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NAC attenuates adriamycin-induced nephrotic syndrome in rats through regulating TLR4 signaling pathway

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Abstract. – OBJECTIVE: Nephrotic syndrome (NS) is a detrimental renal disease that affects a large population. It is suggested that Toll-like Receptor 4 (TLR4) signaling pathway plays an important role in NS. The aim of this study was to evaluate the immunosuppressive effect of N-acetylcysteine (NAC) in the treatment of NS elucidate its interaction with TLR4 pathway in a rat model.

MATERIALS AND METHODS: Rat NS model was constructed using the Bertain method by injecting adriamycin (4.5 mg/kg) intravenously at day 1, and injecting 2 mg/kg adriamycin (ADR) at day 7. NS rats were treatment with NAC of 150 mg/kg daily through gavage. Control rats received equivalent amounts of saline daily. Quantitative Real-time PCR was used to evaluate TLR4 expression in kidney tissues after treatments. Western blot analysis was used to evaluate NF-κBp65 expression. ELISA was used to evaluate the expression of immunological factors, including TNF-α, IL-6, and IL-1β.

RESULTS: Rat NS models demonstrated higher protein levels in urine, accompanied by an increased in the TLR4 level. After NAC treatment, TLR4 level was reduced. NAC treatment also attenuated the NF-κBp65 overexpression in NS rats. Concomitantly, TNF-α, IL-6, and IL-1β levels, which are indicators of immunological and informatory responses, were also decreased after NAC treatment.

CONCLUSIONS: NAC treatment ameliorated nephrotic syndrome in NS rat models by suppressing TLR4 signaling, as well as immunological and inflammatory responses.

Key Words:
NAC, TLR4, Kidney syndrome, Rat.

Introduction

Nephrotic syndrome (NS) is characterized by a group of clinical symptoms including massive proteinuria, hypoalbuminemia, oedema and hypercholesterolemia. NS constitutes the predominant renal disease among children, and severely diminishes the quality of life for millions of people[1,2]. Two major factors that contribute to NS are excessive oxidative stress[3,4] and dysregulated immunological and inflammatory responses[5]. Oxidative stress occurs when the generation of oxidants or reactive oxygen species (ROS) outstrips local antioxidant capacity, leading to the oxidation of essential biological macromolecules, including lipids, carbohydrates, and DNA[6]. The damages to glomerular cells, such as podocytes, lead to the loss of glomerular basement membrane integrity and contribute to proteinuria. Further, the reiterative renal immunological responses and inflammation result in overproduction of cytokines, which activates glomerular cell proliferation, extracellular matrix components expansion and prolonged renal fibrosis. Therefore, therapy that relieves the oxidative stress in kidney, as well as suppressing immunological and inflammatory responses, is urgently needed for the treatment of NS. Emerging studies have demonstrated the effects of antioxidants in the treatment of NS[7,8]. N-acetylcysteine (NAC) is a potent antioxidant, which acts by promoting glutathione (GSH) synthesis to neutralize oxidants, and scavenging ROS[9]. It has been conventionally used against chronic pulmonary diseases, and demonstrated efficacy in reducing lung-function decline[10]. Recently, the efficacy of NAC against NS has been explored[11]. Also, the ability of NAC in improving renal perfusion and decreasing cell apoptosis makes it a promising drug for NS treatment. However, the current evidence on the immunosuppressive function of NAC is conflicting, and whether NAC has an immunosuppressive role in NS, it deserves further clarification[12-14]. Toll-like

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receptor 4 (TLR4) signaling pathway is involved in ADR-induced NS[15,16]. TLR4 signaling pathway is an important constituent of the innate immunity. In NS, TLR4 is activated, leading to the secretion of nuclear factor kappa B p65 (NF-κBp65), a key regulator of the immune response[15]. NF-κB is a heterodimer, consisting of p50 and p65 subunits, which upon activation, translates into nucleus and bind to specific DNA, thereby resulting in the heightened immune and inflammatory responses. Herein, we strived to evaluate the immunosuppressive effect of NAC in NS rats and delineated the role of NAC in regulating TLR4 signaling pathway. A NS rat model was constructed using Adriamycin (ADR)[17]. ADR was conventionally used as an anticancer drug, the high-risk of which in inducing NS limits the application of this drug. The results corroborated the ameliorating effect of NAC in proteinuria, and demonstrated the capability of NAC in inhibiting TLR4 signaling pathway. The role of NAC in regulating immunological and inflammatory responses in kidney enhances its potential in the treatment of NS.

