Effects of Artesunate prevent nephritis via the Toll-like receptor 4/nuclear factor-κB signaling pathway in rats

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Abstract. The active ingredient in *Artemisia carvifolia*, artemisinin, may alleviate inflammation and toxicity. Artemisinin and its derivatives are first-line anti-malarial drugs currently, which have rapid effects on fever caused by malaria parasites with fewer side effects. The present study investigated the effects of Artesunate in a mouse nephritis model. Mice were injected intraperitoneally with 500 μl pristine to induce nephritis, and were treated with 28.8 mg/kg Artesunate. Subsequently, proteinuria, renal function, and tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels were assessed to evaluate the effects of Artesunate on nephritis. Western blot analysis was used to measure the protein expression levels of α-smooth muscle actin (SMA), TLR4, myeloid differentiation primary response gene 88 (MyD88), NF-κB p65 and transforming growth factor (TGF)-β1 to investigate the underlying mechanisms of Artesunate on nephritis. The results demonstrated that Artesunate reduced proteinuria and preserved renal function in nephritis mice. Artesunate attenuated TNF-α and IL-6 levels, suppressed α-SMA, TLR4, MyD88, NF-κB p65 and TGF-β1 protein expression, and decreased caspase-3 activity in nephritis mice. These results indicated that the effects of Artesunate may prevent nephritis and inhibit inflammation via the TLR4/NF-κB signaling pathway in mice. Therefore, Artesunate may be a potential therapeutic agent to prevent nephritis.

Introduction

Glomerular nephritis is a type of diffuse or focal autoimmune disease characterized by glomerular damage (1). Clinical manifestations include edema, abnormal urine (proteinuria, hematuria), anemia, renal dysfunction and difficult treatment; with disease migration, most patients may develop chronic renal failure, and the prognosis is poor (2). Current research has indicated that the prevalence of chronic kidney disease will increase rapidly in the next 20 years; the proportion of adults with chronic kidney disease will increase from current 13.2 to 14.1% in 2020, and to 16.7% in 2030 (3). The development of effective therapies and treatment methods for the prevention and treatment of kidney disease is particularly important, and the studies have suggested that kidney-associated diseases will be an important direction for future research of novel drugs (4).

Present research into nephritis has suggested that inflammatory responses are associated with genetic, immune and cytokine immune complex deposition factors (2). Both natural and acquired immune responses against pathogens are mediated by Toll-like receptors (TLRs) (5). After TLRs recognize pathogen-associated molecular patterns, signals are transmitted into the cells via the Toll-interleukin (IL)-1 receptor homology region (TIR), to activate nuclear factor (NF)-κB transcription factors, causing the release of various immune-associated cytokines, including IL-1, IL-6, IL-8 and TNF-α, thus causing an inflammatory response (5). Apart from exogenous factors, the products decomposed by the extracellular matrix (ECM), including hyaluronic acid, acetyl heparin, fibrinogen or fibronectin EDA domain, and other endogenous ligands, may act via TLR4 to trigger immune and inflammatory responses (6).

In previous years, research on Artesunate (Fig. 1) has made great progress in anti-inflammatory immune activity (7). Artesunate exhibits significant anti-inflammatory activity and immunosuppressive effects *in vivo* and *in vitro* (7). Artesunate reduces the generation of various inflammatory cytokines including tumor necrosis factors (TNFs), namely α-induced IL-1β, IL-6 and IL-8 generated in synovial cells; IL-4, IL-5, IL-13 and eosinophil activating chemokines secreted in BALB/c mice broncho-alveolar caused by ovalbumin; interferon-γ, IL-17 and TNF-α induced by trinitrobenzene sulfonic acid in mice colitis; the expression levels of IL-1β, IL-17 and TNF-α, and the activity of metalloproteinase-9 induced by collagen protein in a mouse arthritis model (8). Artesunate may inhibit the immune response mediated by T auxiliary cells 1/T and 17, to reduce the expression levels of TLR 4 and TLR 9. The anti-inflammatory activity and immune inhibition function of Artesunate suggest therapeutic prospects for chronic inflammation and autoimmune diseases (9). Clinical and animal experiments have demonstrated Artesunate has good therapeutic effects on rheumatoid arthritis, asthma and systemic lupus erythematosus (10). Artesunate is a compound

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with the best solubility among all the artemisinin derivatives, which is simple to use clinically, so research on its pharmacological activity is extensive, and has been in relation to anti-tumor, anti-fibrosis and anti-parasitic disease effects (10). These results suggest that Artesunate may be a promising therapeutic agent for the prevention of nephritis by inactivation of the TLR4/NF-κB signaling pathway in mice.

