Effect of arsenic trioxide on immune function and renal histopathological changes in MRL/lpr mice

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Abstract: OBJECTIVE To investigate the effect of arsenic trioxide (As_2O_3) on immune function and renal pathology in MRL/lpr mice. **METHODS** Forty-five MRL/lpr mice were divided into control, As_2O_3 0.8 mg·kg^{-1} (ip, once a day) and cyclophosphamide 50 mg·kg^{-1} (ip, once a week) groups. After continuously administration for 2 months, the serum level of anti-double stranded-DNA (dsDNA) autoantibody, interferon-γ (IFN-γ) and interleukin-12 (IL-12) of mice was measured with ELISA. The subsets of the spleen lymphocytes were detected with flow cytometry. The kidney was removed for periodic acid Schiff dyeing. The expression of IgG and complement C_3 in the nephridial tissue was observed by immunofluorescence assay. **RESULTS** Two months after therapy, compared with that of before treatment, the anti-dsDNA antibody level in normal control group increased from 1.18 ± 0.26 to 1.80 ± 0.26 ($P < 0.01$), while it significantly decreased from 1.14 ± 0.58 to 0.92 ± 0.06 in As_2O_3 group and from 1.09 ± 0.22 to 0.67 ± 0.14 in cyclophosphamide group, respectively ($P < 0.05$, $P < 0.01$). Compared with normal control group: ① the serum levels of the anti-dsDNA antibody, IFN-γ and IL-12 in As_2O_3 and cyclophosphamide groups were lower than those of normal control group ($P < 0.05$, $P < 0.01$), and the anti-dsDNA antibody level was much lower in cyclophosphamide group than As_2O_3 group ($P < 0.01$); ② percentage of $CD3^+$, $CD19^+$ and $CD3^+CD4^+$ cells in As_2O_3 group was lower than normal control group ($P < 0.01$), the percentage of $CD3^+$, $CD3^+CD8^+$ and $CD19^+$ cells in cyclophosphamide group was much lower than normal control group ($P < 0.01$), $CD3^+CD4^+$ cells in As_2O_3 group were fewer than cyclophosphamide group ($P < 0.01$); ③ the glomerulus cell count per glomerular cross-sections and the integral of activity in As_2O_3 and cyclophosphamide groups were less than normal control group ($P < 0.05$, $P < 0.01$), while there was no significant difference between As_2O_3 and cyclophosphamide groups; ④ IgG deposition along the glomerular mesangium and capillary loop in As_2O_3 and cyclophosphamide groups was much less than normal control group ($P < 0.05$), and there was no difference in complement C_3 expression among the three groups. **CONCLUSION** As_2O_3 can decline the level of anti-dsDNA antibody and the activation and proliferation of T cells, B cells and T subsets in MRL/lpr mice. It can also decrease the serum level of IFN-γ and IL-12 hence suppress kidney lesions.

Key words: arsenic trioxide; lupus erythematosus, systemic; antibodies, antinuclear; cytokines; mice, inbred MRL lpr

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by increase in B cell activity, impairment of T lymphocyte regulation, production of autoantibodies[1-2] and a wide spectrum of organ involvement, such as lupus nephritis. SLE is currently treated with corticosteroids and cytotoxic or immunosuppressive drugs. These therapies prolong survival but are associated with severe side effects, particularly infection, therefore discovery for a new effective drug is of great significance.

Arsenic trioxide (As_2O_3) is the main component of a traditional Chinese materia medicia, which is called white arsenic. It was once used to treat rheumatic disease, asthma, tumor and so on, and for the past few years it has been found to be effective in the treatment of both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL), although the molecular mechanism in detail is yet un-

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known[3]. We have already confirmed that As_2O_3 could prolong life expectancy of lupus mice and had certain therapeutical effect on them[4-5], while the specific mechanism is still unknown.

MRL/lpr mice which can spontaneously develop hypergammaglobulinemia and high levels of autoantibodies, including anti-double stranded-DNA (dsDNA) antibody, associated with immune-complex-mediated glomerulonephritis and vasculitis[6-7]. Because of the resemblance between the murine and human diseases, MRL/lpr mice have been used extensively to attempt to determine SLE etiology and to evaluate therapy. So the effect of As_2O_3 on the level of autoantibody, lymphocyte subsets, cytokines and glomerulonephritis in MRL/lpr mice was investigated in this study.

