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ORIGINAL ARTICLE

Salvianolic acid A alleviates renal injury in systemic lupus erythematosus induced by pristane in BALB/c mice

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KEY WORDS

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SLE;
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Abstract The purpose of this study was to investigate the effects of salvianolic acid A (SAA) in systemic lupus erythematosus (SLE) induced by pristane in BALB/c mice. Lupus mice were established by confirming elevated levels of autoantibodies and IL-6 after intraperitoneal injection of pristane. Mice were then treated with daily oral doses of SAA for 5 months in parallel with mice treated with prednisone and aspirin as positive controls. The levels of autoantibodies were monitored at monthly intervals and nephritic symptoms observed by hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) staining. Western blot analysis of renal tissue was also employed. SAA treatment caused a significant reduction in the levels of anti-Sm autoantibodies and reduced renal histopathological changes and pathological effects. SAA treatment also significantly inhibited the phosphorylation of IKK, IκB and NFκB in renal tissues of lupus mice. In conclusion, the results suggest that SAA alleviates renal injury in pristane-induced SLE in BALB/c mice through inhibition of phosphorylation of IKK, IκB and NFκB.

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1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the appearance of autoantibodies which react with many organs and systems, including the skin, kidneys, heart, blood vessels, and nervous system. The pathogenesis involves a complicated interaction between genetic and environmental factors and the innate and acquired immune systems[1–3]. Although survival of SLE patients has improved remarkably over the last few decades, the accumulation of disease- and therapy-related complications continues to place an enormous economic burden on both patients and society[4].

The course of SLE is not entirely clear with periods of flares interspersed with periods of less severe disease[5]. Renal damage is one of the most common and serious consequences of SLE[6] with lupus nephritis causing significant morbidity and mortality if left untreated[7]. In fact, the Lupus Foundation of America[4] has established that morbidity in female patients exceeds 90%, most of whom range in age from 15 to 45. Protecting the kidneys is therefore an important factor in the pharmacotherapy of SLE.

As an animal model of SLE, pristane-injected mice broadly simulate the clinical symptoms of the disease and have been used to investigate the effects of potential pharmacotherapies. In 1984, a depside of D-(+)-β-(3,4-dihydroxyphenyl)-lactic acid and 2-(3,4-dihydroxyphenylethenyl)-caffeic acid named salvianolic acid A (SAA, Fig. 1), was isolated from the dried roots of *Salvia miltiorrhiza* Bge.[8]. It was confirmed to scavenge lipid free radicals and inhibit lipid peroxidation as effectively as vitamin E[9]. More importantly, it has been shown to protect the myocardium[10,11], blood vessels[12,13] and injured hepatocytes[14]. Due to these multiple pharmacological effects, we sought to evaluate its ability to decrease the levels of serum autoantibodies and protect the kidneys in lupus induced by pristane in BALB/c mice.

2. Materials and methods

2.1. Animals

Sixty female BALB/c mice (age 2 months, weight 18 ± 2 g) were obtained from Vital River Laboratories, Beijing, China. The animals were housed in plastic cages under controlled humidity and temperature and exposed to a 12-h/12-h light/dark cycle with free access to purified water and a standard diet. All experimental protocols in this study were approved by the Laboratories Institutional Animal Care and Use Committee of the Chinese Academy of Medical Science and Peking Union Medical College.

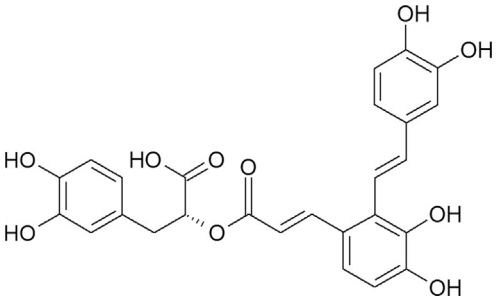

Figure 1 Chemical structure of Salvianolic acid A.

