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ORIGINAL ARTICLE

Reno-Protective Effect of Realgar Nanoparticles on Lupus Nephritis of MRL/Lpr Mice through STAT1

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ABSTRACT

Background: Realgar, an arsenic tetrasulfide compound, is a highly recognized traditional Chinese medicinal prescription that has been widely used to treat various diseases such as inflammatory diseases. However, there are still some problems in the clinical treatment of Realgar, such as large oral dose and high potential toxicity. **Objective:** To evaluate effects of Realgar nanoparticles on lupus nephritis (LN) *in vivo* in MRL/lpr mice. **Methods:** Ten-week mice were orally administered every day for eight consecutive weeks except the mice of normal model groups. The serum levels of anti-ds-DNA antibody IgG, IgM, IFN-γ, Creatinine (Cr), and blood urea nitrogen (BUN) were determined, and 24-hour urine protein was also measured. Renal inflammatory pathology analysis was assessed by hematoxylin-eosin (H&E) staining. The expression of phosphorylated signal transducer and activator of transcription 1 (*p*-STAT 1) and Janus Kinase 1 (JAK 1) in kidney tissue was determined by direct reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC). **Results:** The mice treated with Realgar nanoparticle in the high dose-treated (Realgar HD, 0.03 g/kg/d) group exhibited significantly reduced serum levels of anti-dsDNA ($p<0.01$), IgG ($p<0.01$), IgM ($p<0.01$), BUN ($p<0.01$), Cr ($p<0.01$), and inflammatory cytokine IFN-γ ($p<0.01$) as well as proteinuria ($p<0.01$) compared to the untreated model MRL/lpr mice. Additionally, high doses of Realgar nanoparticles significantly suppressed the phosphorylations of STAT 1 ($p<0.01$) and the renal pathological changes. **Conclusions:** The study indicates that Realgar nanoparticles may be a potential agent to treat LN, and the down-regulated *p*-STAT1 expression suggests that it may be one of the LN treatment targets for Realgar nanoparticles.

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Keywords: JAK1/STAT1 Signaling Pathway, Lupus Nephritis, MRL/lpr Mice, *p*-STAT 1, Realgar Nanoparticle

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune chronic inflammatory connective tissue disease. SLE is more common in young women, and its etiology has not been clarified (1). Currently, it is argued that the pathogenesis is not only an inherent factor such as genetics and sex hormones, but also a factor related to the environment and drugs. Its clinical expression is complex and diversiform so that it can encroach upon each viscera of the whole body. Most of the patients exhibited insidious onset and began to show some symptoms such as mild arthritis, rash, insidious nephritis, and thrombocytopenic purpura. Some patients were stable in the subclinical state or mild lupus for a long time, some patients could suddenly change from light to severe lupus, and more patients gradually showed multiple system damage from light. There were also some patients who were involved in multiple systems and even exhibited lupus crisis (2). Lupus nephritis (LN) is one of SLE diseases with visceral damage, and renal damage is one of the most common clinical manifestations of SLE. According to statistics, the incidence of renal damage with confirmed diagnosis of SLE was 24.24%, 72.4% two years later, and 92.3% four years later (3,4). Lupus nephritis is one of the leading causes of death in SLE. In addition to the systemic manifestations of SLE, the main clinical manifestations are hematuria, proteinuria, and renal insufficiency. At present, the drugs for lupus nephritis are mainly glucocorticoid and immunosuppressive agents, with adverse side effects and easy dependence (5,6). Therefore, we hope to develop a new drug with therapeutic or protective effects on patients with LN in clinical treatment and experiment. Realgar, an arsenic tetrasulfide compound, is a highly recognized traditional Chinese medicinal prescription that has been widely used to treat various diseases (such as rheumatism) for 1,500 years. However, there are still some problems in the clinical treatment of realgar, such as high oral dose, and high potential toxicity. Accordingly, the use of nanotechnology to solve its insolubleness, improves its bioavailability to reduce toxicity and side effects, being one of the hotspots of realgar research. In the early stage, we prepared with an average particle size of, 100 nm of realgar nanoparticles by high energy ball milling, and found that some of their characteristics may reduce the difference between *in vivo* and *in vitro* to achieve good therapeutic effects. Therefore, conducting further studies in this regard is worthwhile (7). In addition, our preliminary work has found that realgar nanoparticles can prolong MRL/lpr mice survival time (8). Accordingly, the main purpose of our study is to explore the potential protective effect of realgar nanoparticles on renal inflammation and the pathological and inhibitory effect of nano-realgar on inflammatory response in LN MRL/lpr mice. To the best of our knowledge, there is no research on the mechanism of the protective effect of nano-realgar on lupus nephritis *in vivo*. Thus, this experiment aims to explore the potential protective mechanism of nano-realgar on lupus nephritis.