1 MATERIALS AND METHODS

1.1 Antibodies and reagents

The following mouse cytokine specific monoclonal antibodies (mAb) and isotype-matched control mAb were purchased from eBioscience Company. PE-Cy5.5-anti-mouse CD3, RPE-anti-mouse CD19, FITC-anti-mouse CD4, APC-rat IgG_1 isotype control, PE-rat IgG_1 isotype control, anti-mouse Fc-g receptor, APC-anti-mouse interferon-γ (IFN-γ), PE-anti-mouse interleukin-12 (IL-12) and FITC-anti-mouse IgG: sodium arsenite, Harbin Yida Pharmaceutical Co., Ltd.. Mouse IgG, Southern Biotechnology Associates. Inc.. Salmon sperm DNA, Sigma. HRP-goat anti-mouse IgG, Beijing Zhong Shan Biological Technology Co., Ltd.. Mouse cytokine ELISA kits, Shenzhen Jingmei Biological Engineering Co., Ltd.. Bovine serum albumin (BSA), Shanghai Yubo Biological Technology Co., Ltd..

1.2 Animal and treatment

Forty-five 3-month-old MRL/lpr mice (weighting 37 – 44 g) were bought from Shanghai SLAC Animal Laboratory (SCXK2007-0005) and bred in our pathogen-free animal facility. Forty-five MRL/lpr mice were divided into control group (ip given normal saline, once a day), As_2O_3 0.8 $mg \cdot kg^{-1}$ (ip, once a day) and cyclophosphamide 50 $mg \cdot kg^{-1}$ (ip, once a week) groups. After continuous administration for 2 months, the blood samples were taken at the starting point and the end point of the experiment.

1.3 ELISA for anti-dsDNA autoantibody

Ninety six-well plates were coated with 100 $mg \cdot L^{-1}$ salmon-milt DNA (100 μl). After blocking with 1% BSA for 10 h, the mouse serum (1∶100) was added in triplicate for 90 min in 37℃. After washing, the bound IgG anti-DNA was detected with HRP labeled goat anti-mouse IgG antibody. The absorbance was determined at 450 nm ($A_{450\ nm}$).

1.4 ELISA for cytokines

Serum levels of IFN-γ and IL-12 assayed using the ELISA kits, following the manufacturer's instructions. $A_{450\ nm}$ was determined and the IFN-γ and IL-12 contents were calculated according to the standard curve.

1.5 Flow cytometry for splenocyte subsets

The spleens were removed and gently homogenized in germ free condition, and the cells washed in PBS twice. The 1×10^6 splenocytes were incubated with PE-Cy5.5-anti-mouse CD3, FITC-anti-mouse CD4, Lyt22 PE-anti-mouse CD8, APC-IgG1 isotype control, PE-IgG1 isotype control, and RPE-anti-mouse CD19 (1∶1000) antibodies for 15 min in dark at room temperature. After being washed the resuspended cells were fixed with 300 μl of 1% paraformaldehyde. Fifty thousands cells were analyzed by flow cytometry.

1.6 Periodic acid Schiff (PAS) staining for kidney tissue pathological changes

The kidneys were fixed in 10% Formalin for 3 h at 4℃, then dehydrated, and paraffin embedded. Paraffin sections (4 μm) were stained with PAS reagent. Glomerular pathological change was evaluated by assessing 20 glomerular cross-sections (GCS) per kidney and scored each glomerulus on a semiquantitative scale. 0: Normal (35 – 40 cells per GCS); 1: mild [glomeruli with a few lesions, with slight proliferative changes, and mild hypercellularity (41 – 50 cells per GCS), and/or minor exudation]; 2: moderate [glomeruli with

moderate hypercellularity (50 – 60 cells per GCS), including segmental and/or diffuse proliferative changes, hyalinosis, and/or moderate exudates〕; and 3: severe〔glomeruli with segmental, or global sclerosis, and/or exhibiting severe hypercellularity (60 cells per GCS), necrosis, crescent formation, and/or heavy exudation〕. Damaged tubules (percentage; consisting of dilation and/or atrophy and/or necrosis) were determined in 200 randomly selected renal cortical tubules per kidney (× 400). Perivascular cell accumulation was determined semiquantitatively by scoring the number of cell layers surrounding the majority of vessel walls on a 0 – 3 scale[8].

1.7 Immunofluorescence assay for IgG and complement C_3 expression in kidney tissue

Kidney cryostat cross-sections (4 μm thick) were stained with FITC-conjugated goat anti-mouse IgG (1∶200) and FITC-conjugated goat IgG fraction of mouse complement C3 for 30 min at 37℃. After washing the sections were observed under fluorescent microscope. The fluorescence intensity within the peripheral glomerular capillary walls and the mesangium was scored on a scale of 0 – 3 (0: none; 1: weak; 2: moderate; 3: strong). At least 10 glomeruli per section were analyzed.

