Article

A Pilot Study of the Therapeutic Efficacy and Mechanism of Artesunate in the MRL/lpr Murine Model of Systemic Lupus Erythematosus

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Recent evidence indicates that artesunate has immunomodulatory properties that might be useful for treating autoimmune disease. In this study, we conducted a pilot study and explored the effect and mechanism of artesunate on the treatment of systemic lupus erythematosus using an MRL/lpr murine model. MRL/lpr mice were divided into control, cyclophosphamide (CTX) and artesunate treatment groups. Blood was collected to measure serum levels of creatinine, antinuclear antibody (ANA) and anti-double-stranded DNA (anti-dsDNA) antibody. Twenty-four-hour urine was collected to measure levels of proteinuria. The concentration of monocyte chemotactic protein-1 (MCP-1) in serum and urine was measured. The expression of MCP-1 in kidney was detected by Western blot and immunohistochemistry assay, respectively. The expression of B cell activating factor (BAFF) in spleen was determined by real time-PCR and immunoblotting. We found that artesunate significantly increased the survival rate, body weight and blood leukocyte counts, and reduced the serum levels of ANA and anti-dsDNA antibody titer, 24 h urinary protein, and serum creatinine. Our results indicated that artesunate could decrease MCP-1, major pro-inflammation cytokine, in serum, urine and kidney. We also found that the level of BAFF, the major B cell activation factor, was decreased in artesunate treated MRL/lpr mice. Its efficacy was comparable with that of CTX in this study. Taken together, we have demonstrated that artesunate can inhibit the progression of disease and reverse the pathologic lesion of lupus nephritis. *Cellular & Molecular Immunology*. 2009;6(6):461-467.

Key Words: artesunate, systemic lupus erythematosus, lupus nephritis, MRL/lpr mice

Introduction

System lupus erythematosus (SLE) is a multisystem autoimmune disease with significant morbidity and mortality. Among many organs affected in SLE, the kidney may be most frequent and severely injured. The incidence of lupus nephritis (LN) is 40%~80%, and LN remains a major cause of morbidity and mortality in patients with SLE (1). Treatment of SLE is difficult as it is hard to balance the efficacy and safety of drugs. Glucocorticoid combined with immunosuppressive drugs is the standard therapy for LN. However, the usage of these medications often results in serious side-effects and the disease often relapses after the therapy withdrawed (2). Recently, biologic therapies have been introduced and their use in LN is an area of intense research. However, the expenses associated with the various biologic therapies proposed for SLE preclude their general use in both developing and underdeveloped countries.

In China, traditional Chinese medicine like Tripterygium wilfordii, is used to treat LN. However, this medication has significant urinary and hematological adverse effects in substantial numbers of treated patients (3, 4). Therefore, it is necessary to explore a new traditional Chinese medicine which could be both efficacious and less side-effect.

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Received Jul 20, 2009. Accepted Nov 11, 2009.

Abbreviations: SLE, systemic lupus erythematosus; LN, lupus nephritis; MCP-1, monocyte chemotactic protein-1; BAFF, B cell activating factor; CTX, cyclophosphamide; ANA, antinuclear antibody; anti-dsDNA, anti-double-stranded DNA.

Arteannuin, extracted from the caudex of one feverfew-southernwood, has been commonly used as an antimalarial agent (5, 6). Recent studies have shown that arteannuin and analogues such as SM735 and dihydro- artemisinine have immunomodulatory properties that might be useful for treating autoimmune diseases including SLE (7). They inhibit the proliferation and antibody production of B lymphocytes. In lupus-prone BXSB mice, dihydro- artemisinine has been shown to promote the multiplication of $CD8^+$ T cells, to decrease concentrations of anti-double-strained DNA (anti-dsDNA) antibodies and TNF-α levels in serum, to reduce the formation of immune complexes and the deposition of auto-antibodies and complement components in kidney, and to prevent or reverse pathologic changes in renal tissues (8, 9). In addition, the combined usage of arteannuin and Chinese caterpillar fungus prevents the relapse of LN and the deterioration of renal function (10).

In present study, the efficacy of artesunate on lupus-like disease of MRL/lpr mice, especially on the renal damage, was investigated. The change of autoantibody and pro-inflammation cytokine levels in serum and urine, the monocyte chemotactic protein-1 (MCP-1) expression in kidney and the B cell activating factor (BAFF) (11-15) expression in spleen, all of which may play a role in the development of lupus nephritis, was also determined to explore the mechanism of action of artesunate.