MATERIALS AND METHODS

Animals. Female MRL/lpr mice (age: 10 weeks old; weight: 23.5 ± 2.0 g) and sex- and age-matched WT C57BL/6 mice (weight: 21.5 ± 1.0 g) were provided by the Model Animal Research Center of JiangXi University of Traditional Chinese Medicine (NanChang, China). The experimental animals were placed in a special pathogen-free environment (24 ± 1°C, 50 ± 5% relative humidity and normal 12-h light/12-h dark cycle)

in the Animal Experiment Center of JiangXi University of Traditional Chinese Medicine. Rats were free and consumed standard food, and disinfectant was provided by the factory. All the mice were kept in controlled conditions for one week before the experiment. All the study experiments were conducted with the approval of the Animal Experiment Ethics Committee of Jiangxi University of Traditional Chinese Medicine (JXUTCM, Protocol Number: 2016043) and in line with the guidelines of the National Institute of Health (National Research Council of USA, 1996). Our test program conformed to the approved criteria.

Preparation Methods of Realgar Nanoparticles. Realgar nanoparticles were prepared using high-energy ball milling, and the average particle size of 88 ± 8.3 nm was obtained through the optimized preparation process. For detailed preparation methods, refer to Tian *et al.* (8). The chemical basis of realgar nanoparticles was neutral, and it was hydrophobic. Realgar nanoparticles were prepared with an average particle size of 100 nm and the LD50 of nanoparticles was 22 $g.kg^{-1}$. Realgar nanoparticles were prepared into suspension with olive oil. The doses of realgar nanoparticles were determined based on the result of previous experiments.

Experimental Design. After 1 week acclimatization, twelve WT C57BL/6 mice were used as control group (10 ml/kg, 0.9 % saline). 48 MRL/lpr mice were randomly divided into 4 groups, including group A: Model (10 ml/kg, 0.9 % saline), group B: Prednisone (Beijing Union Pharmaceutical Company, China) treated (5 $mg.kg^{-1}/d$, positive contro1), group C: Treated with realgar nanoparticles at a high dose (Realgar HD, 0.03 g/kg/d), group D: Treated with realgar nanoparticles at a low dose (Realgar LD, 0.01 g/kg/d), each experimental group consisted of 12 mice. Furthermore, the doses of realgar nanoparticles were determined based on the result of previous experiments (7). The experimental protocols used in the present study were approved by the Animal Ethical Committee of Jiangxi University of Traditional Chinese Medicine. All the mice were continuously administrated for 8 weeks by gavage with the same volumes (0.2 ml). All the mice were killed by the cervical dislocation method, and the kidney tissues of the fresh mice were aseptically isolated on the day after the last intragastric administration. The amount of 24 h proteinuria were respectively obtained from mice using metabolic cages, and was assessed at weeks of 0, 4 and 8 respectively. Serum was obtained by the centrifuge at 3000 rpm for 10 min at 4°C, and stored at -20°C before use. Renal tissues were fixed in 4% neutral-buffered formalin and embedded in paraffin for histopathological and immunohistochemistry (IHC) analysis.

Measurement of Renal Function. The level of serum BUN and Cr were measured in 8 weeks using the commercial ELISA kit (Jiancheng Institute of Biological Engineering, Nanjing city, Jiangsu province, China). The test was conducted according to the reagent specification, and the relevant operation steps were performed strictly. The content was calculated as mg/dL.

Measurement of the Urinary Protein. To investigate the protective effects of realgar nanoparticles on the progression of renal disease, one time per four weeks and with minimal animal disturbance, the mice were individually transferred to a steel grid inserted within a sterilized plastic mouse cage, without feed or water, for two hours, during which a few drops of urine were voluntarily released on to the cage floor. Urine collection commenced in late morning, with the prospect of sufficient time for urination (100 - 200 µl, then frozen at -20°C). The collected urine samples were centrifuged at 3000 rpm for 5 min to remove all the particulates, then the supernatant was collected and frozen at -

20°C before use. The 24-hour urinary protein was measured by the BCA protein assay kit (Zhongshan Institute of Biotechnology, Beijing, China).

Measurement of Inflammatory Cytokines in Peripheral Blood. Serum IFN-γ was detected using the commercial ELISA kit (Jiancheng Institute of Biological Engineering, Nanjing city, Jiangsu province, China) based on the manufacturer's instructions. The test was conducted according to the reagent specification, and the relevant operation steps were performed strictly. The content was calculated as mg/dL.

Measurement of serum levels of anti-dsDNA antibody, IgG, IgM and complement C3. The levels of anti-ds-DNA antibody, IgG, and IgM (Oumeng Company, Germany) were determined using the dot immunobinding assay method according to the reagent specification. Normal mouse IgG was considered negative control. The complement C3 in serum was determined using an appropriate commercial ELISA kit (Diaclone Company, France) according to the reagent specification.

