N-acetylcysteine effectively alleviates systemic lupus erythematosus in mice via regulation of oxidative stress

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Abstract

Purpose: To study the influence of N-acetylcysteine (NAC) on systemic lupus erythematosus (SLE) mice, and the mechanism(s) involved.

Methods: Fourteen MRL/lpr SLE mice aged 5 weeks (mean weight = 20.35 ± 2.12 g) were divided into two 7-mouse groups: SLE (control) and treatment groups. The control group comprised healthy female SPF-grade C57BL/6 mice (n = 7). The treatment group mice received intraperitoneal injection of NAC at a dose of 250 mg/kg daily for 8 weeks. The serum levels of malondialdehyde (MDA) and nitric oxide (NO), and activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD), were assayed using standard methods. The level of urine protein and activity of anti-double stranded (ds) DNA antibody were determined using their respective enzyme-linked assay (ELISA) kits.

Results: The spleens of mice in SLE mice were significantly enlarged, relative to control mice, but they were reduced significantly by NAC (p < 0.05). N-Acetylcysteine (NAC) also significantly reduced the serum levels of MDA and NO in SLE mice, but significantly increased the serum activities of superoxide dismutase and GPx. Moreover, urine protein concentration and activity of anti-dsDNA antibody in SLE mice significantly increased, but reduced significantly by NAC treatment (p < 0.05).

Conclusion: These results suggest that NAC effectively alleviates SLE in mice via regulation of oxidative stress. Thus, NAC has the potentials for development into a therapy for the management of SLE.

Keywords: Anti-dsDNA antibodies, Antioxidant enzymes, N-acetylcysteine, Oxidative stress, Systemic lupus erythematosus

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects the skin, joints, kidneys, brain, as well as cardiac and pulmonary tissues [1]. The disease manifests in tiredness, raised body temperature, rashes and pain in the joints. Since SLE is not curable, emphasis is placed on minimizing the excruciating symptoms experienced by patients. The treatment involves changes in lifestyle, such as staying away from the sun and regulation of dietary habit. Other strategies used include drugs such as anti-inflammatory medications and steroids [2].
Oxidative stress occurs when the rate of generation of harmful free radicals overwhelms the capacity of endogenous antioxidants to neutralize them. It is characterized by infiltration of inflammatory neutrophils, increased protease secretion and production of high levels of oxidative intermediate products [3]. Oxidative stress has been shown to contribute to the pathogenesis of SLE [4]. In SLE, oxidative stress results in loss of immunoregulation, uncontrolled activation and handling of cell-death signals, generation of autoantibodies, and deadly comorbidities [4]. The causes of SLE are unknown. However, the pathogenesis of SLE is attributed, at least in part, to compartmentalized oxidative stress within the immune system.

N-Acetylcysteine (NAC) is an acetyl derivative of cysteine. It functions as a precursor of the antioxidant glutathione, thereby participating in the modulation of glutamatergic, neurotropic and inflammatory pathways [5]. This study investigated the influence of NAC on SLE mice, and the mechanism involved.

EXPERIMENTAL

Materials

Low-temperature high-speed centrifuge was purchased from Sigma-Aldrich Co. Ltd. (Germany), and low-speed centrifuge was obtained from Changsha Xiangrui Centrifuge Co. Ltd. Physiological saline injection was a product of Chengdu Qingshan Likang Pharmaceutical Co. Ltd. Urine protein ELISA kit was purchased from Thermo Fisher Scientific Co. Ltd. (Shanghai). Refrigerator was bought from Qingdao Haier Group. N-Acetylcysteine (NAC) injection was obtained from Hainan Lingkang Pharmaceutical Co. Ltd. Malondialdehyde (MDA), GPx and SOD assay kits were products of Shanghai Ruisai Biotechnology Co. Ltd. Mouse anti-ds-DNA antibody ELISA kit was obtained from Jianglai Biotechnology Co. Ltd. This research received approval from Animal Ethical Group of Taizhou People’s Hospital (approval no. 2019862528) and was conducted according to the guidelines of Principles of Laboratory Animal Care from World Health Organization [6].