Materials and Methods

Reagents

Artesunate was purchased from Guilin Pharmaceutical Co., Ltd (Guangxi Province, China). Test kits for ANA antibody, anti-dsDNA antibody and MCP-1 assays were purchased from Euroimmun Corporation (Germany). Primers for PCR and SYBR Premix Ex Taq were purchased from Takara Company (Japan). The BAFF and MCP-1 antibody were purchased from Santa Cruz Company (USA). All other reagents were of the highest grade and commercially available.

Animals

Ten weeks old female MRL/lpr mice were purchased from Shanghai Experimental Animal Corporation. They were housed in SPF rooms at 22°C and 40% humidity under a 12 h light, 12 h dark cycle. The local research ethics committee gave approval for the study. The mice were divided into three groups: control group (n = 5) (physiologic saline 2 ml/d intragastric administration (*i.g.*) for 16 weeks), artesunate treatment group (n = 5) (artesunate 125 mg/kg·d solved in 2 ml physiologic saline *i.g.* for 16 weeks) and cyclophosphamide (CTX) treatment group (n = 4) (CTX 100 mg/kg·d intravenous injection (*i.v.*) for 2 days). The treatment was started at 16 weeks when the concentration of urine protein exceeded 100 mg/ml. The body weight of the mice was measured every 4 weeks. All mice were sacrificed at 32 weeks old.

Routine laboratory measures

Titers of antinuclear antibody (ANA) and anti-dsDNA antibody in serum were measured by indirect immuno-fluorescence method. The concentration of creatinine in the serum was measured by bitterness acid method. The quantity of urine protein was detected by Coomassie brilliant blue method. The concentration of MCP-1 in serum and urine was measured by ELISA method.

Renal pathology

At 32 weeks, the mice were anesthetized by ketamine injection. Kidneys were perfused with 4°C physiologic saline and then vertically split through the renal hilum. One part of each kidney was fixed in liquid nitrogen. Another part was fixed in 10% formalin and dehydrated. Then the tissue was imbedded in paraffin and sliced into 4 μm sections. The sections were stained with PAS for microscopic pathological diagnosis.

Western blot assay

Protein of spleen and kidney were lyses with SDS lysis buffer. Protein was separated in acryl/bisacrylamide gel, and then transferred to a polyvinylidene fluoride membrane. After saturation, primary antibody (BAFF and MCP-1) and secondary horseradish peroxidase-conjugated antibody were sequentially added. Revelation was obtained by enhanced chemiluminescence.

Immunohistochemistry assay

MCP-1 protein expression was detected by immuno-histochemistry assay. Briefly, the blocked kidney sections were stained with monoclonal anti-MCP-1 antibody by incubation overnight at 4°C. After washed with PBS, the slides HRP conjugated rabbit-anti-mouse IgG was added at 37°C for 30 min. After washed in PBS, the reaction products

Table 1. The comparison among various groups in 24 h urine protein (mg)

Group	Mice age (weeks old)			
	20	24	28	32
Control	3.69 ± 0.57	8.98 ± 1.32	9.30 ± 0.63	10.02 ± 1.61
CTX	3.34 ± 0.35	5.10 ± 1.01*	7.74 ± 1.05	9.56 ± 1.91
Artesunate	3.13 ± 0.55	4.91 ± 1.17*	5.25 ± 1.01*#	6.37 ± 1.19*#

*$p < 0.01$ *vs* control group; #$p < 0.05$ *vs* CTX group.

were visualized after incubation with DAB. Under the optic microscope, the positive cells expressed brown granules.

BAFF gene expression

The expression of BAFF in spleen of 32 weeks old mice was determined by real-time PCR. Total RNA of spleen fixed in liquid nitrogen was isolated by Trizol reagent. cDNA was synthesized following the manufacturer's instructions. BAFF and β-actin sequences were amplified by real-time PCR using the following gene-specific primers: BAFF (159 bp), forward, 5'-TGG TGA CCC TGT TCC GAT GTA-3' and reverse, 5'-AGA AGG TGT CGT CTC CGT TGC-3'; β-actin (211 bp), forward, 5'-CCT CTA TGC CAA CAC AGT GC-3' and reverse, 5'-GTA CTC CTG CTT GCT GAT CC-3'. For quantitative detection of BAFF mRNAs, the templates and primer sets were mixed with SYBR Premix Ex Taq, and real-time PCR was performed using Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The expression of mRNA was normalized to β-actin, respectively.