Materials and methods

Animal model of nephritis. Female BALB/c mice (weight, 11-14 g; age, 5 weeks; n=66) were purchased from the Experimental Animal Centre of Laboratory Animal Sciences, Tianjin Medical University (Tianjin, China) and housed in a specific pathogen-free facility at 23±2˚C and 50-60% relative humidity, with a 12-h day/night cycle and free access to food and water. The experimental protocol was proved by the Ethics Committee of Tianjin Nankai Hospital. After being acclimated for 1 week, mice were injected intraperitoneally (i.p.) with 500 μl pristine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as the nephritis model (n=60), or were injected i.p. with 500 μl PBS as sham (n=6). After three months, nephritis model mice were randomized into two groups: Nephritis model (n=30) and 28.8 mg/kg artesunate (n=30). The nephritis model and artesunate groups received either, 100 μl of PBS or 28.8 mg/kg artesunate, respectively, orally by gavage each day for 6 weeks.

Proteinuria and renal function. Blood samples (50 μl) were obtained from the tail tip under ether anesthesia (35 mg/kg pentobarbital; Sigma-Aldrich; Merck KGaA) to examine the levels of blood urea nitrogen (BUN) and serum creatinine with a commercial autoanalyzer (Beckman Coulter, Inc., Brea, CA, USA).

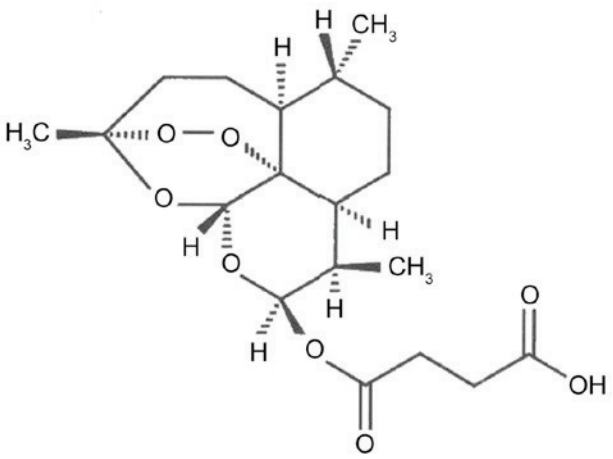

Figure 1. The constitutional formula of Artesunate.

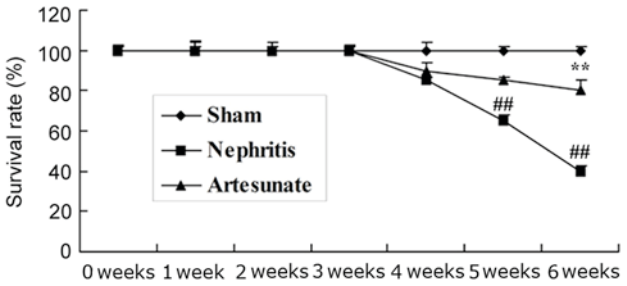

Figure 2. Effects of Artesunate enhance survival rate of nephritis mice. Survival rate of rats in the sham, nephritis and Artesunate groups over a 6-week period. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. sham; **P<0.01 vs. nephritis.

Enzyme-linked immunosorbent assay (ELISA). Following animal sacrifice using decollation under 35 mg/kg pentobarbital anesthesia, kidney tissues from the three groups were harvested, washed with PBS, then homogenized using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min at 4˚C. Homogenates were centrifuged at 1,000 x g for 10 min at 4˚C and collected to measure TNF-α (cat. no. H052) and IL-6 (cat. no. H007) levels using commercial ELISA kits (Nanjing Jianc heng Bioengineering Institute, Nanjing, China). Optical densities were measured at a wavelength of 450 nm with an ELISA plate scanner (CA94089; Molecular Devices, LLC, Sunnyvale, CA, Canada).