Mice

Seven healthy female SPF-grade C57BL/6 mice and 14 MRL/lpr SLE mice aged 5 weeks (mean weight = 20.35 ± 2.12 g) were obtained from Shanghai Ruitaimosi Biotechnology Co. Ltd. Healthy female SPF-grade C57BL/6 mice (n = 7) served as control group. The MRL/lpr SLE mice were divided into two groups (7 mice/group): SLE and treated groups. Mice in the treatment group received intraperitoneal injection of NAC at a level of 250 mg/kg body weight daily for 8 weeks, while the control mice were given commensurate amount of normal saline intraperitoneally, instead of NAC.

Blood sample collection

Following 8-week treatment duration, apical blood specimens obtained from orbital vascular plexus of mice were centrifuged at 15,000 rpm for 15 min to obtain serum samples which were frozen at -80 °C prior to use for biochemical analysis.

Measurement of oxidative status in SLE mice

Oxidative stress indices

Malondialdehyde (MDA) levels were measured using the thiobarbituric acid method. Serum (1mL) was put into a test tube covered tightly with a plastic wrap, and a puncture was made. After incubating in a water bath at 95 °C for 90 min, the test tube was cooled with tap water and centrifuged. The absorbance of the supernatant was read at 532 nm. The activity of glutathione peroxidase (GSH-Px) was assayed with colorimetric method. The supernatant (1 mL) was pre-heated in a test tube in a 37 °C water bath for 5 min. Then, metaphosphoric acid precipitation buffer was added and the tube was allowed to stand before it was centrifuged. A chromogenic solution was added to the supernatant, and the absorbance was read at 550nm.

The activity of superoxide dismutase (SOD) was measured using enzyme rate method. Serum (1mL) was put into a test tube, shaken for 5min in a 37 °C water bath, and then placed in water bath for 40 min, with thorough shaking. The serum was kept at room temperature for 5 min, followed by absorbance measurement at 550nm. Nitric oxide (NO) level was determined using nitric acid reductase method. The absorbance of supernatant was read at 530nm, and NO activity was determined using the absorbance value. All indicators were measured strictly in accordance with the operating instructions.

Anti-ds-DNA antibodies

The ds-DNA antibody levels were determined using enzyme-linked immunosorbent assay (ELISA) kits in line with the manufacturer’s instructions. Serum samples were diluted and incubated at room temperature for 30 min. Then,
alkaline phosphatase-labeled anti-human IgG was added, followed by incubation at room temperature for 30 min. Following rinsing, chromogenic solution was added, and then dilute sulphuric acid was added to terminate the reaction. The absorbance was measured at 450 nm in a microplate reader.

**Urinary protein**

Urine protein level and activity of anti-ds DNA antibody were assayed using ELISA. Sample diluent and serum (50 µL each) were added to reaction and sample wells, respectively. Then, 50 µL biotin-labeled antibody was added and incubated at 37°C for 1 h. Thereafter, 80 µL streptomycin-horseradish peroxidase was added to each well and incubated at 37°C for 30 min. The wells were washed thrice with phosphate-buffered saline-Tween (PBS-T) to remove unbound molecules. Substrates A and B, each at a volume of 50 µL were added to the wells and incubated at 37°C in the dark for 10 min. The reaction was stopped by the addition of 50 µL termination solution. Optical density was read at 450 nm.

**Statistics**

The results are presented as mean ± SEM, and were analyzed using SPSS (21.0). Groups were compared using Chi-Squared test. Statistical significance was assumed at \( p < 0.05 \).

**RESULTS**

**General characteristics of mice**

There were no marked variations in general data of mice among the groups (\( p > 0.05 \); Table 1).