Statistical analysis

Data were expressed as mean ± SD. Unpaired ANOVA and *t* test were used to compare the values. $p < 0.05$ was considered as statistically significant.

Results

Artesunate decreased the mortality of mice

In the control group, two mice (40%) were dead at 28 weeks old. In the two treatment groups, all mice survived until 32 weeks when they were sacrificed.

Artesunate treatment increased body weights

The body weights of mice of the three groups were compared. In the control group, body weights increased very slowly after 24 weeks old. In the artesunate and CTX treatment groups, the body weights steadily increased in accordance to the time. At 32 weeks old, the mean weight of mice in the artesunate treated group (36.33 ± 5.48 mg) was significantly higher than the control group (32.80 ± 3.00 mg) ($p < 0.05$). There was no significant difference between CTX treated and the control group.

Artesunate treatment decreased the titer of serum ANA and anti-dsDNA antibodies

The titers of ANA and anti-dsDNA antibodies in the sera of 28-week-old mice were determined. Although the mean titer of ANA and anti-dsDNA antibodies in artesunate and CTX treatment groups were slightly lower than that in the control group, the difference was not statistically significant ($p = 0.23$, and $p = 0.15$) (Figure 1).

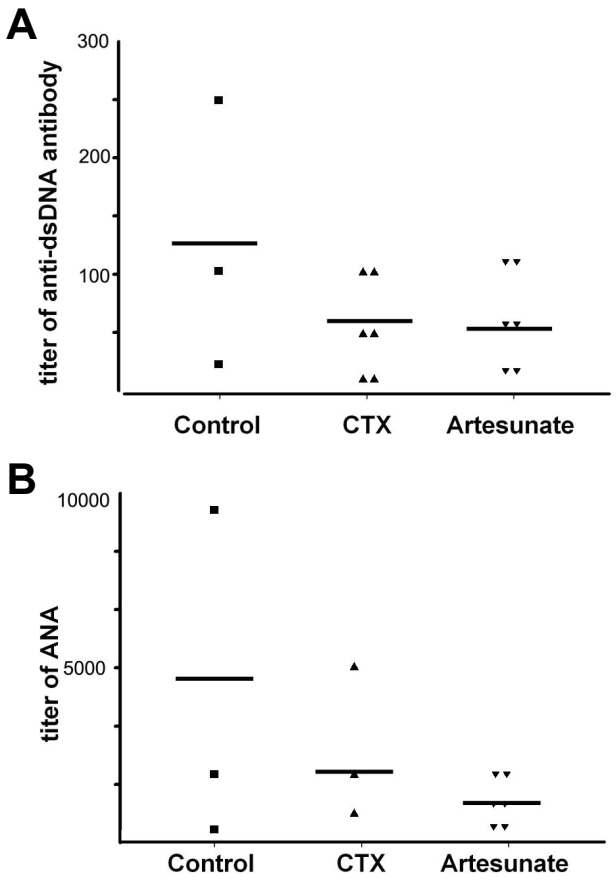

Figure 1. Artesunate and CTX decreased the titer of serum ANA and anti-dsDNA antibodies in MRL/lpr mice. The titer of the two antibodies in the serum of 28 weeks old mice was detected by indirect immunofluorescence method. ■, ▲ and ▼ denote the titer of the antibody in the mice of control, CTX and artesunate groups, respectively.

Artesunate reduced the amount of protein uria

The concentrations of urine protein in mice of the three groups increased with age. In the early stage of treatment the

Table 2. Changes of serum creatinine and blood counts in 32 weeks old mice

Group	Cr (umol/L)	WBC ($\times 10^9$/L)	Hb (g/L)	Plt ($\times 10^9$/L)
Control	20.8 ± 5.1	4.1 ± 0.7	156.4 ± 15.1	643.8 ± 71.3
Artesunate	15.9 ± 2.4*	7.0 ± 0.4*	178.6 ± 15.3	749.0 ± 60.9
CTX	16.0 ± 3.3*	4.4 ± 1.0	153.4 ± 13.6	659.2 ± 68.6

*$p < 0.05$ *vs* control.