Direct Reverse Transcriptase Polymerase Chain Reaction（RT-PCR）of Messenger (M) RNA. Messenger RNA was isolated from kidneys followed by the standard protocol for the QuickPrep Micro mRNA purification kit (Qiagen, Tokyo, Japan). Sample mRNA levels were quantified by reading the absorbance at 260 nm, and 100 ng of mRNA was analyzed by RT-PCR using the Access RT-PCR System (Promega, Madison, WI, USA). The housekeeping gene encoding mouse β-actin was used as an internal control for semi-quantitative comparison to cytokine transcripts. Using PRIMER 5.0 software design and β-actin specific primers, by searching for gene sequences in dbEST and nr (the non-redundant set of GenBank, EMBL, DDBJ database sequences) databases, it was ensured that the nucleotide sequences used as primers were specific, and that there was no DNA polymorphism. The primers used for RT-PCR were as follows: JAK1 (92pb), sense 5′-CTGGTAGATGGCTACTTC-3′, antisense 5′-GGCTCATAGAGTAGACAG -3′; STAT1 (90pb), sense 5′-TTCTGGCCTI'GGATTGAC-3′, antisense 5′-TCTCAGCAGCCATGACTT-3′; β– actin (90pb), sense 5′-GTG GGC CGC TCT AGG CAC CAA-30, and antisense 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′. The reaction conditions of PCR amplification were as follows: 95℃ 3 min, 95℃ 20 s, 58℃ / 57℃ 20 s, then extended at 72℃ for 30 s, 86℃ / 85℃ 15 s, a total of 40 cycles, and finally extended at 72℃ for 7 min. After the reaction, the computer automatically drew their own standard curves, and the software automatically calculated the exact content of the target gene housekeeper gene in the sample to be tested. The ratio of β-actin content between target gene and housekeeper gene in the same sample was used as an index to evaluate the expression level of the target gene. The experiments were replicated three times in parallel. RT-PCR products were quantified by agarose gel electrophoresis and ethidium bromide, and the visible bands were detected by a UV light transmission apparatus, and the content of RNA was detected by a UV spectrophotometer. RT-PCR products were examined on 1.2% agarose gels in a 1x TAE buffer. Gels were stained with 0.5 mg/ml ethidium bromide. Densitometric analysis of stained bands was performed using the Image Master VDS (Pharmacia Biotech, Uppsala, Sweden).

Histopathological Observation. The HE staining steps were as follows. 1: Fixed, the above appropriate amount of renal tissue was fixed for 24 hours, and then rinsed with running water for 24 hours. 2: Dehydrated and embedded, 95% ALC I 4 h, II 2 h; anhydrous ALC . Xylene + anhydrous ethanol (1/1) for 20 min, Xylene : I 10 min, II 10 min; soft wax (52℃) : I 30 min, II 1 h, Xylene + anhydrous ethanol (1 / 1) for 20 min, Xylene : I 10 min / 10 min (52℃): I 30 min / min). Dipping hard wax (60℃): I 30 min, II 30 min; entrapment. 3:Sliced, repaired, cutted, expanded, pasted and baked (roasted at

55℃ and 60℃) for 5 h. 4:HE stained, Xylene dewaxed, each 5 min; 10 min absolute ethanol : I 5min, II 5 min; 95% ethanol : I 5min, II 5 min >80% ethanol 5 min; 70% ethanol 5 min; D.W. 5 min; Harris hematoxylin solution was washed with 5 min; water, 0.5% hydrochloric acid alcohol was separated for 10s, and observed under a microscope. Washed and blued 30 min; 70% ethanol 5 min; 80% ethanol 5 min; eosin solution (95% ethanol solution) 30s; 95% ethanol: I 1 min, II 5 min; anhydrous ethanol : I 5 min, II 5 min; Xylene + ethanol (1 /1) 5 min; Xylene:I 5 min, II 5 min, III 5 min; sealed with neutral gum, and then was observed under an ordinary optical microscope.

Immunohistochemistry（IHC） Analysis. The specimens were fixed with 4% neutral buffered formaldehyde and embedded in paraffin. 5μm continuous section, immunohistochemistry using the Envision two-step method, microwave thermal repair, DAB color staining, hematoxylin contrast staining, neutral gum seal with the same section. Sections of renal tissue were analyzed by immunohistochemistry using monoclonal antibodies against JAK1 (abcam, US) and p-STAT1 (abcam, US), respectively. The staining steps were performed in strict accordance with the instructions, the PBS solution was used instead of the first antibody as the negative control, and the positive film provided by the company was used as the positive control.

Statistical Analysis. The results of the measurement data were expressed by mean ± standard deviation (SD), and the differences between the two groups were compared by *t-test* and SPSS 21.0 software. Statistical analysis indicated that $p<0.05$ was statistically significant.

RESULTS

Realgar nanoparticles treatment reduced 24 h proteinuria.

Proteinuria is a major symptom indicative of the development of renal disease in MRL/lpr mice. As Table 1 shows, the amount of 24-hour proteinuria exhibited a progressive rise in the model group, compared to the WT C57BL/6 control group. While the content of 24-hour proteinuria dramatically decreased in realgar nanoparticle-treated groups in week 4, compared to the model group ($p<0.01$, Table 1). In week 8, the mice treated with realgar nanoparticles had significantly less 24-hour proteinuria than the model group mice ($p<0.01$, Table 1). Obviously, the content of 24-hour proteinuria in the realgar LD-treated mice decreased from 0.88 ± 0.09 g/24 h (week 0) to 0.31 ± 0.08 g/24 h (week 8) ($p<0.01$, Table 1), while in the realgar HD group, the content of 24-hour proteinuria was 0.84 ± 0.08 g/24h in day 0, and the number significantly declined to 0.41 ± 0.07g/24h by week 4 and continued to decline to 0.29 ± 0.09 g/24h by week 8 ($p<0.01$, Table 1). Overall, realgar nanoparticles demonstrated a significant proteinuria reduction in the progression of SLE in MRL/ lpr mice with time dependence.