1.8 Statistical analysis

All data were expressed as $\bar{x} \pm s$. Statistical significance was analyzed using ANOVA and/or Mann-Whitney-Wilcoxon. Statistical difference was accepted at $P < 0.05$.

2 RESULTS

2.1 Effect of As_2O_3 on serum anti-dsDNA autoantibody in MRL/lpr mice

There was no significant difference among these three groups in anti-dsDNA autoantibody levels before treatment. Two months later, the serum level of anti-dsDNA antibody in control group obviously increased ($P < 0.01$) and those in As_2O_3 and cyclophosphamide groups decreased ($P < 0.05$, $P < 0.01$) compared with before treatment. Compared with control group after treatment, As_2O_3 and cyclophosphamide could decline anti-dsDNA autoantibody levels ($P < 0.01$). As_2O_3 group had higher level of anti-dsDNA antibody than cyclophosphamide group ($P < 0.01$) (Tab. 1).

Tab. 1 Effect of arsenic trioxide (As_2O_3) on serum level of anti-double stranded-DNA (dsDNA) antibody in MRL/lpr mice

Group	Anti-dsDNA antibody ($A_{450\ nm}$)	
	Before treatment	After treatment
Normal control	1.18 ± 0.26	1.80 ± 0.26 **
Cyclophosphamide	1.09 ± 0.22	0.67 ± 0.14 $^{**\#\#}$
As_2O_3	1.14 ± 0.58	0.92 ± 0.06 $^{*\#\#\triangle\triangle}$

Forty-five MRL/lpr mice were divided into control group (ip given normal saline, once a day), As_2O_3 0.8 mg·kg^{-1} (ip, once a day) and cyclophosphamide 50 mg·kg^{-1} (ip, once a week) groups. The mice were administrated for 2 months. The serum level of anti-dsDNA antibody was detected with ELISA. $\bar{x} \pm s$, $n = 15$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with before treatment; $^{\#\#}P < 0.01$, compared with control group after treatment; $^{\triangle\triangle}P < 0.01$, compared with cyclophosphamide group after treatment.

2.2 Effect of As_2O_3 on serum level of IFN-γ and IL-12 in MRL/lpr mice

The level of IFN-γ and IL-12 in both As_2O_3 and cyclophosphamide groups was dramatically lower than those in control group ($P < 0.05$, $P < 0.01$). There were no significant differences of IFN-γ and IL-12 levels between As_2O_3 and cyclophosphamide groups (Tab. 2).

Tab. 2 Effect of As_2O_3 on serum levels of interferon-γ (IFN-γ) and interleukin-12 (IL-12) in MRL/lpr mice

Group	IFN-γ/ng·L^{-1}	IL-12/ng·L^{-1}
Normal control	17.8 ± 2.8	103.1 ± 13.0
Cyclophosphamide	13.0 ± 2.0$^{\#\#}$	82.2 ± 10.6$^{\#}$
As_2O_3	11.7 ± 2.2$^{\#\#}$	84.1 ± 11.8$^{\#}$

See Tab. 1 for the mouse treatment. Two months after treatment, serum level of IFN-γ and IL-12 was detected with ELISA. $\bar{x} \pm s$, $n = 15$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with normal control group.

2.3 Effect of As_2O_3 on percentage of splenic lymphocyte subsets in MRL/lpr mice

The percentage of $CD3^+$, $CD3^+CD4^+$ and $CD19^+$ cells in As_2O_3 group was lower than control group ($P < 0.01$). The percentage of $CD3^+$, $CD3^+CD8^+$ and $CD19^+$ cells in cyclo-

phosphamide group was much lower than normal control group ($P < 0.01$). $CD3^+CD4^+$ cells in As_2O_3 group were fewer than cyclophosphamide group ($P < 0.01$) (Tab. 3).

2.4 Effect of As_2O_3 on renal histopathological changes in MRL/lpr mice

Compared with normal control group, capillary endothelial cell and mesangial cell proliferation were reduced, the membrane thickening was lesser, and perivascular infiltration with lymphocytes was decreased under microscope in glomeruli in As_2O_3 and cyclophosphamide groups (Fig. 1). The glomerulus cell count (numbers of cells per glomeruli) and the total activity score in As_2O_3 and cyclophosphamide groups were less than control group ($P < 0.05$) (Tab. 4). However, no significant difference could be seen between As_2O_3 and cyclophosphamide groups.