2.2. Materials

Materials [suppliers] were as follows: SAA (purity 98%) [Department of Medicinal Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College]; aspirin, 2,6,10,14-tetramethylpentadecane (pristane), calf thymus DNA (dsDNA), histone H1 human antigen (histone) and bovine serum albumin (BSA) [Sigma–Aldrich Co., St. Louis, MO, USA]; prednisone [National Institutes for Food and Drug Control, Beijing, China]; native bovine Sm antigen (Sm) [Raybiotech Inc., Norcross, GA, USA]; mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit and mouse IgG total ELISA kits [Affymetrix Ebioscience Inc., San Diego, CA, USA]; bicinchoninic acid kit for protein determination [Applygen Technologies Inc., Beijing, China]; antibodies against phosphorylated inhibitor of nuclear factor kappa-B kinase (p-IKK), phosphorylated inhibitor of NFκB (p-IκB) and phosphorylated nuclear factor κB (p-NFκB), GAPDH and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate [Cell Signaling Technology Inc., Beverly, USA]; horse anti-mouse IgG (biotin conjugate), goat anti-mouse IgM (biotin conjugate) and streptavidin (HRP conjugate) [Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China]. All other reagents were analytical grade and used as received.

2.3. Experimental protocol

SAA, prednisone and aspirin were administered to animals as saline solutions. A total of 60 female BALB/c mice were randomly divided into five equal groups: control, model, SAA, prednisone and aspirin ($n = 12$ per group). On the first day, an intraperitoneal (i.p.) injection of 0.5 mL normal saline (NS) was administered to the control group, and an i.p. injection of 0.5 mL pristane (0.783 g/mL at 20 °C) administered to all other groups. Mice in the control and model groups were given NS each day by gavage while mice in the SAA, prednisone and aspirin groups were administered SAA (5 mg/kg/d), prednisone (5 mg/kg/d) and aspirin (300 mg/kg/d) by gavage. The latter two groups served as positive controls. Blood samples were collected from the tail vein before treatment (0) and 1, 2, 3, 4, and 5 months after treatment and serum analysed for the levels of autoantibodies. After collecting the last sample, all mice were euthanized and kidneys removed for pathological examination and Western blotting.

2.4. ELISA for autoantibodies

Anti-dsDNA, histone and Sm autoantibodies (IgG or IgM) were investigated by ELISA as described recently[15]. ELISA plates separately coated with 5 μg/mL dsDNA, 0.5 μg/mL histone or 0.5 μg/mL Sm antigens were incubated overnight at 4 °C. The following day, plates were washed with PBS–Tween-20 and incubated with blocking buffer (PBS–2% BSA) for 2 h at 37 °C. They were then incubated for 3 h at 25 °C with murine serum diluted 1:2000, 1:200 and 1:400 after which plates were washed again and incubated for 1 h at 37 °C with biotin-conjugated horse-antimouse IgG or biotin-conjugated goat-antimouse IgM antibodies diluted 1:1000 with 2% BSA. Subsequently, plates were washed and incubated for 0.5 h at 37 °C with horseradish peroxidase–labeled streptavidin diluted 1:1000 with 2% BSA.

The plates were then washed again and after adding 50 μL of substrate (TMB) to the wells were maintained for 30 min at 25 °C. The reaction was terminated by adding 50 μL 2 mol/L H_2SO_4 to each well and absorbance read at 450 nm using a microplate reader. The mean enzyme indices (EI) of the samples in the different groups were calculated as:

$$\text{EI}\ (\%) = \text{OD}_{450}\ \text{of samples}/(\text{Mean}\ \text{OD}_{450}\ \text{of control mice} + 3 \times \text{SD}) \times 100$$

2.5. *ELISA analysis for IL-6 and total IgG*

The concentrations of interleukin-6 (IL-6) and total IgG in the supernatant of serum were measured using appropriate commercial ELISA analysis for the murine form of IL-6 and total IgG, according to manufacturer's instructions. The intensity of each sample was read at 450 nm in a microplate spectrophotometer.

2.6. *Histopathological analysis*

Kidneys were fixed for 24 h in 4% formalin prior to paraffin embedding. Sections were stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) and then examined under a Nikon inverted microscope (ECLIPSE Ti-U) for the presence of glomerulonephritis, renal tubular lesions and interstitial inflammation.