Western blot analysis. Kidney tissues from the three groups were harvested, washed with PBS, homogenized using RIPA buffer (Cell Signaling Technology, Inc.) for 30 min at 4˚C and homogenates were centrifuged at 1,000 x g for 10 min at 4˚C. Protein was quantified with a bicinchoninic acid assay following centrifugation at 1,000 x g for 10 min at 4˚C. Proteins (50-80 μg) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology, Haimen, China) or 5% skimmed milk powder in TBS with Tween-20 and incubated with the following rabbit primary antibodies at a 1:500 dilution: Anti-mouse α-smooth muscle actin (SMA; sc-53142), TLR4 (sc-10741), myeloid differentiation primary response gene 88 (MyD88; sc-11356), NF-κB p65, transforming growth factor (TGF)-β1 (sc-9043) and GAPDH (sc-367714), all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) at 4˚C overnight. Following this, membranes were washed with TBST (TBS with 0.1% Tween-20) and incubated with anti-rabbit or anti-mouse IgG, horseradish peroxidase-conjugated antibodies (cat. nos. 7074 and 7076, respectively; 1:5,000; Cell Signaling Technology, Inc.) for 1 h at 37˚C. Proteins were visualized using an Enhanced Chemiluminescence detection system (EMD Millipore). Protein expression was quantified using ImageJ analysis software version 1.37 (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. One-way analysis of variance and a post hoc Tukey test were used for multiple comparisons using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Artesunate enhances survival rate. The survival rate of mice in the sham, nephritis and Artesunate groups was assessed

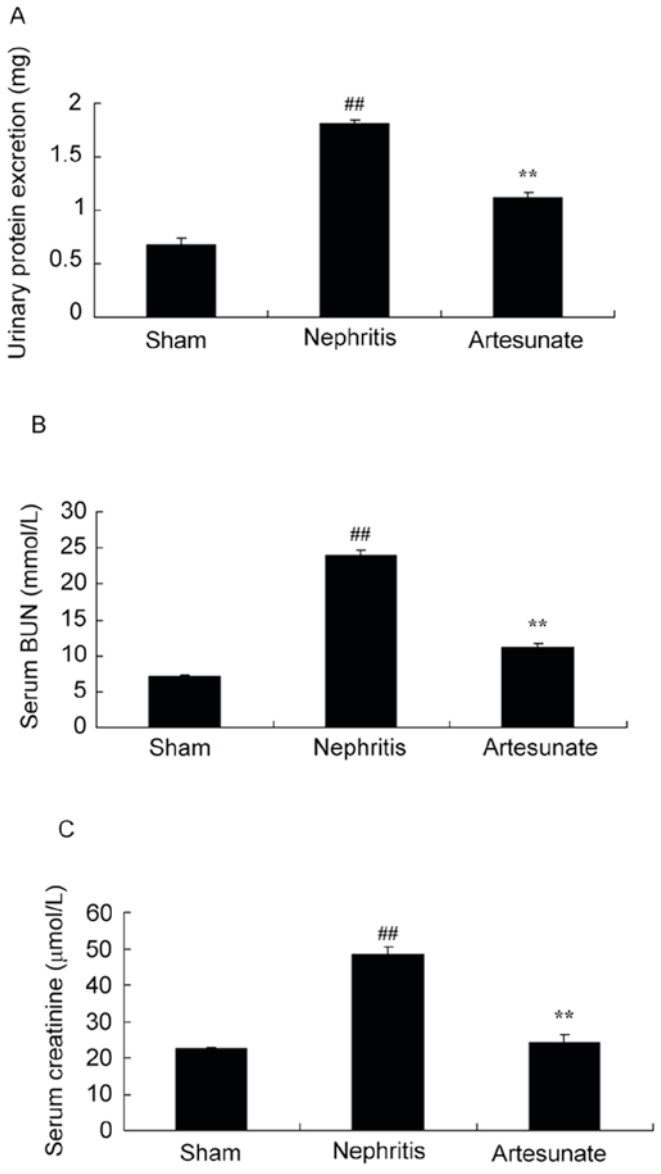

Figure 3. Artesunate prevents nephritis and improves renal function. (A) Urinary protein, (B) serum BUN and (C) creatinine levels in mice in the sham, nephritis and Artesunate groups. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. BUN, blood urea nitrogen.