2.5 Effect of As_2O_3 on IgG and C3 complement expression in kidney tissue in MRL/lpr mice

As shown in Fig. 2 and Fig. 3, the staining intensity of IgG in the kidneys of As_2O_3 and cyclophosphamide groups was comparatively less than normal control group ($P < 0.05$), while there was no significant difference among the three groups in complement C_3 deposition. The immunofluorescence staining pictures of complement C_3 were omitted.

Tab. 3 Effect of As_2O_3 on percentage of splenocyte subsets in MRL/lpr mice

Group	$CD3^+$/%	$CD3^+CD4^+$/%	$CD3^+CD8^+$/%	$CD19^+$/%
Normal control	59.1 ± 5.2	30.5 ± 3.4	28.7 ± 4.9	38.7 ± 2.5
Cyclophosphamide	48.3 ± 6.6 **	28.2 ± 4.2	20.1 ± 3.1 **	32.6 ± 2.3 **
As_2O_3	44.0 ± 4.1 **	19.9 ± 3.9 **##	24.1 ± 3.2	31.7 ± 1.9 **

See Tab. 1 for the mouse treatment. Two months after treatment, the percentage of splenocyte subsets was determined by flow cytometry. $\bar{x} \pm s$, $n = 15$. ** $P < 0.01$, compared with normal control group; ## $P < 0.01$, compared with cyclophosphamide group.

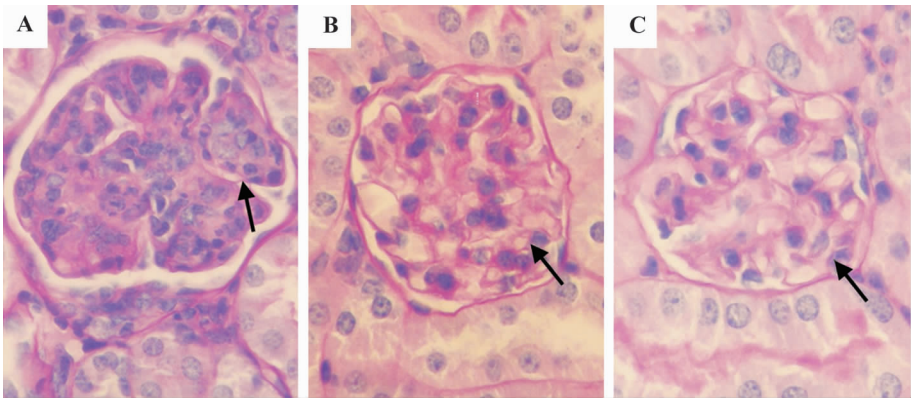

Fig. 1 Effect of As_2O_3 on proliferation of mesangial cells and mesangial matrix in glomerulus of MRL/lpr mouse kidneys (PAS ×400). See Tab. 1 for the mouse treatment. Two months after treatment, the glomerulus image was detected by microscope after periodic acid-Schiff stain (PAS) staining. A: normal control group; B: As_2O_3 group; C: cyclophosphamide group. ↑: proliferation of mesangial cells and mesangial matrix.

Tab. 4 Effect of As_2O_3 on total activity score and glomerulus cell count in renal tissue in MRL/lpr mice

Group	n	Total activity score	Glomerulus cell count (per GCS)
Normal control	8	12 (8 – 14)	67 (41 – 58)
Cyclophosphamide	9	4 (3 – 7) **	47 (42 – 55) *
As_2O_3	10	5 (3 – 8) **	48 (41 – 58) *

See Tab. 1 for the mouse treatment. Two months after treatment, glomerular pathological change was evaluated by assessing 20 glomerular cross-sections (GCS) per kidney and scored each glomerulus on a semiquantitative scale (0 – 3). Perivascular cell accumulation was determined semiquantitatively by scoring the number of cell layers surrounding the majority of vessel walls on 0 – 3 scale. * $P < 0.05$, ** $P < 0.01$, compared with control group.