2.7. *Western blotting*

Kidney tissue was homogenized in ice-cold RIPA cell lysis buffer and, after centrifugation, soluble proteins were quantified using the bicinchoninic acid (BCA) protein assay as described previously[16]. Phosphorylated levels of IKK, IκB and NFκB were determined by Western blotting after mixing with loading buffer and boiling for 10 min. Bands were quantified by Quantity One software (Bio-Rad, Richmond, CA, USA) and normalized to GAPDH as an internal control.

2.8. *Statistical analysis*

Results are expressed as mean ± SEM. The significance of differences between groups was determined by unpaired *t* test or one-way ANOVA followed by Dunnett's multiple comparison test. $P < 0.05$ was regarded as statistically significant. Figures in this article are depicted using GraphPad Prism6 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. *Pristane-induced lupus mice*

To confirm the establishment of pristane-induced lupus, serum autoantibodies and IL-6 were measured by ELISA. On day 30, the levels of anti-dsDNA, anti-Sm autoantibodies, IL-6, and total IgG in pristane injected groups were notably higher than corresponding levels in the control group (Fig. 2). Mice were considered positive for lupus if EI > 100% for anti-dsDNA and Sm autoantibodies (IgG or IgM). Taking the concentrations of IL-6 and total IgG as auxiliary reference, mice with EI < 100% (including IgG and IgM) were rejected. The lupus positive mice were randomly divided into the model, SAA, prednisone and aspirin groups.

On day 15 after administration of pristane, depletion of facial hair (Fig. 3A) and abdominal trichomadesis (Fig. 3C) were observed in the model group, but not in the control group. Finally, when mice were sacrificed, lipogranuloma (Fig. 3E) was observed in the peritoneal cavity of the model group but not in the control group (Fig. 3B, D and F).

3.2. *Serum autoantibody levels*

SLE is characterized by a large and increasing array of auto-antibodies to cellular constituents[17]. The levels of anti-dsDNA, anti-histone and anti-Sm autoantibodies (IgG and IgM) in the

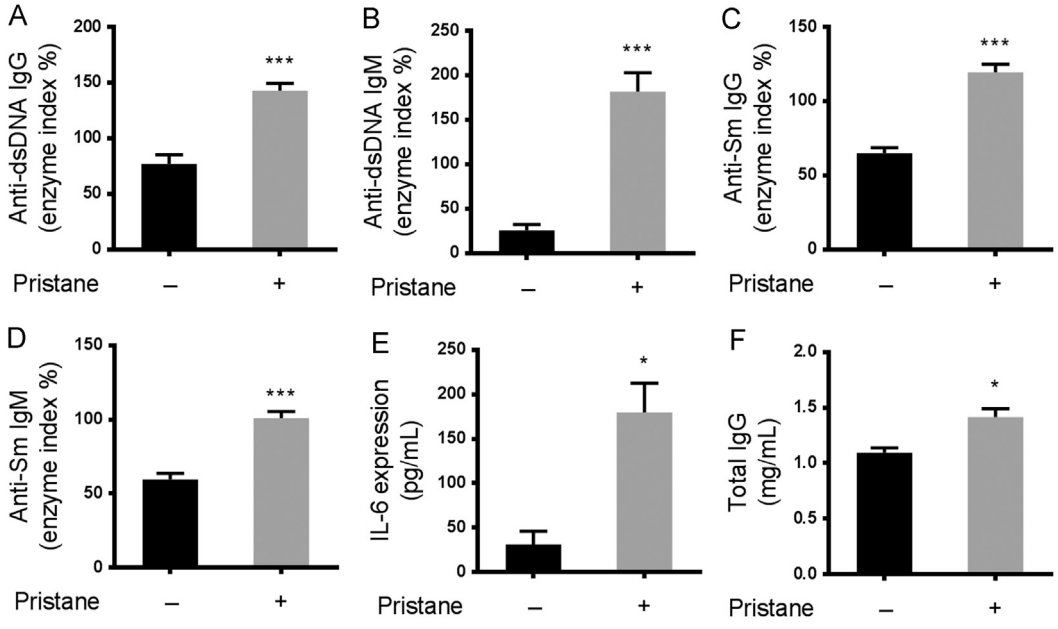

Figure 2 Peripheral blood was collected from BALB/c mice on day 30 after intraperitoneal injection of pristane (+) or NS (–) and the levels of (A) dsDNA-IgG; (B) dsDNA-IgM; (C) Sm-IgG; (D) Sm-IgM; (E) IL-6 and (F) total IgG determined by ELISA. Values are means ± SEM, $^{*}P < 0.05$, $^{***}P < 0.001$ *vs*. the control group.