over a 6-week period. As presented in Fig. 2, the survival rate of nephritis model group was reduced compared with the sham group. However, treatment with 28.8 mg/kg Artesunate significantly increased the survival rate of the nephritis mice, compared with the nephritis model group (Fig. 2).

Artesunate prevents nephritis and improves renal function. The effect of Artesunate on nephritis was investigated by blood content analysis. Urinary protein (Fig. 3A), serum BUN (Fig. 3B) and creatinine (Fig. 3C) levels in the nephritis model group were significantly increased compared with the sham group. However, 28.8 mg/kg Artesunate treatment significantly inhibited urinary protein, serum BUN and creatinine levels in nephritis mice (Fig. 3).

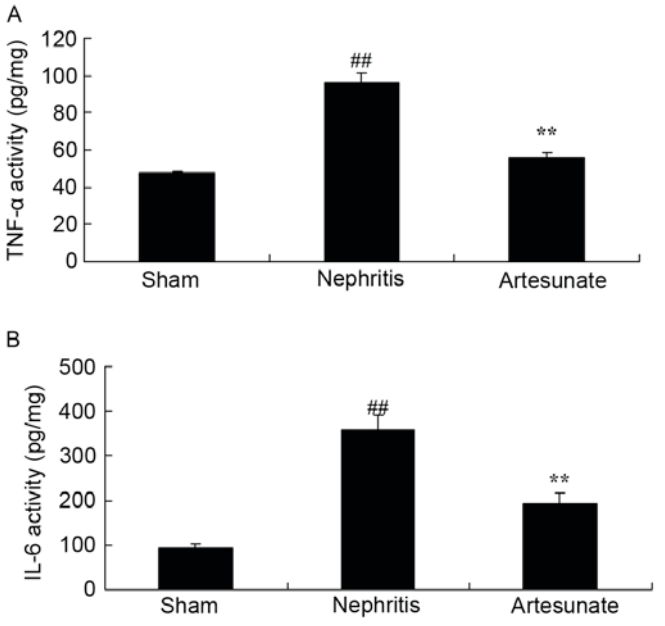

Figure 4. Artesunate inhibits TNF-α and IL-6 levels. (A) TNF-α and (B) IL-6 levels in sham, nephritis and Artesunate groups, as assessed by ELISA. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

Artesunate inhibits TNF-α and IL-6 levels. The effect of Artesunate on inflammation in nephritis mice was investigated by ELISA. As expected, a significant increase in TNF-α (Fig. 4A) and IL-6 (Fig. 4B) levels were observed in the nephritis model group compared with the sham group. Treatment with Artesunate significantly decreased TNF-α and IL-6 levels in nephritis mice, compared with non-treated animals (Fig. 4).

Artesunate inhibits α-SMA protein expression. The effect of Artesunate on α-SMA protein expression in nephritis mice was investigated by western blotting. The protein expression of α-SMA in the nephritis model group was significantly increased compared with the sham group (Fig. 5). However, a notable reduction of α-SMA protein expression following Artesunate treatment was observed in nephritis mice, compared with the nephritis model group (Fig. 5).

Artesunate inhibits TLR4 protein expression. A similar effect on TLR4 protein expression by Artesunate treatment was observed. The results demonstrated a significant increase in TLR4 protein expression in the nephritis model group compared with the sham group (Fig. 6). Treatment with Artesunate significantly suppressed TLR4 protein expression in nephritis mice compared with the nephritis model group (Fig. 6).

Artesunate inhibits MyD88 protein expression. Artesunate was administrated in order to observe its influence on MyD88 protein expression in nephritis mice. The results demonstrated that MyD88 protein expression in the nephritis model group markedly increased compared with the sham group (Fig. 7). Artesunate (28.8 mg/kg) treatment obviously decreased MyD88 protein expression in nephritis mice compared with the nephritis model group (Fig. 7).