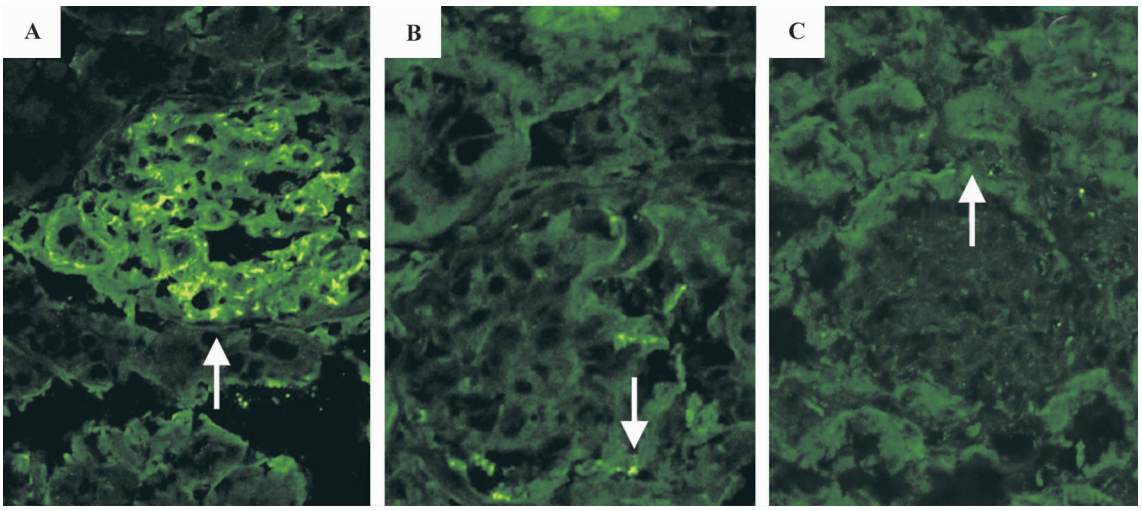

Fig. 2　Effect of As_2O_3 on IgG deposition in kidney tissue in MRL/lpr mice (×400). See Tab. 1 for the mouse treatment. Two months after treatment, IgG expression in kidney tissue was tested by immunofluorescence assay. A: control group; B: As_2O_3 group; C: cyclophosphamide group. ↑: IgG deposition.

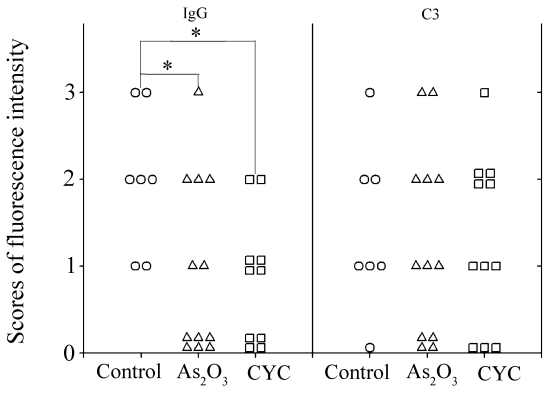

Fig. 3　Effect of As_2O_3 on expression of IgG and complement C_3 in kidney tissue in MRL/lpr mice. See Tab. 1 for the mouse treatment. Two months after treatment, the histologic examination was determined by semiquantitative analysis of fluorescence spectrometry. CYC: cyclophosphamide. $^*P<0.05$, compared with normal control group.

3　DISCUSSION

The pathogenesis of SLE is multifactorial and polygenic. T-helper ($CD4^+$ T) cells are considered to be significant in the immunopathogenesis of SLE, and lots of studies showed that the development of SLE is usually associated with disorder of cytokine network and imbalance of Th1/Th2[9]. It demonstrated that Th1 cytokine such as IFN-γ and IL-12 may be responsible for tissue damage and severe inflammatory response both in human and mice[10-11]. In MRL/lpr mice, the concentration of IFN-γ and IL-12 gradually rises over a prolonged period[12]. Moreover, IFN-γ administration exacerbates the disease whereas MRL/lpr mice, with defective IFN-γ or IFN-γ receptor expression, develop less severe forms[13-14].

As_2O_3 acts on signaling caspases and apoptosis[15], cellular redox, and cellular responses to stress[16]. Although mostly focused on the APL response to As_2O_3, it's already been examined the therapeutic impact of As_2O_3 on the severe autoimmune disorders manifested in lupus mice thereby predict its potential as a novel therapeutic agent for autoimmune disease[4-5,17].