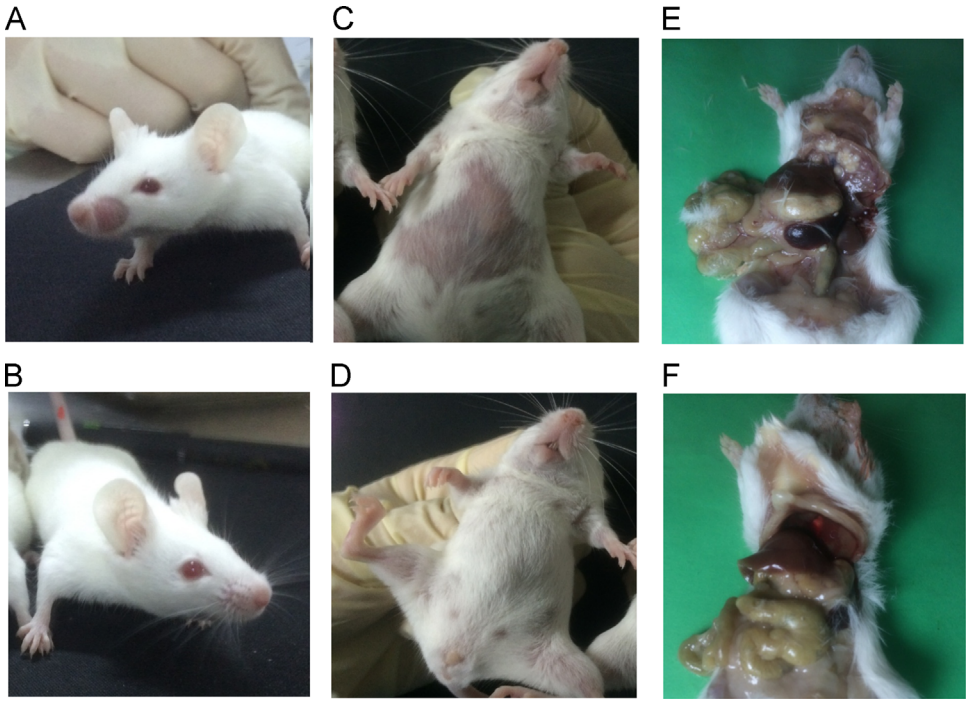

Figure 3 Appearance of BALB/c mice on day 30 after intraperitoneal injection of pristane to the model group showing (A) depilation of facial hair; (C) abdominal trichomadesis; (E) lipogranuloma in the peritoneal cavity. Appearance of BALB/c mice on day 30 after intraperitoneal injection of NS to the control group showing (B) normal hair on the face; (D) normal hair on the abdomen; (F) the normal peritoneal cavity without foreign matter.

pristane-injected groups were all significantly different from the level in the control group. The concentration of total IgG was clearly elevated at month 1 (Fig. 4).

The levels of anti-dsDNA IgG autoantibodies in pristane-injected groups decreased at month 1, reached a peak at month 2 and then declined (Fig. 4A). The levels of anti-dsDNA IgM autoantibodies decreased from the initial level and subsequently remained at a low level. However, in the SAA group, levels of anti-dsDNA IgG and IgM autoantibodies were not significantly different from those in the model group.

The levels of anti-histone IgG autoantibodies began to increase in the pristane-injected groups, reached a peak at month 1 and then declined (Fig. 4B) Anti-histone IgM levels also peaked at month 1 then decreased at month 2 before increasing slightly over subsequent months. Statistical analysis showed that at months 2 and 5 the levels of anti-histone IgG in the SAA treated group were significantly lower than in the model group. The data also show that SAA treatment decreased the level of anti-histone-IgM autoantibodies after month 2.