**Table 1**: General physical characteristics of mice in each group (mean ± SD, n = 7)

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (weeks)</th>
<th>Body weight (g)</th>
<th>Hair loss</th>
<th>Rash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>18.37 ± 1.34</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>SLE</td>
<td>5</td>
<td>19.16 ± 1.17</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td>19.33 ± 1.36</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

**Effect of NAC on spleen size and weight**

As shown in Table 2, the spleens of mice in SLE group were significantly enlarged, relative to control group, but they were reduced significantly by NAC.

**Table 2**: Influence of NAC on spleen size and weight (mean ± SD, n = 7)

<table>
<thead>
<tr>
<th>Group</th>
<th>Spleen size (cm)</th>
<th>Spleen weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.78 ± 0.57</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>SLE</td>
<td>14.46 ± 1.23a</td>
<td>0.34 ± 0.08a</td>
</tr>
<tr>
<td>Treatment</td>
<td>13.31 ± 1.01ab</td>
<td>0.19 ± 0.04ab</td>
</tr>
<tr>
<td>( F )</td>
<td>13.28</td>
<td>32.17</td>
</tr>
<tr>
<td>( P )-value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**Effect of NAC on indices of oxidative stress in SLE mice**

N-Acetylcysteine (NAC) significantly reduced the serum levels of MDA and NO in SLE mice, but markedly raised the activities of SOD and GPx (\( p < 0.05 \); Table 3).

**Table 3**: Effect of NAC on indices of oxidative stress in SLE mice (mean ± SD, n = 7)

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/mL)</th>
<th>NO (µmol/L)</th>
<th>SOD (U/mL)</th>
<th>GPx (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.27 ± 1.88</td>
<td>31.37 ± 18.57</td>
<td>93.26 ± 21.45</td>
<td>222.32 ± 23.84</td>
</tr>
<tr>
<td>SLE</td>
<td>19.13 ± 3.21a</td>
<td>63.64 ± 23.26a</td>
<td>60.11 ± 21.42a</td>
<td>181.61 ± 22.13a</td>
</tr>
<tr>
<td>Treatment</td>
<td>12.24 ± 1.65ab</td>
<td>45.29 ± 19.36ab</td>
<td>75.42 ± 20.53ab</td>
<td>196.35 ± 23.03ab</td>
</tr>
<tr>
<td>( F )</td>
<td>60.91</td>
<td>4.36</td>
<td>4.31</td>
<td>5.62</td>
</tr>
<tr>
<td>( P )-value</td>
<td>&lt; 0.001</td>
<td>0.029</td>
<td>0.029</td>
<td>0.013</td>
</tr>
</tbody>
</table>

**Effect of NAC on urine protein level and activity of anti-dsDNA antibody in SLE mice**

The level of urinary protein and activity of anti-dsDNA in SLE mice were significantly increased, but they were reduced significantly by NAC treatment (\( p < 0.05 \)). These results are shown in Table 4 and Figure 1 and Figure 2.

**Table 4**: Effect of NAC on urine protein level and activity of anti-dsDNA antibody in SLE mice (mean ± SD, n = 7)

<table>
<thead>
<tr>
<th>Group</th>
<th>Urine protein (µg/mL)</th>
<th>Anti-dsDNA antibody (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.58 ± 1.22</td>
<td>19.03 ± 4.17</td>
</tr>
<tr>
<td>SLE</td>
<td>12.64 ± 2.13a</td>
<td>56.33 ± 3.72a</td>
</tr>
<tr>
<td>Treatment</td>
<td>8.47 ± 2.21ab</td>
<td>37.41 ± 5.11ab</td>
</tr>
<tr>
<td>( F )</td>
<td>32.11</td>
<td>127.42</td>
</tr>
<tr>
<td>( P )-value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**Effect of NAC on splenic size and weight**

As shown in Table 2, the spleens of mice in SLE group were significantly enlarged, relative to control group, but they were reduced significantly by NAC.
DISCUSSION

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disorder characterized by total absence of autoimmunity and presence of immune response to antigens normally present in tissues. Innate immunity is important for the abnormal SLE-associated immune adaptations [7, 8]. The pathogenesis of SLE is complex and yet to be fully understood. Results from studies using animal models have revealed some of the mechanisms involved in the etiology of SLE. It has been reported that oxidative stress and endosome recycling are complementary mechanisms involved in reorganizing the T-cell receptor signaling complex in SLE activation and processing of cell-death signals, autoantibody production and fatal comorbidities [4].