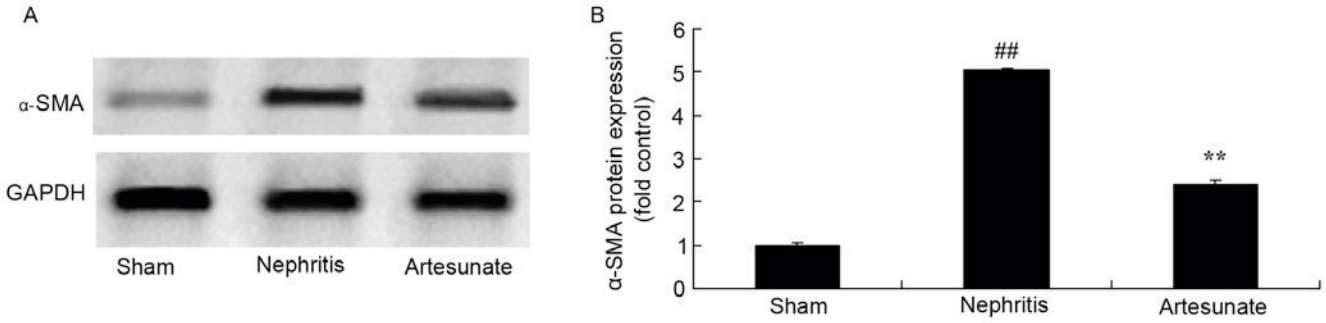

Figure 5. Artesunate inhibits α-SMA protein expression. Representative western blot (A) images and (B) quantification of α-SMA protein expression levels. GAPDH served as an internal control. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. α-SMA, α-smooth muscle actin.

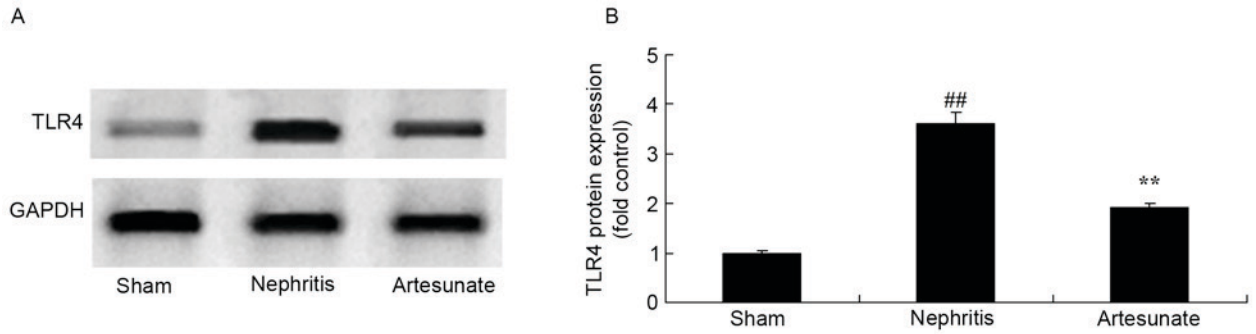

Figure 6. Artesunate inhibits TLR4 protein expression. Representative western blot (A) images and (B) quantification of TLR4 protein expression levels. GAPDH served as an internal control. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. TLR4, toll-like receptor 4.