The levels of anti-Sm IgG autoantibodies increased after administration of pristane, reached a peak at month 1, decreased until month 3 and then increased again (Fig. 4C). The levels of anti-Sm IgM autoantibodies decreased at month 1 and then increased until reaching a plateau at month 3. The levels of anti-Sm IgG autoantibodies in the SAA treated group were significantly different from those in the model group at months 1, 4 and 5 and levels of anti-Sm IgM autoantibodies were clearly lower at months 2 and 5. The data show that SAA treatment continued to reduce the levels of anti-Sm IgG and IgM autoantibodies through to the last stage.

The concentrations of total IgG in pristane-injected groups were increased at month 1 and reached a peak at month 4 (Fig. 4D). The results showed that total IgG was obviously decreased by SAA treatment at month 3 and reached its lowest level at month 4.

3.3. *Renal histopathology*

As shown by H&E and PAS staining, pristane-induced lupus resulted in immunocomplex-mediated glomerulonephritis. In the control group, the size and cell counts of glomeruli were normal and the glomerular capillary wall was thin (Fig. 5A and F). Histologic sections from the model group exhibited severe renal damage characterized by mesangial broadening and increasing cell layers, glomerular atrophy with capillary dilation and thickening of capillary walls (Fig. 5B), basement membrane thickening, mesangial matrix expansion, and dilation of renal tubules (Fig. 5G). These renal histopathological changes were diminished in the SAA treatment group, particularly the glomerular lesions (Fig. 5C and H). Similarly, in both the prednisone and aspirin treated groups, renal pathological changes were significantly less (Fig. 5D–E and I–J).

3.4. *Phosphorylation of IKK, IκB and NFκB in kidney*

Because SAA treatment provided good protection of kidney in lupus mice, Western blot analysis was employed to determine whether the protection was related to inhibition of the activation of IKK (Fig. 6A), IκB (Fig. 6B) and NFκB (Fig. 6C) in renal tissues. In the model group, p-IKK, p-IκB, p-NFκB were clearly activated in renal tissues but in the SAA-treated group, phosphorylation of IKK, IκB and NFκB was significantly inhibited compared with the model group.