The total loss of autoimmunity in SLE might be due to exacerbated levels of ROS which result in induction of apoptosis, upregulation of antibodies and symptoms of SLE. Reactive oxygen species (ROS) not only promote apoptosis, they also slow down the removal of apoptotic bodies, thereby enhancing the levels of neo-epitopes due to reaction between ROS and nuclear debris. The neo-epitopes induce production of broad range of autoantibodies, resulting in inflammatory responses and tissue damage. The noxious impact of ROS triggers a shift in intracellular redox condition (decreased proportion of reduced/oxidized glutathione), oxidative changes in proteins and DNA, and genetic induction of oxidative stress.

When oxidative reactions exceed normal levels, superoxide anions ($O_2^-$) are elevated. Under the action of various stimulating factors, endothelial cells generate high amounts of oxygen free radicals [$O_2^-$, singlet oxygen (‘$O_2$), hydroxyl radical ($OH$) and perhydroxyl radical (HO$_2^-$)], which in turn cause tissue damage [9,10].

Malondialdehyde (MDA), the final product of peroxidation of membrane lipids, aggravates membrane damage. Its level in tissues is used as an index of oxidative injury [11].

Superoxide dismutase (SOD) which dismutates superoxide radical into molecular oxygen and hydrogen peroxide, has reduced activity in SLE patients [12]. The enzyme GPx converts H$_2$O$_2$ to molecular oxygen and water. It is ubiquitously expressed in cells. The active center of GPx is selenium, and its catalytic action results in the transformation of reduced glutathione (GSH) to oxidized glutathione (GSSG) [13].

Nitric oxide (NO) is a ubiquitous signaling compound in cells and tissues. Under condition of oxidative stress, the level of NO is significantly elevated, thereby leading to lipid peroxidation and ultimately tissue damage [14]. The results of this investigation revealed that NAC markedly reduced the levels of MDA and NO in SLE mice, but it markedly increased the activities of SOD and GPx. These results suggest that oxidative stress may be involved in the pathogenesis of SLE, and they are in agreement with reports of previous studies [4]. N-Acetylcysteine (NAC) is a sulfhydryl-containing compound that promotes the synthesis of GPx. A mucolytic and antioxidant drug, NAC influences several inflammatory pathways and also functions as a sulfhydryl group donor. N-Acetylcysteine (NAC) gives rise to GSH, and it acts as a direct ROS scavenger, thereby influencing cellular redox condition [15]. It may also induce SOD synthesis.

As an antigen, DNA may occur as dsDNA or as single-stranded (ss) DNA. Due to the fact that DNA epitopes indicate the repetitive charges on the nucleic acid molecule, synthesized polynucleotide products are frequently recognized by anti-dsDNA antibodies. High levels of anti-dsDNA antibodies are almost only found in SLE, which makes it a specific biomarker for SLE [16]. In this study, the level of urine protein and activity of anti-dsDNA in SLE mice were significantly increased, but they were reduced significantly by NAC treatment. Similarly, the spleens of mice in SLE group were significantly enlarged relative to control group, but were reduced significantly by treatment with NAC. Thus, it is likely that NAC is effective in alleviating SLE in mice.

CONCLUSION

The results obtained in this study show that NAC effectively alleviates SLE in mice via regulation of oxidative stress. The elucidation of the
mechanism through which NAC alleviates SLE is beneficial for the development of anti-SLE drugs.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Feng Lu; Feng Lu, Bingxin Liu, Hui Zhao collected and analyzed the data, while Feng Lu wrote the manuscript. All authors read and approved the manuscript for publication.

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