Effect of Realgar nanoparticles in renal function in MRL/lpr mice.

There was a considerable reduction in renal function in the realgar nanoparticle-treated MRL/lpr mice ($p<0.01$, Table 2), particularly in the realgar HD group ($p<0.01$, Table 2). While there was also a significant decrease in the prednisone group compared to the model group ($p<0.01$, Table 2). The results demonstrated that realgar nanoparticles could improve the renal function of MRL/LPR mice.

Table 1. Realgar nanoparticles reducing 24-hour proteinuria in the serum of MRL/lpr mice（n=12, Mean ± SD).

Groups	Proteinuria（g/24h）		
	0 day	4 week	8 week
Realgar HD	0.84 ± 0.08**	0.4 1± 0.07**	0.29 ± 0.09**
RealgarLD	0.88 ± 0.09**	0.53 ± 0.07**	0.36 ± 0.06**
Prednisone	0.91 ± 0.11**	0.45 ± 0.06**	0.39 ± 0.05**
Model	0.8 7± 0.10	1.13 ± 0.17	1.03 ± 0.15
Control	0.11 ± 0.02	0.13 ± 0.02	0.12 ± 0.02

Twenty-four hour urinary protein was detected by Coomassie Brilliant Blue test at weeks 0, 4 and 8. Values are presented as mean ± SD. ** indicates p<0.01.

Table 2. Effect of Realgar nanoparticle in renal function in MRL/lpr mice (n=12, Mean ± SD).

Groups	BUN (mg/dL)	Cr (mg/dL)
Realgar HD	9.53 ± 0.47**	10.86 ± 0.91**
Realgar LD	12.63 ± 0.45**	11.65 ± 1.39**
Prednisone	13.28 ± 1.48**	12.43 ± 2.56**
Model	21.83 ± 3.43##	19.72 ± 2.53##
Control	9.81 ± 1.41	9.62 ± 1.54

Values represent the mean ± S.D. The one-way ANOVA was performed on the raw data. ##Significant difference at p<0.01 levels compared with the control group. **Significant difference at p<0.01 levels compared with the model group.

Effect of Realgar nanoparticles on the serum inflammatory cytokine.

Compared to the control group, the expression level of IFN-γ in the serum of the model group was significantly higher than that of the control group (p<0.01, Table 3). Realgar nanoparticles could significantly reduce the expression of interferon-γ (p<0.01, Table 3). Similarly, there was a significant decrease in the prednisone group (p<0.01, Table 3). The level of inflammatory cytokines in the realgar HD group was significantly lower than that in the model group (p<0.01, Table 3). There was also a significant decrease in the prednisone-treated group (p<0.01, Table 3). However, realgar HD group showed no significant decrease in inflammatory cytokines, compared to the Prednisone group (p>0.05, Table 3).

Table 3. Effect of Realgar nanoparticles on the serum inflammatory cytokine.

Groups	IFN-γ (ng/L^{-1})
Realgar HD	21.83 ± 3.43##
Realgar LD	25.83 ± 3.86##
Prednisone	21.93 ± 3.45##**
Model	38.83 ± 4.46##
Control	2.83 ± 0.46

Values represent the mean ± S.D. The one-way ANOVA was performed on the raw data. ##Significant difference at p<0.01 levels compared with the control group. **Significant difference at p>0.05.

Effect of Realgar nanoparticles on the levels of anti-ds-DNA, IgG, IgM and complement C3 in the serum.

As Table 4 shows, the anti-dsDNA antibody in serum was not detected in the control group. In other words, there was no LN changes, whereas the anti-dsDNA antibody levels in the model group mice exhibited an obvious evidence of LN ($p<0.01$, Table 4). Furthermore, realgar treatment significantly decreased the production of anti-dsDNA（$p<0.01$）, IgG, and IgM, particularly in the HD group ($p<0.01$, Table 4). Moreover, the level of the serum complement C3 in the model group was significantly higher than that in the control group ($p<0.01$, Table 4). The level of the complement C3 in the realgar HD group was significantly lower than that in the model group ($p<0.01$, Table 4).

Table 4. Effect of Realgar nanoparticles on the level of anti-ds-DNA, IgG, IgM, and C3 in serum (n=12, Mean ± SD).

Groups	ds-DNA (relative titers)	IgG/ng.L^{-1}	IgM/ng.L^{-1}	C3 (10^{-2}g.L^{-1})
Realgar HD	4.50 ± 0.40**	13.86 ± 0.91**	9.03 ± 1.27**	22.54 ± 1.67**
Realgar LD	14.50 ± 0.45**	18.65 ± 1.39**	11.23 ± 1.76**	34.56 ± 2.63**
Prednisone	12.20 ± 1.40**	13.43 ± 2.56**	9.68 ± 3.88**	23.76 ± 4.37**
Model	24.80 ± 1.40##	26.72 ± 2.53##	15.68 ± 2.75##	42.57 ± 3.26##
Control	0.80 ± 0.05	0.62 ± 0.04	5.68 ± 0.75*	1.66 ± 0.32

Values represent the mean ± S.D. The one-way ANOVA was performed on the raw data. ##Significant difference at $p<0.01$ levels compared with the control group. **Significant difference at $p<0.01$ levels compared with the model group.