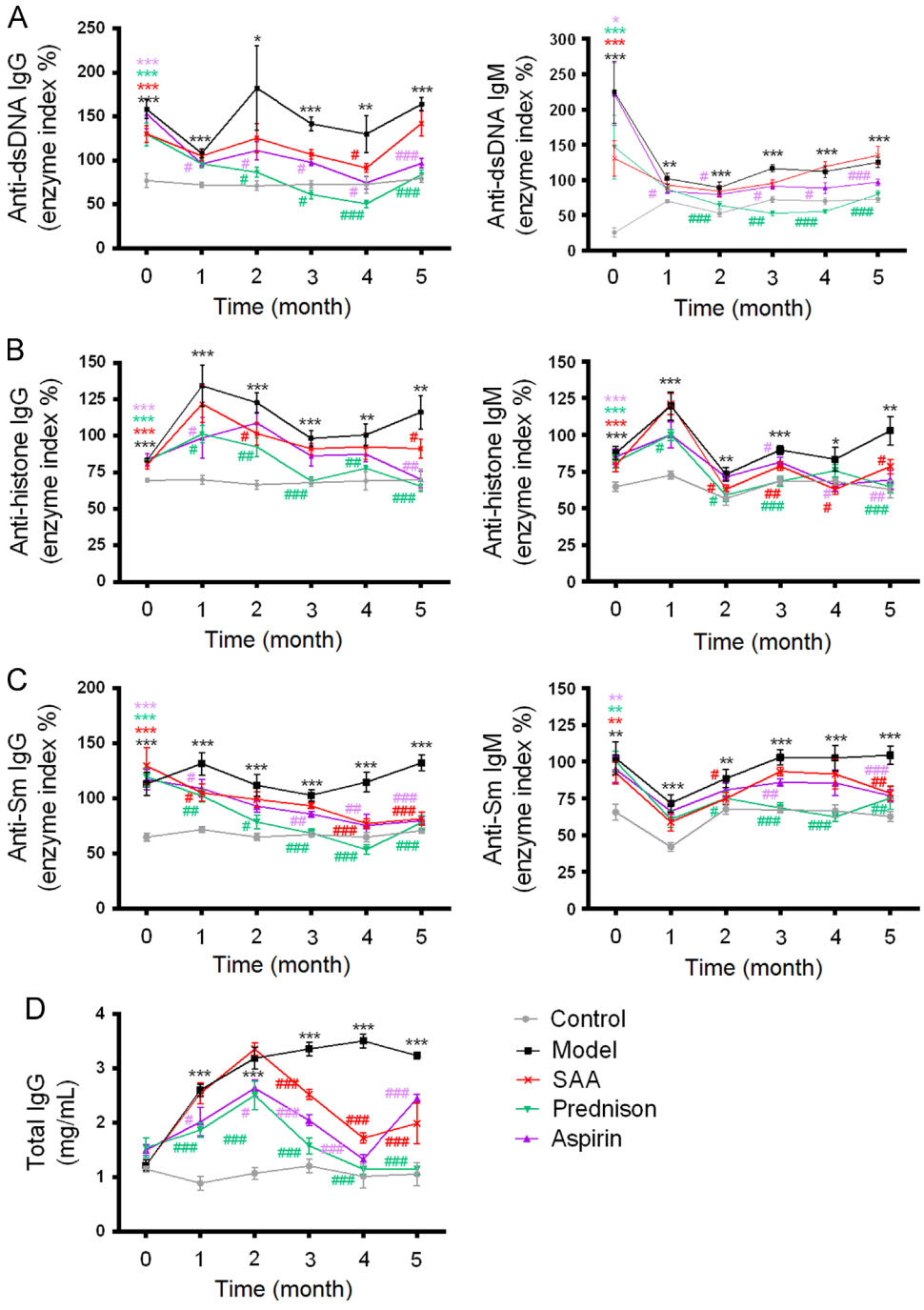

Figure 4 SAA treatment reduces serum levels of (A) anti-dsDNA; (B) anti-histone and (C) anti-Sm autoantibodies, as well as (D) total IgG in mice with pristane-induced lupus. Serum samples from mice were collected before treatment (0) and at 1, 2, 3, 4 and 5 months after treatment and the levels of autoantibodies determined by ELISA based on optical density (OD) at 450 nm. Values are means ± SEM from twelve independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* model group.

4. Discussion and conclusions

There remain many questions relating to the aetiology and pathophysiology of SLE. First, SLE is characterized by a variety of clinical manifestations and large increases in autoantibody levels that have led some to argue that it shares pathogenic mechanisms with a diverse range of diseases[18,19]. Secondly, the pathogenesis of SLE is characterized by a broad range of pathogenic events that likely reflect the heterogeneity of the disease[20]. Thirdly, the immunomodulators aspirin, corticosteroids, antimalarials and immunosuppressants have been the mainstay of pharmacotherapy for SLE but, because of the complexity of the disease and the side effects of drugs, treatment remains unsatisfactory[4,21]. Due to this reason, it has been proposed that multiple medications should be used to treat the disease and new procedures are required[3].