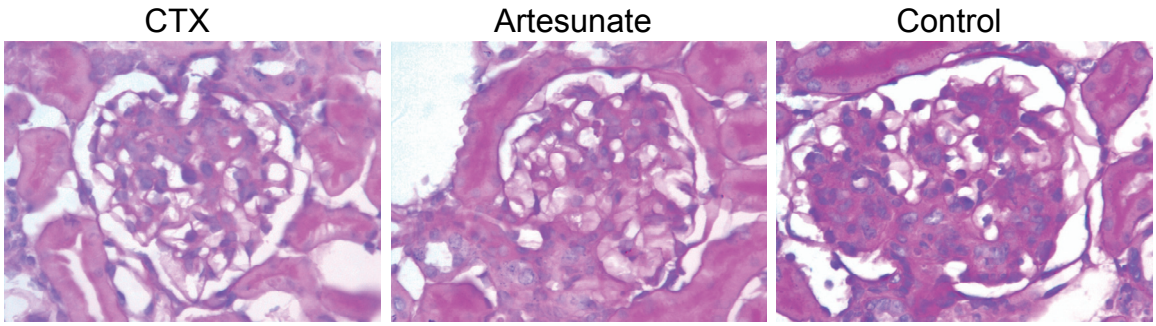

Figure 2. Artesunate improved the renal pathology in 32 weeks old mice. At 32 weeks, the CTX or artesunate treated MRL/lpr mice were anesthetized by ketamine injection. The kidney sections were stained with PAS for microscopic pathological diagnosis. (×400).

urine protein increased more slowlier in the artesunate and CTX treatment groups than in the control group. However, proteinuria in the CTX treatment group increased in an accelerated way after 24 weeks, when the treatment was stopped. At 32 weeks, there was no significant difference in the levels of proteinuria between CTX treated and control groups. In the artesunate group the level of proteinuria was significantly lower than that of the control group ($p < 0.01$) and also CTX treatment group ($p < 0.05$) (Table 1).

Artesunate decreased serum creatinine and increased blood leukocyte count

In 32-week-old mice, the concentration of serum creatinine was much lower in the artesunate treatment group than that in the control group ($p < 0.05$). The number of leucocytes in peripheral blood was much higher in the artesunate treatment group than that in the control group ($p < 0.05$). There were no statistically significant differences in the amount of hemoglobin (Hb) and platelet (Plt) counts among the various