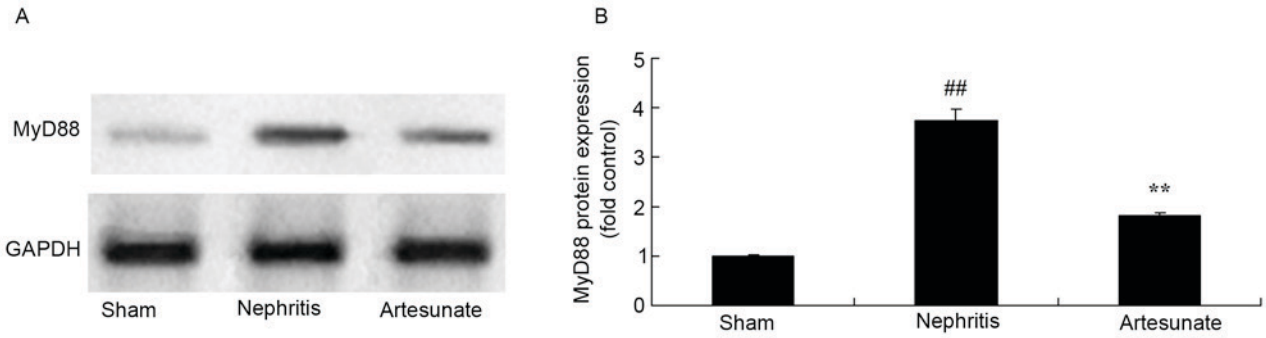

Figure 7. Artesunate inhibits MyD88 protein expression. Representative western blot (A) images and (B) quantification of MyD88 protein expression levels. GAPDH served as an internal control. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. MyD88, myeloid differentiation primary response gene 88.

Artesunate inhibits NF-κB p65 protein expression. NF-κB p65 protein expression levels are closely associated with pro-inflammatory cytokine levels in nephritis models. The results demonstrated that NF-κB p65 protein expression markedly increased in nephritis the model group compared with the sham group (Fig. 8). Artesunate (28.8 mg/kg) treatment significantly decreased NF-κB p65 protein expression in nephritis mice compared with the nephritis model group (Fig. 8).

Artesunate inhibits TGF-β1 protein expression. The effects of Artesunate on TGF-β1 protein expression in a nephritis model were examined by western blot analysis. The results demonstrated that TGF-β1 protein expression markedly increased in the nephritis model group compared with the sham group (Fig. 9). Artesunate treatment significantly inhibited TGF-β1 protein expression in nephritis mice compared with the nephritis model group (Fig. 9).

Artesunate inhibits caspase-3 activity. To further evaluate the effects of Artesunate on apoptosis in a nephritis model, caspase-3 activity was measured by ELISA. Caspase-3 activity in the nephritis model group as significantly increased compared with the sham group. Artesunate treatment significantly decreased caspase-3 activity in nephritis mice compared with the nephritis model group (Fig. 10).

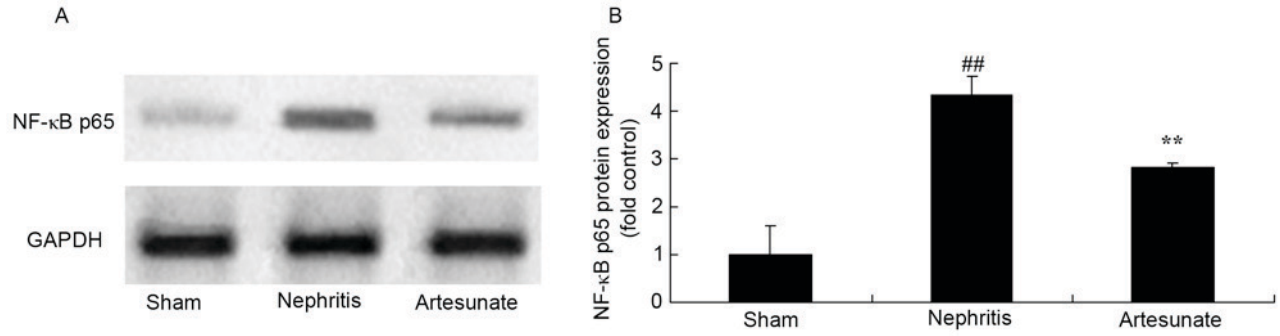

Figure 8. Artesunate inhibits NF-κB protein expression. Representative western blot (A) images and (B) quantification of NF-κB protein expression levels. GAPDH served as an internal control. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. NF-κB, nuclear factor-κB.