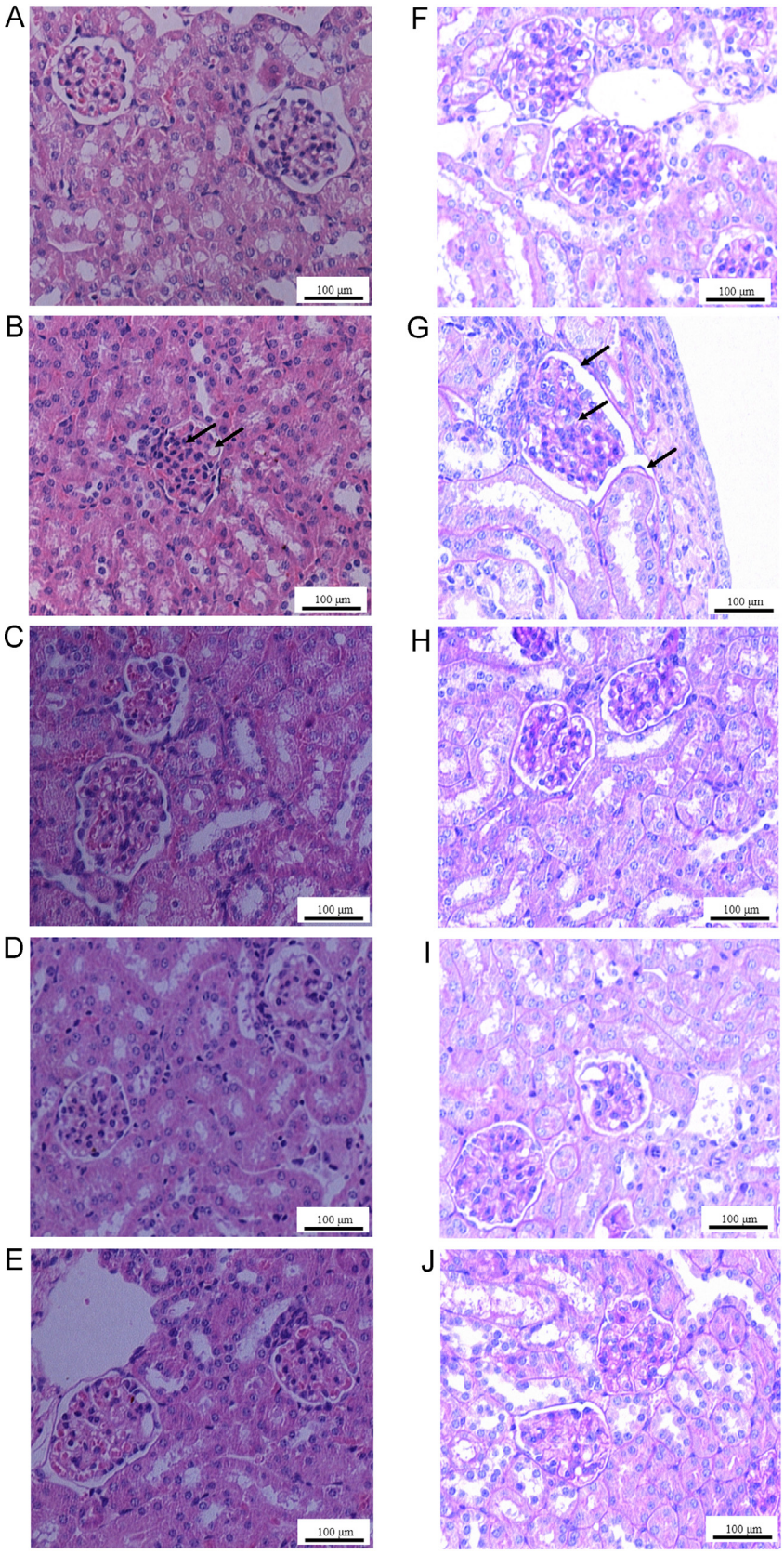

Figure 5 Histology of kidney tissue collected at the time of euthanasia stained with (A–E) H&E and (F–J) PAS. (A) and (F) control group; (B) and (G) model group; (C) and (H) SAA group; (D) and (I) prednisone group; (E) and (J) aspirin group.