Materials and Methods

Rat NS Model

Thirty-six healthy male Wistar rats, with the body weight of 170-210 g, obtained from the Experimental Animal Center of Suzhou Aiermaite Technology Co. Ltd. (Suzhou, Jiangsu, China). Each rat was placed in a separate metabolic cage, and housed in a SPF-level room maintained at 18-30°C, 48%-80% humidity, with natural air circulation. The rats were separated into the following three groups: control group, which received saline daily; NS group, which were NS rats constructed by injecting ADR at day 1 and day 7; NAC group, which were NS rats that received NAC of 150 mg/kg daily through gavage. For Western blotting and ELISA analysis, mice were sacrificed at day 14, and kidney tissues were collected, snap frozen in liquid nitrogen, and stored in -80°C. The protein concentration in urine was determined by spectrophotometry after 3% sulfosalicylic acid precipitation of urine samples.

Measurement of Inflammatory Cytokines Levels

The kidney tissues were homogenized in physiological saline solution and centrifuged at 3000 r/min for 10 min at 4°C. The supernatant was collected and the levels of Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Nanjing Jiancheng Co., Nanjing, Jiangsu, China).

RT-PCR

Kidney tissues were recovered from -80°C fridge, followed by total RNA extraction using the Trizol RNA isolation kit (Thermo Fisher, Rockville, MD, USA). Synthesis of cDNA was performed with the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher, Rockville, MD, USA). RT-PCR was performed using 1 μg of cDNA. Primers used in this study included: 5'- CCAGAGCCGTTGGTGTAT-3' (sense) and 5 '-GCCCTGTGAGGTCGTTGA-3' (antisense) for TLR4; 5 '-TCAGGTCATCACTATC-GGCAAT-3' (sense) and 5 '-AAAGAAAGGGTGTAAAA-CGCA-3' (antisense) for β-actin. The polymerase chain reaction (PCR) product is 432 bp, which was loaded in 1.5% agarose gel for electrophoresis. The gel was stained with ethidium bromide and imaged with GL2200 Pro system (Carestream Molecular Imaging, Rochester, NY, USA). The mRNA level of TLR4 was quantified by calculating the ratio of TLR4 band intensities and β-actin band intensities.

Western Blotting Analysis

Kidney tissue was lysed using T-PER tissue lysis buffer (Thermo Fisher, Waltham, MA, USA) and measured for protein concentrations with BCA analysis (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, Jiangsu, China). Equivalent proteins were loaded onto precast gels from Bio-rad (Hercules, CA, USA) and resolved using SDS-PAGE. Proteins were then transferred onto a PVDF membrane (Thermo Fisher, Waltham, MA, USA) at 100 V for 30 min. Membranes were then blocked with 5% non-fat milk dissolved in Tris-buffered saline (TBS) solution for 3 h at room temperature with gentle shaking, and then washed three times with Tris-buffered saline-tween (TBST) buffer for 3 times. Rabbit anti-rat β-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA, 1:200 dilution), or rabbit anti-rat NF-κBp65 antibody, dissolved in Tris-buffered saline-tween (TBS-T) was applied to the membrane and incubated overnight at 4°C. The membranes were then washed with Tris-buffered saline-tween (TBST) for three times. HRP-conjugated anti-rabbit antibody (Zhongshan Biotechnology, Co., Ltd, Beijing, China) was subsequently added to the membrane and incubated at room temperature for 1 h.

