is abundant in the synovial tissue of RA patients [34]. Therefore, the inhibitory effect of AR-6 on the proliferation of FLSCs stimulated by TNF-α was evaluated. AR-6 significantly inhibited the proliferation of cytokine-stimulated FLSCs, suggesting an anti-inflammatory activity of AR-6 against RA synoviocytes.

In summary, AR-6 significantly inhibited the symptoms and serum inflammatory mediators (TNF-α, IL-1β, IL-6) in AA rats and the TNF-α-stimulated hyperplasia of FLSCs, AR-6 down-regulated the productions of TNF-α and NO in LPS-stimulated macrophages. While other triterpene saponin may share some of anti-inflammatory properties of AR-6, the absence of apparent toxicity, the preventive effect, and the availability of therapeutic of this molecule further enforce its interest, compared to most current immunosuppressor agents. Taken together, these results suggest that AR-6 may be effectively applied to inflammatory diseases at the level of proinflammatory cytokine and mediator regulation. However, further basic investigation of AR-6 at several levels is imperative for its application to RA in the future.

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Conflict of interest statement The authors declare that they have no conflict of interest to the publication of this manuscript.

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