Effect of Realgar nanoparticles on the gene expression spectrum of JAK1, STAT1 in renal tissue.

STAT1 gene expression spectrum was evidently decreased in the mice treated with realgar nanoparticles ($p<0.01$, $p<0.05$, Figure 1), particularly in the realgar HD group ($p<0.01$, Figure 1). Furthermore, the gene expression spectrum of STAT1 in the prednisone treated group mice reduced compared to the saline control group ($p<0.01$). However, the gene expression spectrum of JAK1 in all groups of mice did not reduce compared to the control group ($p>0.05$).

Table 5. Effect of Realgar nanoparticle on the gene expression spectrum of JAK1, STAT1 in renal tissue.

Groups	JAK1/β-action	STAT1/β-action
Realgar HD	35.68 ± 1.08*	17.74 ± 1.23**
Realgar LD	32.28 ± 2.58*	29.36 ± 2.76#
Prednisone	34.38 ± 1.58*	17.47 ± 1.69**
Model	35.38 ± 3.48	37.38 ± 3.88##
control	1.28 ± 0.08	1.48 ± 0.09

Values represent the mean ± S.D. The one-way ANOVA was performed on the raw data. ##Significant difference at $p<0.01$ levels compared with the control group.**Significant difference at $p<0.01$ levels compared with the model group. *Significant difference at $p>0.05$ levels compared with the model group. # Significant difference at $p<0.05$ levels compared with the model group.

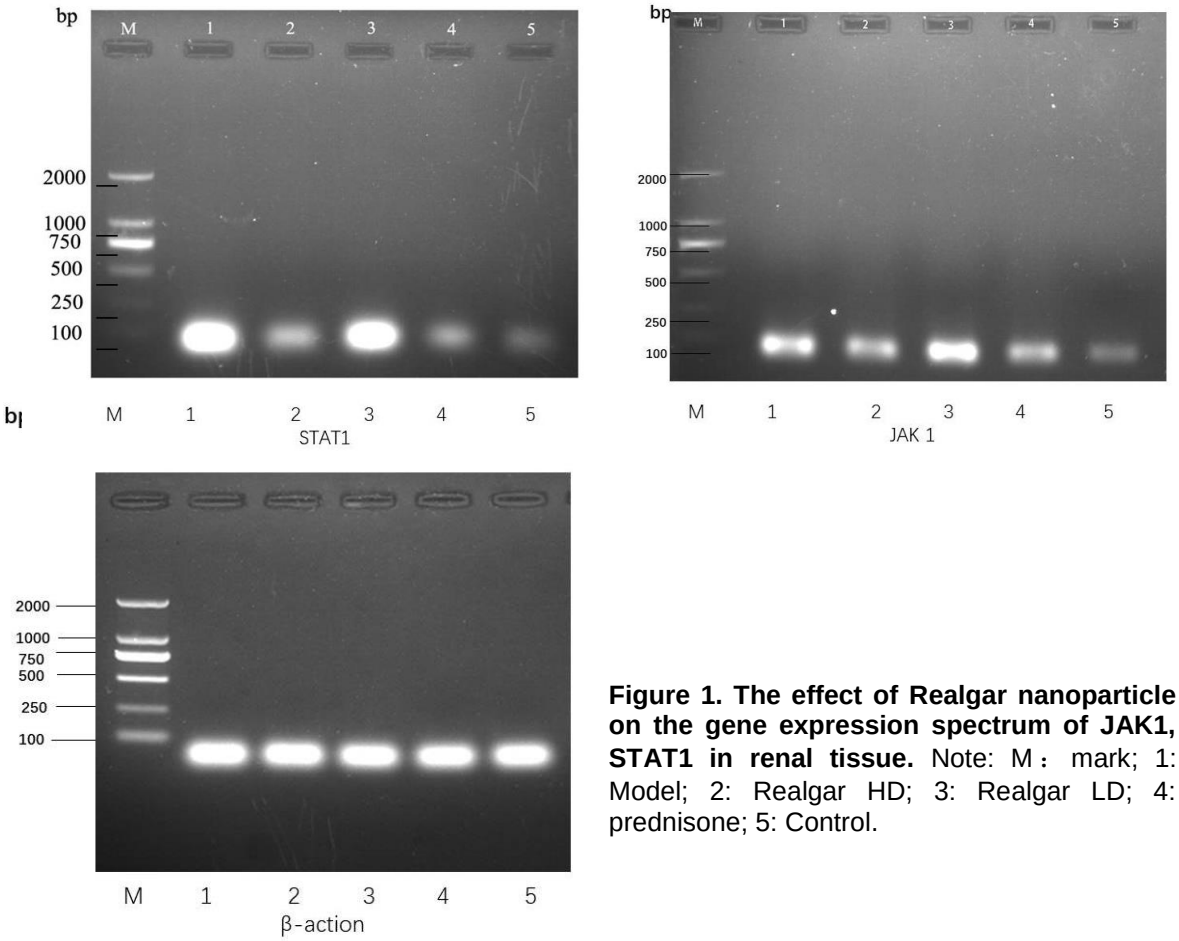

Figure 1. The effect of Realgar nanoparticle on the gene expression spectrum of JAK1, STAT1 in renal tissue. Note: M： mark; 1: Model; 2: Realgar HD; 3: Realgar LD; 4: prednisone; 5: Control.