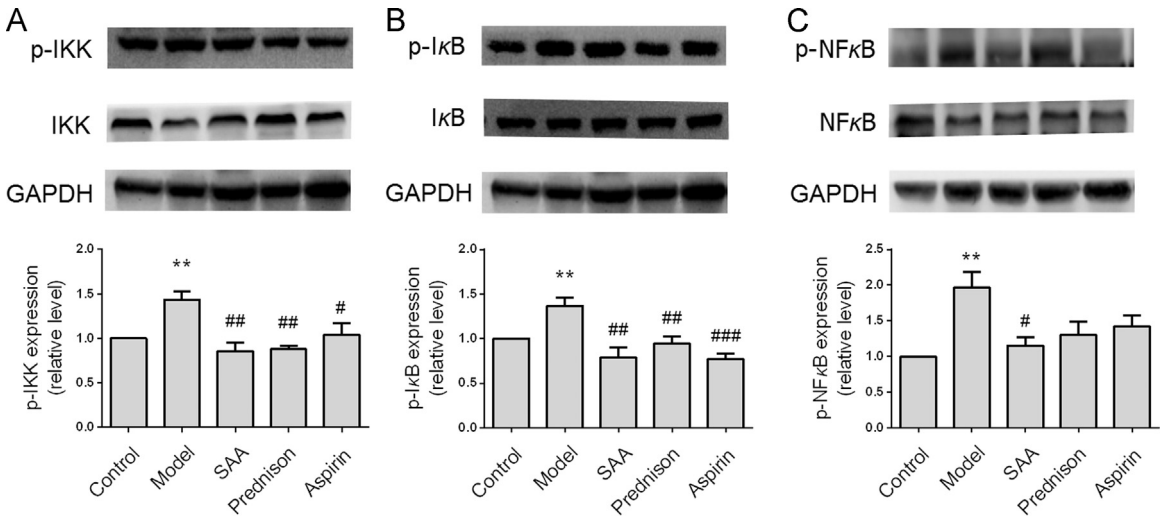

Figure 6 Chronic treatment with SAA suppresses the activation of the p-IKK, p-IκB, p-NFκB signaling pathways. Crude proteins obtained from kidney tissues were analyzed by Western blotting using specific antibodies against (A) p-IKK; (B) p-IκB; and (C) p-NFκB. Quantitative analysis was performed by densitometry using an internal control (GAPDH). Values are means ± SEM from six independent experiments. $^{**}P<0.01$ *vs.* control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* model group.

Pristane-treated mice develop clinical manifestations of SLE, including arthritis, immune complex-mediated glomerulonephritis and vasculitis, as well as increases in autoantibodies[22,23]. In this study, we consistently observed depilation of facial hair, abdominal trichomadesis and lipogranuloma in the model group and noted the depilation displayed a similar manifestation to that observed in SLE patients. Furthermore, we found increasing levels of autoantibodies and renal lesions in pristane-injected mice that comprehensively simulate clinical manifestations of the disease. On this basis, we maintain we established a reliable and useful animal model of SLE to investigate its pharmacotherapy.

SLE is associated with increased cardiovascular incidence and fatality due to premature atherosclerosis that leads to the prophylactic use of aspirin for the primary and secondary prevention of coronary heart disease[21]. Oral corticosteroids, most commonly prednisone, are the main therapeutic choice for SLE and to treat lupus nephritis generally[24,25]. However, the current drugs for SLE suffer from major reason side effects which limit their use[25,26]. In consideration of lack of satisfactory clinical drug and their different use as two types of anti-inflammatory drugs in treating SLE, mice treated with prednisone and aspirin served as positive controls in the present study.

A rise in anti-Sm autoantibodies is well recognized as the most specific feature of SLE and a criterion of the American College of Rheumatology for the diagnosis of the disease[27]. We observed SAA treatment caused a significant reduction in the level of anti-Sm autoantibodies including those of IgG and IgM and also reduced the levels of total IgG. Some studies have indicated an association between anti-Sm autoantibodies and some particular disease manifestations such as lupus nephritis[27]. Because these positive effects of SAA treatment translated into improved renal function and the attenuation of histological damage, we conducted further experiments to elucidate the mechanism of renal protection and found that SAA inhibited the levels of phosphorylation of IKK, IκB and NFκB in renal tissues. Thus we maintain that SAA plays a therapeutic role in SLE by blocking the IKK/IκB/NFκB-mediated inflammatory response.

In conclusion, we have demonstrated that SAA plays a protective role against the elevations in autoantibody levels and renal lesions in a mouse model of SLE. Furthermore we have shown that the renal protection is mediated by inhibiting the IKK/IκB/NFκB signaling pathway. However, given that SAA exerts multiple pharmacological effects, we anticipate it can alleviate other debilitating symptoms of SLE and further research is needed to confirm if this is the case.

Acknowledgments

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