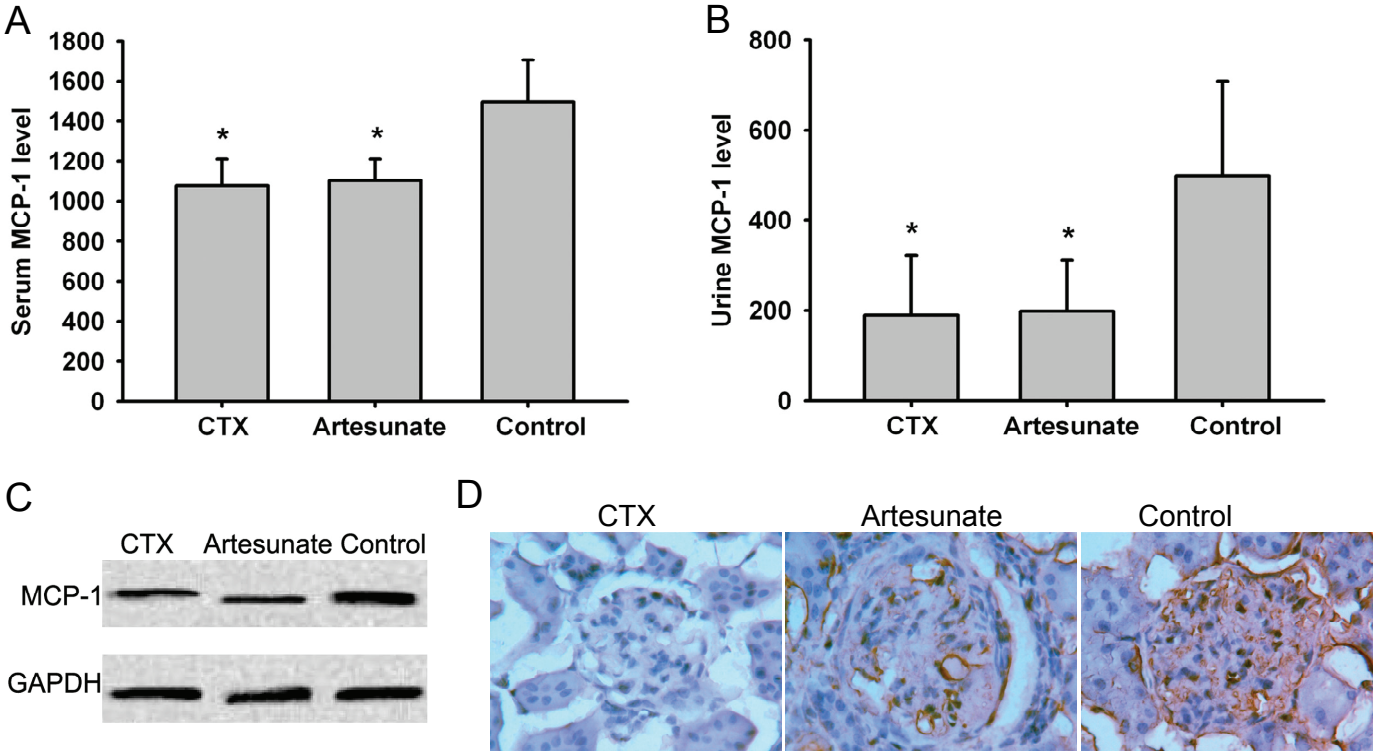

Figure 3. Artesunate and CTX reduced the level of MCP-1 in MRL/lpr mice. The MCP-1 levels of serum (A) or urine (B) in CTX or artesunate treated mice were measured by ELISA. (C) The expression level of MCP-1 in kidney of CTX or artesunate treated mice was assayed by Western blot. GAPDH was used as a loading control. (D) The localization of MCP-1 in the kidney of CTX or artesunate treated mice was analyzed by immunochemical staining. $^{*}p < 0.05$ *vs* the control group.

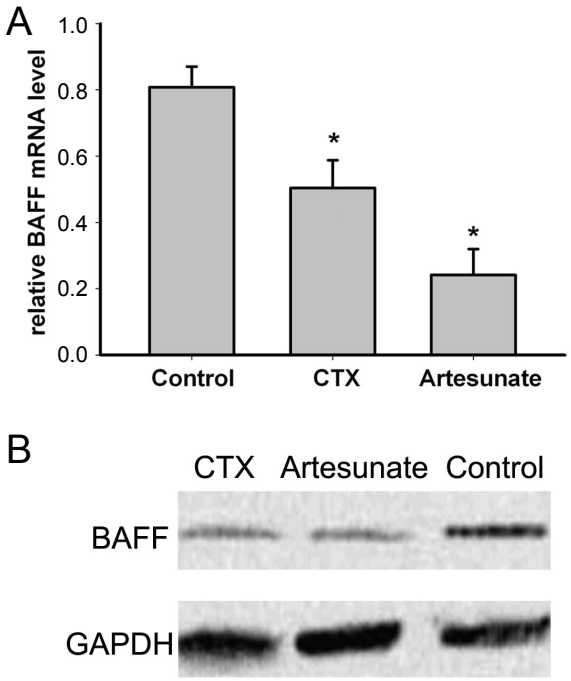

Figure 4. Artesunate and CTX reduced the mRNA and protein level of BAFF in the spleen of MRL/lpr mice. The expression of BAFF in spleen of 32 weeks old mice was determined by real-time PCR (A) or Western blot (B). β-actin (PCR) and GAPDH (Western blot) were used as a loading control. $^{*}p < 0.05$ *vs* the control group.

three groups. CTX had no effect on the level of serum creatinine or on the blood cell counts (Table 2).

Artesunate improved the histopathologic changes of glomerulonephritis

In the control group, glomerular mesangial cell proliferation, matrix deposition, capillary vessel hyperplasia and capillary vessel loops with thrombosis were found in 32-week-old mice. Crescent formation and significant lymphocytes infiltration appeared in the renal cortex. In the artesunate treated group these changes were much less than observed in the control group. Capillary vessel loop thrombosis and crescent formation were not found. Sclerosis of glomeruli and interstitial fibrosis were also lessened. In the CTX treatment group a few crescents were found in the glomeruli and local capillary vessel loop thrombosis was seen (Figure 2).

***Artesunate reduced the level of MCP-1 in* MRL/lpr mice**

In 32-week-old mice, the concentration of serum MCP-1 in artesunate and CTX treatment groups was much lower than that of the control group ($p < 0.05$), but there was no differences between the artesunate treated and CTX treated groups (Figure 3A). As the serum, urine MCP-1 showed the similar pattern (Figure 3B). The protein level of MCP-1 in kidney was analyzed by immunoblotting. The MCP-1 protein level was significantly decreased in artesunate and CTX treatment groups, compared with the control group (Figure 3C) ($p < 0.05$). Immunochemical staining indicated that the MCP-1 was located in glomerular mesangium, renal interstitium and renal tubule. The positive areas in artesunate and CTX treatment groups were significantly decreased than the control group (Figure 3D).