Histopathological assay.

At the end of the study, HE staining was used to assess the severity of kidney inflammation in the lupus mice. Compared to the model group, the mesangial proliferation of the mice was gradually alleviated in the high and low dose groups of realgar nanoparticles. Meanwhile, the tissue necrosis of renal tubular epithelial cells decreased gradually, and the infiltration of inflammatory cells around small blood vessels in renal interstitial decreased gradually (figure 2A, B, C), being similar to the prednisone group (Figure 2D).

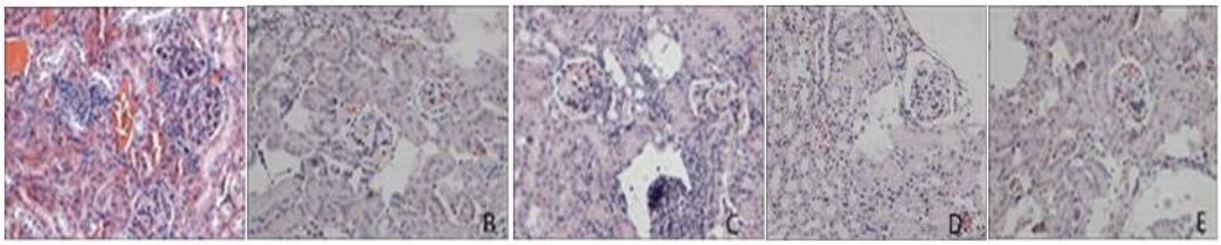

Figure 2. The H&E (×400) of the kidney sections in MRL/lpr mice. A. Model group; B. Realgar HD group; C. Realgar LD group; D. prednisone group; E. Control group.

Effect of Realgar nanoparticles on the expression of renal JAK1, p-STAT 1 protein in MRL/lpr mice.

In renal tissue sections, p-STAT 1 was expressed in mesangial cells, glomerular endothelial cells, and some tubular epithelial cells in the MRL/LPR group (Figure 3A). In accordance with the RT-PCR results, the expression of *p*-STAT1 protein was detected by immunohistochemistry in the model group, and the expression of p-STAT 1 protein was consistent with that of RT-PCR (Figure 3A).

Table 6. Effect of Realgar nanoparticles on inhibition of *p*-STAT 1 phosphorylation and reduction of JAK1 expression in kidney tissue (n=12, Mean ± SD).

Groups	p-STAT1 (Mean density)	JAK1 (Mean density)
Realgar HD	$0.13 \pm 0.04^{**}$	$0.31 \pm 0.02^{*\$}$
Realgar LD	$0.23 \pm 0.05^{\#}$	$0.30 \pm 0.04^{*\$}$
Prednisone	$0.15 \pm 0.03^{**}$	$0.30 \pm 0.06^{*\$}$
Model	$0.33 \pm 0.06^{\#\#}$	$0.32 \pm 0.03^{\#\#}$
Control	$0.03 \pm 0.01^{*}$	$0.04 \pm 0.02^{*}$

Values represent the mean ± S.D. The one-way ANOVA was performed on the raw data. $^{\#\#}$Significant difference at $p<0.01$ levels compared with the control group. **Significant difference at $p<0.01$ levels compared with the model group. *Significant difference at $p>0.05$ levels compared with the model group. $^{\#}$ Significant difference at $p<0.05$ levels compared with the model group.$^{\$}$ No significant difference at $p>0.05$ levels compared with the Prednisone group. Mean density of p-STAT 3 was measured by Image-Pro Plus v 6.0.

On the contrary, the expression of p-STAT1 in mice treated with realgar nanoparticles significantly decreased ($p<0.01$, $p<0.05$, Figure 3B, C), particularly in the realgar HD group ($p<0.01$, Figure 3B). Compared to the model group, the level of p-STAT1 in the prednisone group was significantly lower ($p<0.01$, Figure 3D). However, the expression of JAK-1 did not significantly decrease compared to the model group ($p>0.05$, Table 6), indicating that the expression of *p*-STAT 1 in Realgar nanoparticle groups was blocked. It is suggested that *p*-STAT-1 may be one of the targets of realgar nanoparticles in the treatment of LN.

DISCUSSION

In this study, we selected MRL/lpr mice to examine the potential therapeutic role of realgar nanoparticles in SLE. Autoantibody production is closely associated with LN activity (9,10), therefore, the serum levels of anti-dsDNA antibodies and complement C3 were determined. The study was designed to treat the MRL/lpr mice at the age of 24 weeks, since all mice at this time had positive anti-dsDNA antibody titers, and more than 90% of the mice had detectable proteinuria, indicating that autoimmune nephritis was established before starting the treatment. The study results show that the mice in the realgar nanoparticle-treated group exhibited significant reduced serum levels of anti-dsDNA, IgG, IgM, BUN, Cr, and proteinuria.