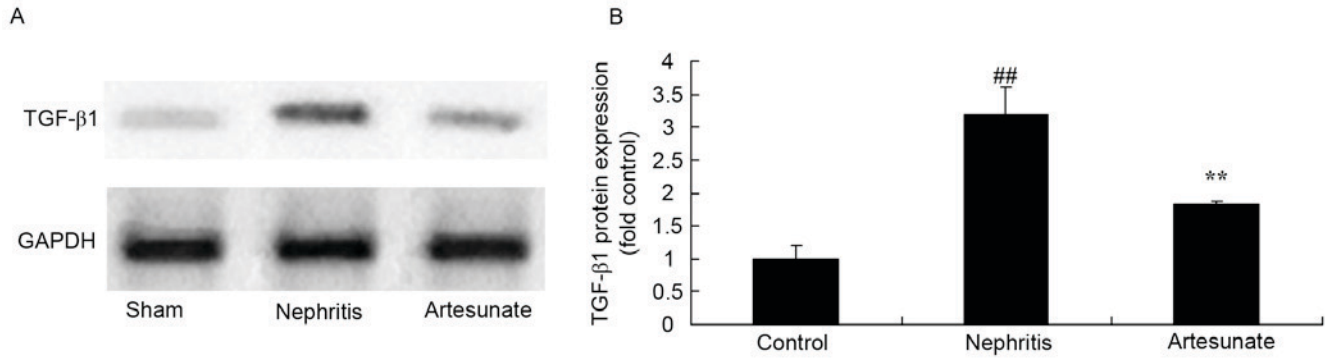

Figure 9. Artesunate inhibits TGF-β1 protein expression. Representative western blot (A) images and (B) quantification of TGF-β1 protein expression levels. GAPDH served as an internal control. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. TGF-β1, transforming growth factor-β1.

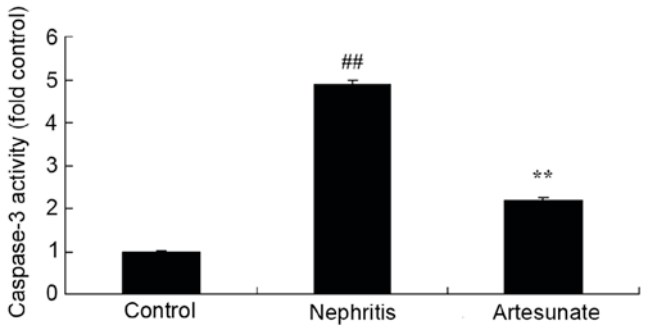

Figure 10. Artesunate inhibits caspase-3 activity. Caspase-3 activity in the sham, nephritis and Artesunate groups, as assessed by ELISA. Data are presented as the mean ± standard deviation. ##P<0.01 vs. sham; **P<0.01 vs. nephritis. miR, microRNA.

Discussion

Glomerular nephritis, also known as chronic nephritis, occurs in bilateral kidneys, and has complex etiology and multiple pathology types. It is a diffuse or focal autoimmune disease primarily manifested by glomerular damage (11). Clinical manifestations include edema, abnormal urine (proteinuria, hematuria), high blood pressure, anemia, renal dysfunction and difficult treatment. As the disease migrates, most patients develop chronic renal failure, and the prognosis is poor (12). For early chronic nephritis, appropriate treatment should be given according to the pathological type, to suppress immune-mediated inflammation, inhibit mesangial cell proliferation and alleviate the kidney hardening, in order to prevent or slow the progressive deterioration of renal function, and improve or relieve symptoms and complications. The following measures may be adopted for comprehensive treatment (13). The results of the present study indicated that Artesunate significantly increased the survival rate, inhibited urinary protein, serum BUN and creatinine levels, and decreased TNF-α and IL-6 levels in nephritis mice. Luo *et al* (8) indicated that Artesunate inhibits the effects of cigarette smoke and inflammation.

The distribution of α-SMA is closely associated with the development, occurrence and prognosis of kidney disease (14). Mesangial cells (MCs) expressing α-SMA are able to contract to reduce the capillary area to influence glomerular hemodynamics, resulting in the occurrence of glomerular sclerosis; in addition, α-SMA induces ECM synthesis and secretion, leading to glomerulosclerosis (15). A previous study demonstrated that MC proliferation, and mRNA and protein expression levels of intracellular α-SMA, are increased significantly in an anti-Thy-1 nephritis model. However, removal of platelets may successfully inhibit these effects (16). A previous glomerulonephritis study has indicated that the expression level of α-SMA is proportional to the degree of MC proliferation (16). Therefore, α-SMA may be an indicator of transformation of MCs from the stationary phenotype to the proliferative/secretory phenotype (14). The results of the present study indicated that Artesunate significantly suppressed α-SMA protein

expression levels in nephritis mice. Xu *et al* (17) suggested Artesunate ameliorates hepatic fibrosis via regulating α-SMA expression in mice.