Artesunate reduced the level of BAFF in spleen

The ratios of BAFF/β-actin in 32-week-old mice of control, CTX and artesunate treatment groups were 0.808 ± 0.062, 0.504 ± 0.084 and 0.2425 ± 0.078, respectively. The mean level of BAFF in artesunate and in CTX treatment groups was lower than that in control group ($p < 0.05$). There was no significant difference in the levels of BAFF between the artesunate and the CTX treatment groups (Figure 4A). The expression of BAFF protein level in spleen was in the same tendency compared with mRNA level (Figure 4B).

Discussion

Studies have shown that arteannuin and its analogues inhibit the progression of LN in BXSB mice (16). Our previous studies have confirmed the therapeutic efficacy of artesunate in MRL/lpr mice, such as improving the histopathologic changes of glomerulonephritis, inhibiting the deposition of C3 in renal glomerulus, as well as suppressing the renal expression of VEGF (17). This pilot study adds further evidence to support a clinical trial of arteannuin in the treatment of SLE. We investigate the mechanism involved in the therapeutic efficacy of artesunate. These effects might be achieved by inhibiting B cells activation as well as down-regulating MCP-1.

Artesunate treatment decreased the mortality of MRL/lpr mice. Body weight and blood leukocyte counts usually decline with the disease progression, but artesunate-treated mice demonstrated stabilization of leukocyte counts and increase in body weights. These two indices of lupus disease activity were superior in the artesunate treated group of mice than in the CTX treated animals. The titers of anti-dsDNA antibodies and ANA in the sera were also reduced by artesunate, although the decrease was not statistically significant compared with the control group. Overall, these data suggest that artesunate treatment could inhibit the activity and progress of SLE and thereby prolong survival.

Artesunate also appears to have a renal protective effect in this MRL/lpr model of lupus nephritis. It decreases proteinuria and maintains normal serum creatinine. Previous study has shown that artesunate could significantly reduce infiltration of inflammatory cells, and suppress the proliferation of glomerular mesangial cells and the formation of glomerular crescents. MCP-1 is secreted by many intrinsic renal cells, including endothelial, mesangial, tubular epithelial and interstitial cells, in response to stimulation with proinflammatory cytokines and immune complexes (18). Anti-MCP-1 gene therapy is specifically effective for local inflammation (19). Our results indicate that artesunate could decrease the expression of MCP-1 level in blood and kidney

of MRL/lpr mice. The improved outcomes could partially explain the effect of the therapy that artesunate inhabits the pro-inflammation signaling, MCP-1 pathways activation in the kidneys.

BAFF is regarded as a key molecule to regulate the auto-antibody production. Studies have shown that the serum BAFF levels were increased in the MRL/lpr mice (20, 21). Using the blocking agent, TACI-Ig or BCMA-Ig could delay the process of SLE by inhibiting the activation of B cells (22). Our results showed that artesunate down-regulates the expression of BAFF in the spleen of MRL/lpr mice. This might contribute to the suppression of the production of ANA and anti-dsDNA antibodies.

CTX is a traditional and effective immunosuppressant to treat SLE and LN. Our research demonstrates that the efficacy of artesunate is comparable to that of CTX. Additionally, the safety of artesunate appears to be greater than that of CTX. For example, leukocyte counts decreased after CTX treatment. The diminished leucocytes could be complicated by serious infection, which is the main cause of lupus-related death. In contrast, our results show that artesunate significantly elevated the number of blood leucocytes. These results suggest that artesunate has not obvious hematologic side-effects.

In conclusion, this pilot study suggests that artesunate may be effective for the treatment of lupus and LN. Artesunate lengthened the life span, raised the body weight, increased the number of blood leucocytes, decreased the titer of serum ANA and anti-dsDNA antibody, and reduced the concentration of urine protein and serum creatinine. These effects of artesunate might be achieved in part by decreasing the inflammation in the kidney and inhibiting B cells activation as well as down-regulating MCP-1, the major pro-inflammation cytokine. Further studies with a larger number of animals in each treatment group will be required to confirm the efficacy and mechanism of action of artesunate, as to provide evidence for its potential clinical application for human disease in the future.

Acknowledgments

The study was supported by grants from Chinese National Natural Science Foundation (No. 30972736), Chinese National 115 Supporting Program (2008BAI59B02), Jiangsu Province 135 Talent Foundation (RC2007002); Jiangsu Province Natural Science Foundation (No. 09KJB320010) and The Six Projects Sponsoring Talent Summits of Jiangsu Province.

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