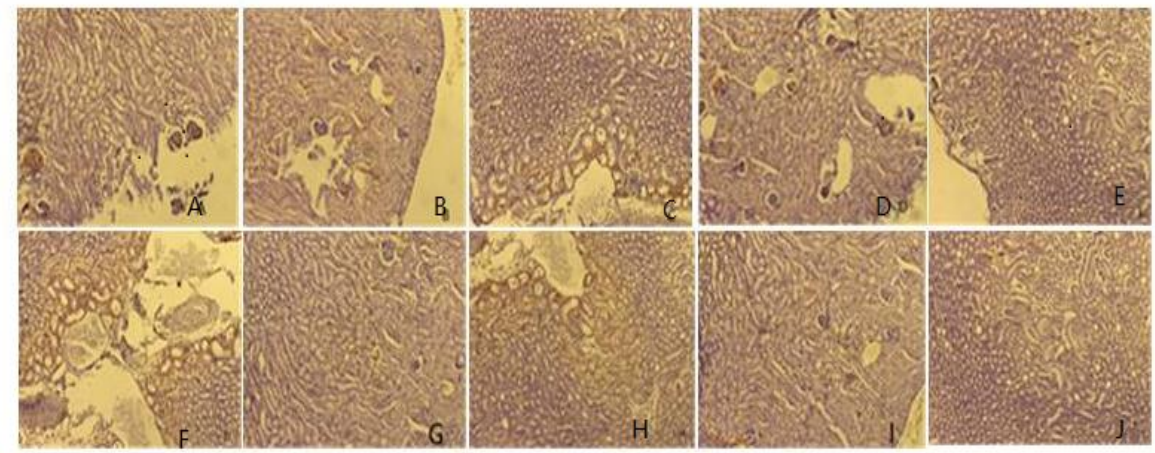

Figure 3. Effect of Realgar nanoparticle on inhibiting phosphorylation of STAT 1 (p-STAT 1) and reducing the expression of JAK1 in kidney tissue. p-STAT 1 and JAK1 were measured on paraffin sections of the kidneys at the end of 8 week by immunohistochemistry (×100). A-E: *p*-STAT1; (A) Model group; (B): Realgar HD group; (C): RealgarLD group; (D): prednisone group. (E): Control. F-J: JAK1; (F) Model group; (G): Realgar HD group; (H): Realgar LD group; (I): prednisone group. (J): Control.

Moreover, realgar nanoparticles significantly suppressed the levels of inflammatory cytokine IFN-γ compared to the untreated MRL/lpr mice , suggesting that realgar nanoparticles exerted therapeutic effects on established lupus nephritis in MRL/lpr mice. Therapeutic trials in MRL/lpr mice frequently involve initiation of treatment before the disease onset (11). Only few agents have demonstrated benefits after the disease onset (12,13). The finding that realgar nanoparticles are effective as a therapy for established MRL/lpr murine lupus suggests that its efficacy maybe more easily translated into treatment of human lupus. Realgar has long been used as a therapeutic agent to treat some diseases in ancient China. However, it is insoluble in water, leading to poor bioavailability and limiting its clinical application. Compared to coarse realgar, the nanoparticle form has been demonstrated to inhibit cell viability more potently and prolong the presence of arsenic in blood (8). However, whether realgar alleviates the LN damage is not clear now. Thus, in this study, we explored the reno-protective effects of realgar nanoparticles on LN MRL/lpr mice. To the best of our knowledge, the present study is the first one to examine the effects of realgar nanoparticle on the treatment of SLE. The therapeutic dose of realgar nanoparticles is determined according to the clinical therapeutic dose. Realgar nanoparticles were also quantitatively evaluated and standardized for their *in vitro* bioactivities and *in vivo* toxicity before being applied to the current studies (8). In the current study, realgar nanoparticles were employed in a therapeutic approach in animals with established diseases, which their histological changes were well correlated with clinical improvement of the kidney disease. The mechanism by which realgar nanoparticles reduced autoimmune nephritis in MRL/lpr mice could be associated with the induction of apoptosis as documented in vitro. *In vitro,* Realgar nanoparticles promote B cell apoptosis through rising Ca^{2+} concentration in B cell (8). In addition, Dan Shi demonstrated that realgar nanoparticles inhibited the proliferation of CML cells and