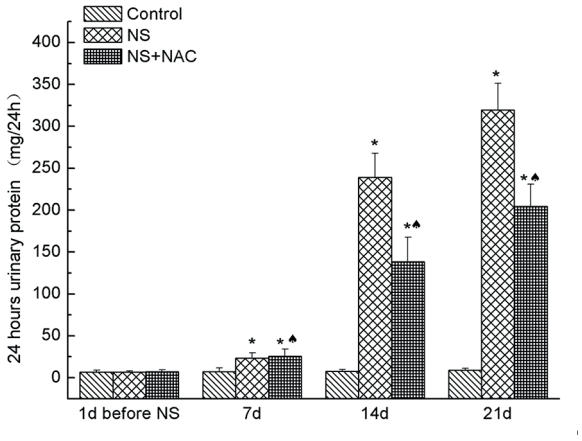

Figure 1. NAC reduced proteinuria in NS rat models. Comparison of urinary protein content of control rats, NS rats, and NS-rats that received NAC treatment at 7, 14 and 21 d, showing the ameliorating effects of NAC in reducing proteinuria in NS rats (N=12). $^{*}p<0.05$ for comparison to control rats; ▲$p<0.05$ for comparison to NS rats that received saline treatment.

After extensive washing with TBS-T, diaminobenzidine (DAB) substrate (Pierce, Rockford, IL, USA) was added for band visualization, followed by scanning with Image-Pro Plus 6.0 (Media Cybernetics, Shanghai, China). The band intensities of NF-κBp65 were normalized to those of β-actin for quantification.

Statistical Analysis

Data statistic analysis was performed by SPSS 19.0 (SPSS Inc. Chicago, IL, USA) and all data were expressed as mean values ± standard deviation. Changes between samples were compared by Student's *t*-test, and differences between groups were compared by the method of one-way ANOVA. LSD test was used to validate ANOVA; $p<0.05$ was considered statistically significant.

Results

NAC Treatment Led to a Decrease in Urinary Protein Content

A rat NS model was constructed using the ADR. Compared to control group, which exhibited no significant difference in 24-hour urinary protein content at the 7th day, 14th day and 21st day, the NS group demonstrated much higher urinary protein content at these time points, which also increased in a time-dependent manner, indicating that ADR administration caused damage to the nephrotic functions ($p<0.05$). Even through the NS rats that received NAC demonstrated higher urinary protein content compared to the control group, the urinary protein content was significant lower than NS rats that received no treatment ($p<0.05$), particularly after 14 days. This indicated that NAC treatment improved the nephrotic functions.

TLR4 level in Kidney was Reduced by NAC Treatment

To evaluate the effect of NAC treatment in TLR4 signaling pathway, we also evaluated TLR4 mRNA levels in rat kidney tissues with RT-PCR analysis. Unsurprisingly, the level of TLR4 increased in NS rats. After NAC treatment, TLR4 level was also significantly reduced compared to NS rats that received no treatments (Figure 2). This indicated that the ameliorating effects of NAC treatment may stem from its role in regulating TLR4 signaling pathway.

NF-κB Level was Reduced by NAC Treatment

NF-κB is a downstream factor of TLR4 that plays an indispensable role in NS. Here we evaluated the level of NF-κB in NS rats in response

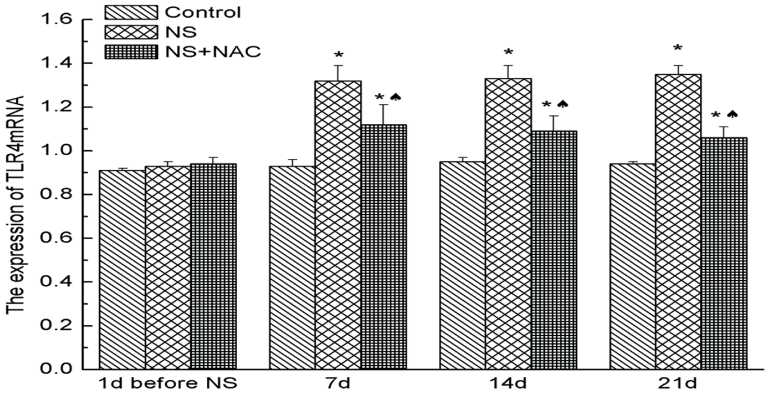

Figure 2. Upregulation of TLR4 in NS rats was attenuated by NAC treatment. RT-PCR analysis showing the relative levels of TLR4 mRNA in control rats, NS rats and NS rats that received NAC treatment at 7, 14, and 21 d (N=12). $^{*}p<0.05$ for comparison to control rats; ▲$p<0.05$ for comparison to NS rats that received saline treatment.