This study demonstrated that anti-dsDNA autoantibody titers rose as the disease evolved, but it could be strongly inhibited by As_2O_3 and cyclophosphamide. There was obvious hyperplasia of lymphocytes in MRL/lpr mice, but As_2O_3 could sharply diminish the number of $CD3^+$ (T), $CD19^+$ (B) cells and $CD3^+CD4^+$ (Th) cells, presenting a little advantage over cyclophosphamide in this aspect. It's supposed that these cells were eliminated by apoptosis. It was found that after treatment the serum concentrations of IFN-γ and IL-12 were markedly higher in control group compared with As_2O_3 group. Meanwhile, control group showed obviously severer inflammation of glomerulus and tubulointerstitial lesion. Therefore, we speculate that As_2O_3 can ameliorate lupus nephritis by suppressing the expression of anti-dsDNA

antibody and inflammatory cytokines such as IFN-γ and IL-12.

As a classic cell cycle nonspecific immunosuppressive agent for SLE, cyclophosphamide has won worldwide acknowledgement for its curative effect on SLE. As_2O_3 seemed to have a similar powerful inhibitory action on auto-antibody, lymphocytes, Th1-type cytokines and renal pathology. These suggested that As_2O_3 be a novel therapeutic agent in changing cytokine and autoantibody production, lymphoid hyperplasia, and mononuclear-cell infiltration and immunocomplex deposition in kidneys. Thus it makes application of As_2O_3 on human lupus or other autoimmune diseases highly promising.

It has been found that arsenic can directly inhibit JAK tyrosine kinase activity thus interfering with the Janus kinase-signal transducer and activator of transcription pathway[18]. It was also reported that the methylation level of promoter, which can be regulated by arsenic, would affect the expression of IFN-γ[19]. These evidences lead us to further verify whether As_2O_3 adjust the activities and differentiation of Th cells via these pathways. Furthermore, exploring the effect of As_2O_3 on B cells and their regulatory factor, which is not involved in this article, is our next effort to exert.

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三氧化二砷对 MRL/lpr 小鼠免疫功能和肾脏组织病理变化的影响

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摘要: 目的 研究三氧化二砷(As_2O_3)对 MRL/lpr 小鼠免疫功能和肾脏组织病理变化的影响。**方法** 45 只 MRL/lpr狼疮小鼠 ip 给予环磷酰胺 50 mg·kg^{-1}(每周 1 次)和 As_2O_3 0.8 mg·kg^{-1},每天 1 次,共 2 个月。用 ELISA 法检测血清抗双链 DNA(dsDNA)抗体、干扰素 γ(IFN-γ)和白细胞介素 12(IL-12)浓度;用流式细胞术测定脾 $CD3^+$, $CD19^+$, $CD3^+CD4^+$ 和 $CD3^+CD8^+$ 细胞亚群的百分比;用 PAS 染色法观察肾组织病理变化;用免疫荧光方法检测肾组织 IgG 和补体 C3 的表达。**结果** 与给药前比较,给药 2 个月后,正常对照组血清抗 dsDNA 抗体水平升高,由给药前1.18 ±0.26升高至 1.80 ±0.26($P<0.01$),As_2O_3 和环磷酰胺组该抗体水平明显降低,分别由给药前 1.14 ±0.58 和 1.09 ±0.22 降低至 0.92 ±0.06 和 0.67 ±0.14($P<0.05$, $P<0.01$)。与正常对照组比较: ① As_2O_3 和环磷酰胺组血清抗ds-DNA抗体、IFN-γ和 IL-12 浓度明显降低($P<0.05$),环磷酰胺组抗 ds-DNA 抗体比 As_2O_3 组显著降低($P<0.01$); ② As_2O_3 组 $CD3^+$, $CD3^+CD4^+$ 和 $CD19^+$ 细胞百分率明显降低($P<0.01$),环磷酰胺组 $CD3^+$, $CD3^+CD8^+$ 和 $CD19^+$ 细胞百分率明显降低($P<0.01$); As_2O_3 组 $CD3^+CD4^+$ 细胞百分率明显降低($P<0.01$); ③ As_2O_3 和环磷酰胺组小鼠肾小球细胞计数和活动度积分明显降低($P<0.05$, $P<0.01$), As_2O_3 和环磷酰胺组无显著差异; ④ As_2O_3 和环磷酰胺组肾 IgG 表达明显降低($P<0.05$),补体 C_3 表达无明显差异, As_2O_3 和环磷酰胺组之间无显著性差异。**结论** As_2O_3 能降低MRL/lpr 狼疮小鼠血清抗 ds-DNA 抗体水平,抑制 T, B 和 Th 细胞活化和增殖,降低血清 IFN-γ 和 IL-12 水平,从而缓解狼疮肾炎的病理变化。

关键词: 三氧化二砷; 红斑狼疮, 系统性; 抗体, 抗核; 细胞因子类; 小鼠, 近交 MRL Lpr

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