degraded the Bcr-Abl oncoprotein, while the underlying mechanism may be associated with apoptosis and/or autophagy (14). Currently, anti-ds-DNA antibody is an important indicator of SLE activity. The present study shows apparent effects on circulating levels of anti-dsDNA antibodies in mice treated with realgar nanoparticles. It is considerable that many other reagents reported to be effective in lupus nephritis mice models have not necessarily had an effect on anti-dsDNA titers, depending on the treatment timing (12,13,15). However, the specific reason is not clear. While immune complex deposition can trigger a series of events that result in kidney inflammation and injury in MRL/lpr mice (16), our study indicated that treatment with realgar nanoparticles significantly reduced the serum levels of anti-dsDNA antibody, complement C3, and decreased renal immune complex deposition in MRL/lpr mice. In addition to the effects on reduced serum levels of anti-dsDNA, IgG, and IgM, treatment with realgar nanoparticles also had a major impact on the the phosphorylations of STAT1 in the kidney. Previous studies in both human and lupus prone mouse models have described that total levels of STAT 1 as well as the activated form of STAT 1 are increased, compared to the healthy controls (17,18). Therefore, the activated *p*-STAT 1 was detected by the IHC method. Realgar nanoparticles have been demonstrated to have several immunosuppressive effects, including inhibition of the phosphorylations of STAT1 and genes encoding other molecules involved in the production of inflammatory mediators. The effect of realgar nanoparticles can be explained by their capacity to induce synthesis of HSP70 and enhance “H0-1” activity in rats under pathological conditions (19). Regardless of the precise mechanism, it is clear that treatment with realgar nanoparticles stabilized renal function and improved renal pathology in MRL/lpr mice.

In conclusion, the study suggests that realgar nanoparticles have potent reno-protective activity in LN MRL/lpr mice. These effects followed by inhibiting the activation of STAT1 phosphorylations, inhibiting the release of inflammatory cytokines, reducing autoimmune activity, and inhibiting the infiltration of macrophages in the kidney of MRL/LPR mice. Thus, realgar nanoparticles could be a valuable alternative medicine for the effective treatment of the SLE disease.

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REFERENCES

1. Rahman A, Isenberg DA. Systemic lupus erythematosus. N Engl J Med. 2008; 358:929-39.
2. Kotzin BL. Systemic lupus erythematosus. Cell. 1996; 85:303–6.
3. Hahn BH, McMahon MA, Wilkinson A, et al. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. Arthritis Care Res. 2012; 64:797–808.
4. Richman IB, Taylor KE, Chung SA, et al. European genetic ancestry is associated with a decreased risk of lupus nephritis. Arthritis Rheum. 2012; 64:3374–82.
5. Hoover PJ, Costenbader KH. Insights into the epidemiology and management of lupus nephritis from the US rheumatologist's perspective. Kidney Int. 2016; 90:487–92.
6. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. Adv Immunol. 1985; 37:269–390.

7. Tian Y, Wang X, Xi R, et al. Enhanced antitumor activity of realgar mediated by milling it to nanosize. Int J Nanomedicine. 2014; 9:745-57.
8. Wei-Dong X, YANG L, Zheng-Feng L, Xiao-Yan L, Xiao-Dong S, Jiang-Ping Y, et al. Realgar nano-particles down-regulated the expression of CD40 in B cells from MRL/lpr mice and promote B cell apoptosis through rising their Ca^{2+} concentration. Chinese journal of immunology. 2013; 29:1249-1253.
9. Ruiz-Irastorza G, Ramos-Casals M, Brito-Zeron P, et al. Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. Ann Rheum Dis. 2010; 69:20–8.
10. Furukawa F, Yoshimasu T. Animal models of spontaneous and drug-induced cutaneous lupus erythematosus. Autoimmun Rev. 2005; 4:345-50.
11. Borel Y, Lewis RM, Andre-Schwatz J, et al. Treatment of lupus nephritis in adult(NZB+NZW)F1 mice by cortisone-facilitated tolerance to nucleic acid antigens. J Clin Invest. 1978, 61:276-286.
12. Hahn BH, Knotts L, Hamilton TR. Influence of cyclophosphamide and other immunosuppressive drugs on immune disorders and neoplasia in NZB/NZW mice. Arthritis Rheum. 1975; 18:145-152.
13. Schiffer L, Sinha J, Wang X, et al. Short term administration of costimulatory blockade and cyclophosphamide induces remission of systemic lupus erythematosus nephritis in NZB/W F1 mice by a mechanism downstream of renal immune complex deposition. J Immunol. 1997; 71:489-505.
14. Shi D, Liu Y, Xi R, Zou W, Wu L, et al. Caveolin-1 contributes to realgar nanoparticle therapy in human chronic myelogenous leukemia K562 cells. Int J Nanomedicine. 2016; 11:5823-5835.
15. Sharabi A, Sthoeger ZM, Mahlab K, Lapter S, et al. A tolerogenic peptide that induces suppressor of cytokine signaling (SOCS)-1 restores the aberrant control of IFN-γ signaling in lupus-affected (NZB × NZW) F1 mice. Clin Immunol. 2009; 133:61-8.
16. Wakeland EK, Liu K, Graham RR, et al. Delineating the genetic basis of systemic lupus erythematosus. Immunity. 2001; 15:397-408.
17. Ghodke-Puranik Y, Niewold TB. Immunogenetics of systemic lupus erythematosus: a comprehensive review. J Autoimmun. 2015; 64:125-1364.
18. Yu SL, Kuan WP, Wong CK, et al. Immunopathological roles of cytokines, chemokines, signaling molecules, and pattern-recognition receptors in systemic lupus erythematosus. Clin Dev Immunol. 2012; 2012:715190.
19. Tang Y, Wang N, Mi S, et al. Effects of Reaglar and presciription containing of reaglar on stress response on proteins HSP70 and HO-1.Pharmacology and Clinics of Chinese Materia Medica, 2008; 24:48-52.