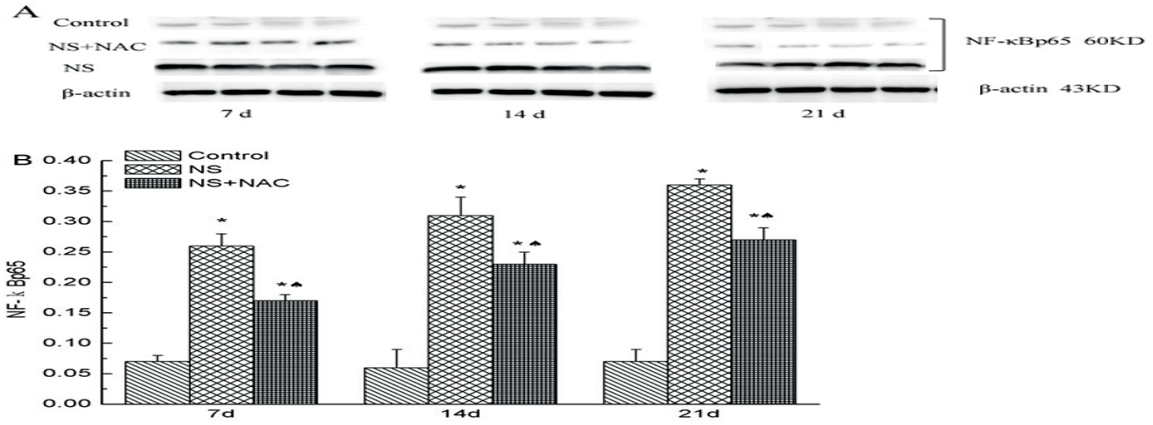

Figure 3. NAC treatment reduced NF-κBp65 expression in kidneys of NS rats (N=12). ***A,*** representative Western blotting analysis on kidney tissues from control rats, NS rats, and NS rats that received NAC treatments. The level of β-actin was used as a loading control. ***B,*** quantification of NF-κBp65 levels in rats as mentioned in A. $^{*}p<0.05$ for comparison to control rats; $^{\blacktriangle}p<0.05$ for comparison to NS rats that received saline treatment.

to NAC treatment. It is indicated that a negligible expression of NF-**κ**B could be observed in normal rats, whereas in NS rats, NF-**κ**B levels were prominently higher ($p<0.05$). A reduction in NF-**κ**B level was seen in rats that received NAC treatment (Figure 3A and B).

NAC Reduces Immunological Responses in Kidney

Dysregulation of immune and inflammatory responses is another contributor to NS. Due to this, we evaluated if NAC treatment alleviated immune and inflammatory responses in NS rats by analyzing levels of key cytokines, including TNF-α, IL-6, and IL-1β. As shown in Figure 4, these cytokines were significantly upregulated in NS rats. However, after NAC treatments, the levels of those cytokines were significantly reduced ($p<0.05$), suggesting that NAC also has an immunosuppressive role in kidney, through which a protective effect can be exerted on kidney.

Discussion

Currently, effective treatment for NS is still lacking. Most therapeutics for NS are aimed at NS-related secondary symptoms, such as hypercholesterolemia, edema, and venous complications, thus failing to prevent further disease progression toward kidney failure. The increasing knowledge of the important roles of oxidative stress and immune response in NS led to the development of a number of novel drugs that potently ameliorate NS[2,7,18-21]. This evidence suggested that a drug that exerts both antioxidant capacity and immunosuppressive activity is highly desirable for the treatment of NS. NAC is a putative antioxidant, which has been used for a wide variety of disorders[22,23]. Despite that the potential of NAC in NS treatment has been demonstrated, yet NAC has to be applied to clinical treatment of NS due to the lack of understanding of its mechanism of action. Apart from the role of NAC as an antioxidant, little is known on its effect in suppressing immunological and inflammatory responses in kidney. Hence, in the present study, we set forth to investigate whether and how NAC regulates NS-related immune responses. TLRs are widely expressed on leukocytes and kidney epithelial cells[24], which act as the first line of innate defense against pathogens[25]. They also respond to endogenous signals of tissue injury. TLR4 is the most extensively studied TLR, the over-activation of which is found to be closely linked to a variety of hepatic, cardiovascular and renal diseases, including NS. Previous evidence indicated that some nephrotoxicities induced by anti-cancer drugs, such as cisplatin[26] and doxorubicin[27], are medicated by TLR4 signaling pathways. Cytokine production, stimulated by TLR4 signaling, and consequently heightened immune and inflammatory responses, are major factors in nephrotic syndrome. Here we demonstrated that NAC reduced proteinuria in ADR-induced NS rat model by attenuating TLR4 signaling. As a result, the downstream signaling pathway, NF-**κ**B pathway, was also suppressed, which led to a decrease in