In pathological process of mesangial proliferative glomerulonephritis, there are many cytokines involved. MCs are stimulated through autocrine and paracrine pathways, and tissue hypertrophy, cell proliferation, and synthesis and accumulation of ECM components, eventually leads to glomerulosclerosis (18). The TGF-β1 signaling pathway is the most active. TGF-β is a multifunctional polypeptide growth factor that regulates cell proliferation, differentiation and apoptosis by complex receptor signaling pathways in the cell surface (19). TGF-β inhibits proliferation of glomerular epithelial cells and endothelial cells; however, it has a dual role on MCs: It stimulates proliferation at a low concentration, and inhibits proliferation at a high concentration (20). MCs have been revealed to secrete TGF-β, and TGF-β regulates the proliferation rate and synthesis of ECM components by binding to the corresponding receptors on MCs (21). The most marked effect of TGF-β on MCs is to stimulate secretion of the ECM, so that ECM components accumulate (21). A previous study in animals and human kidney disease confirmed TGF-β1 may increase ECM synthesis, reduce ECM decomposition, increase and coordinate the expression of integrin, and promote the excessive accumulation of the ECM in the kidneys, eventually leading to glomerulosclerosis (22). Jiang *et al* (23) reported that Artesunate attenuates lung injury through TGF-βl and anti-inflammatory activities. Consistent with this, the results of the present study demonstrated that Artesunate significantly suppresses TGF-β protein expression in nephritis mice.

Previous studies have indicated TLRs are involved in immune and inflammatory response (6,24). In TLR family, TLR4 mediates inflammation, and serves an important role in non-infectious inflammations in liver, lung and kidney ischemic reperfusion injury. Organs may be protected by inhibiting the expression of TLR4 (25). One of the main signal transduction pathways involves binding with the TIR region within TLR cells through an adapter protein (MyD88), which leads to activation of transcription factors (such as NF-κB), thus contributing to synthesis and transcriptional expression of a large number of cellular activity factors, including IL-1, IL-6 and IL-8, and adhesion molecules and inflammatory cytokines, which ultimately affects the immune and inflammatory response (25). There has been increased focus on the role of TLR4 in kidney disease; glomerular disease is a type of immune-mediated inflammation response, and TLR4 is closely associated with inflammation regulation (24). TLR4 expression is increased in glomeruli, which activates renal parenchymal cells and immune cells to promote secretion of large amounts of cytokines and inflammatory mediators, causing persistent inflammation, leading to significantly faster glomerulosclerosis. NF-κB activity is significantly increased, which is induced by MyD88 signal transduction pathways, and activated NF-κB regulates gene transcription of inflammatory cytokines and a variety of fiber-associated factors, including TGF2β1. Fibroblast proliferation and differentiation may also be directly mediated by NF-κB, thereby affecting the development of glomerulosclerosis (26). A previous study confirmed that TLR4 is an important regulatory factor in the inflammatory response activation pathway, and diabetic kidney injury may be associated with increased expression of TLR4 (24). The results of the present study demonstrated that Artesunate significantly decreased TLR4, MyD88 and NF-κB p65 protein expression in nephritis mice. Wang *et al* (27) indicated that Artesunate attenuates lipopolysaccharide-induced inflammation via the TLR4/MyD88/NF-κB signaling pathways in microglial cells (27). The present study indicated that the protective effects of Artesunate on nephritis are partially attributed to inhibition of the TLR4/MyD88/NF-κB p65 signaling pathway.

In conclusion, Artesunate treatment attenuated nephritis by its anti-inflammatory effect, and by inhibiting the TLR4/MyD88/NF-κB p65 signaling pathway in mice. Therefore, Artesunate may be a potential therapeutic agent to prevent nephritis.

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