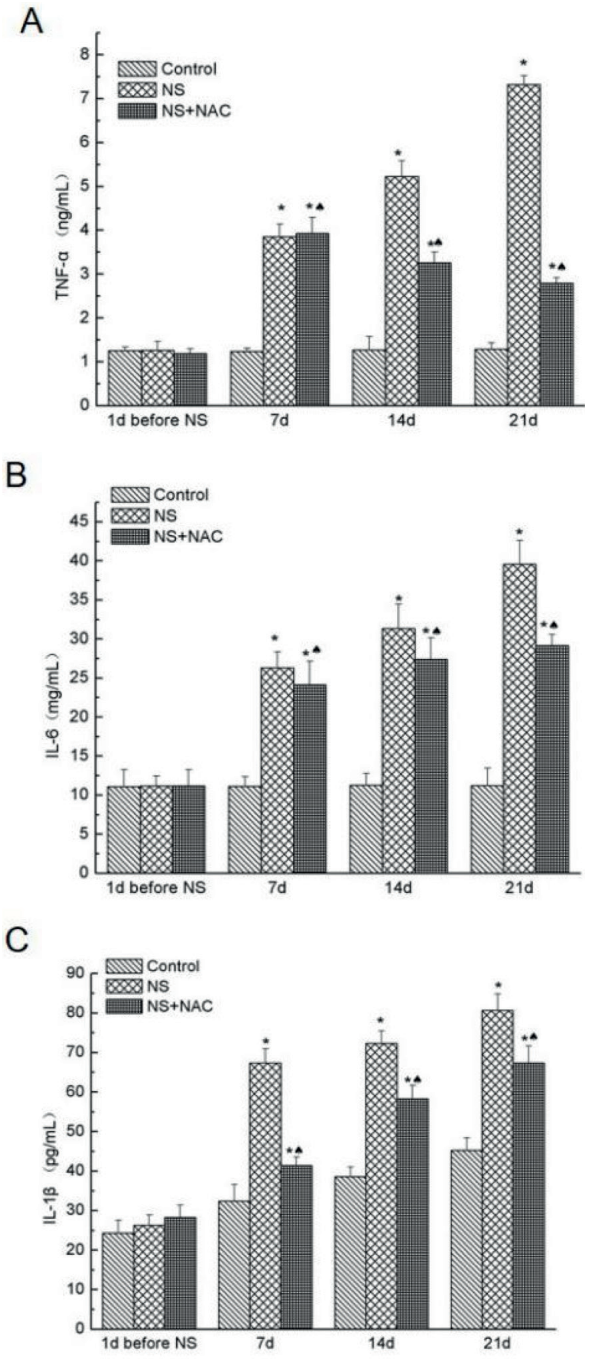

Figure 4. NAC treatment reduced cytokine levels in kidney of NS rats (N=12). Comparison of TNF-α ***(A)***, IL-6 ***(B)*** and IL-1β ***(C)*** levels in kidney of control rats, NS rats, and NS rats that received NAC treatment with ELISA. $^{*}p<0.05$ for comparison to control rats; $^{\blacktriangle}p<0.05$ for comparison to NS rats that received saline treatment.

the production of immunological factors, such as TNF-α, IL-6, and IL-1β. These findings corroborated the potential of NAC in alleviating NS not only as an antioxidant, but also as an immunosuppressant.

Conclusions

We demonstrated the efficacy of NAC in inhibiting TLR4 pathway, NF-κB activation, and cytokine production, through which proteinuria in ADR-induced rats was alleviated. The data presented in this study extend our knowledge about the immunosuppressant role of NAC in treating NS. Further evaluation of NAC in clinical arena is warranted for the broader application of NAC.

Conflict of interest

The authors declare no conflicts of interest.

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