β-Sitosterol improves acute nephritis in rats by inhibiting inflammasome and activating autophagy

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Research Article

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Abstract

β-sitosterol is a compound found in many plants and has a variety of biological activities, including immunomodulatory and anti-inflammatory activities. The purpose of this study is to test its anti-inflammatory ability through a mouse model of acute nephritis.

Methods: Acute nephritis mouse model was established by intravenous injection of antibodies. Pathological examination was used to test the therapeutic effect of β-sitosterol on model rats. Furthermore, the expression of related molecules was detected by ELASA.

Results: β-sitosterol improved acute nephritis by repairing renal function, proteinuria and renal pathology, especially reducing cell crescents, neutrophil influx, glomerular fibrinoid necrosis and glomerulonephritis activity scores. β-sitosterol inhibits NLRP3 inflammasome mediated inflammasome initiation signal, enhances sirtuin 1 (SIRT1)/autophagy axis and inhibits the secretion of NLRP3 inflammasome.

Conclusion: β-sitosterol represents a new drug candidate for the treatment of acute nephritis, which can reduce the kidney damage in model rats by enhancing the SIRT1/autophagy axis and reducing the activation of NLRP3 inflammasome.

Introduction

Inflammation is the body's response to tissue damage, caused by physical injury, ischemic injury (caused by an insufficient supply of blood to an organ), infection, exposure to toxins, or other types of trauma. It is necessary for body to maintain tissue homeostasis [1]. In this process, besides the release of inflammatory mediators, microcirculation processes also occur, including changes in vascular permeability, leukocyte movement, recruitment and accumulation [2]. When inflammation is not effectively treated or excessive inflammation occurs, a variety of inflammatory diseases affecting multiple organs can occur. Acute nephritis is an acute disease, which is often caused by immunity after infection and the condition progressed quickly. It is easy to be complicated with acute renal insufficiency, hypertensive encephalopathy, threating life safety [3]. Effective drugs for the treatment of acute nephritis can reduce patient’s condition and reduce the harm of complications for patients with acute nephritis. Nowadays drug therapy is mainly to solve, prevent or improve the quality of life affected by inflammatory diseases. Some drugs have been designed based on the condition of involved organs and the status of patients, specifically. However, this way of acute nephritis treatment could be expensive, with symptomatic, non-permanent effect, and even side effect. For example, nonsteroidal anti-inflammatory drugs could hurt bone tissue by modulating the proliferation, differentiation, adhesion and migration of osteoblasts [4]; Some drugs are related to upper gastrointestinal injury, including bleeding and ulcers [5]. In addition, another risk to consider is the interaction of anti-inflammatory drugs and other drugs, for example anticoagulants, corticosteroids, serotonin reuptake inhibitors and antihypertensive drugs, when these drugs are intake simultaneously. This complexity of therapy makes it valuable to find new

compounds with the capacity of acute nephritis therapy and less side effects. Plant extracts or plant derived compounds have great potential to achieve these drugs.

β-Sitosterol is a plant derived compound, which is found in rice, wheat, corn, nuts, peanuts and especially in cat claw (rhynchophyllum villosum). Importantly, it is considered to be the reason why cat claw has the abilities to relieve inflammation, virus damage, ulcer and cancer, as well as enhance the immune system [6]. Structurally, β- Sitosterol is similar to cholesterol, but it can be absorbed more slowly in intestine, interfering the absorption of cholesterol and preventing its release to serum [7]. Besides, β- Sitosterol regulates immunity, inflammation and pain feelings by controlling the production of inflammatory cytokines [8]. Our research showed that β-Sitosterol can improve acute nephritis by inhibiting inflammasomes and activating autophagy in rats.

Materials And Methods

1 Mouse model of acute nephritis

Experimental animal protocols were performed in accordance with the ARRIVE guidelines of the Ethnical Committee on Animal Experiments at Animal Ethics Committee of Seventh People's Hospital of Shanghai University. Adult male rats (weighing between 180 and 220 g) were randomly assigned into four groups: normal control group (NC group), acute nephritis group (AC group), low dose group (LD group) and high dose group (HD group). Experimental nephritis (anti-THY1 nephritis) was induced by a single intravenous injection of anti-THY1 antibody. Gavage with purified water or water-soluble β Sitosterol (The dosage was calculated according to 5mg/kg in the LD group and 20mg/kg in the HD group) in rats were started 3 days before the injection of anti THY1 antibody.

2 Blood and Tissue Sampling

Blood and Tissue Sampling as described previously[9]. Briefly, at week 8, the 24 h urine sample was collected with a rat metabolic cage. Urine total protein(TP) concentration was measured using the sulfosalicylic acid precipitation method, and the creatinine concentration was assessed with a creatinine assay kit (Bioassay Systems, Hayward, CA). Albuminuria(ALB) was measured using a rat albumin-specific ELISA kit (Exocell Laboratories, Philadelphia, PA). Commercial assay kits for serum creatinine (CREA) and blood urea nitrogen (BUN) were purchased from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). CREA and BUN concentrations were measured according to the protocols provided by the manufacturers. At week 8, all rats were sacrificed after anesthesia with isoflurane. Select kidney tissue was fixed in 4% paraformaldehyde for histopathologic staining. The remaining tissue was stored in liquid nitrogen. After gradient alcohol dehydration and xylene transparence, the kidney tissue was incorporated into a wax block and cut into 10 μm sections.

3 pathological analysis

Renal sections were fixation overnight in 4% Paraformaldehyde (pH 7.0), then followed by paraffin embedding. The produced samples were sectioned at 10μm thickness. Paraffin tissue sections were deparaffinized in xylene and rehydrated through graded ethanol solutions. Then, the sections were stained with H&E (catalog no. C0105, Beyotime, China), Masson staining and immunohistochemistry (anti-TGF, 1:200; ab215715, abcam, UK) according to the manufacturer's instructions. and the following histological features were examined using light microscopy, including: (a) proliferation of glomerular cells; (b) crescent; (c) neutrophil infiltration; (d) glomerular sclerosis; and (e) peri-glomerular tubulointerstitial inflammation as described previously.

4 inflammation level analysis

To assess the inflammation, the levels of MCP-1, iNOS, TNF-α, IL-1β and IL-6 in serum were determined with ELISA kits (Mlbio, Shanghai, China) were used following the manufacturer's instructions.

5 Flow cytometry

Flow cytometric analysis was performed to evaluate the percentage of apoptotic cells. Flow cytometry as described previously [10]. Briefly, The cells were treated with or without 20uM cisplatin for 24 h. Cells were digested with trypsin for 2 minutes and centrifuged at 1500 rpm for 5 minutes. According to the manufacturer's instructions, the density of cells used was 106 cells/mL after addition of 400 uL Annexin V binding fluid. Cells were re-stained with 10uL PI for 5 minutes and lightly placed at 4 °C in dark before being immediately measured with a laser eight-color flow cytometer (FACSVerse, BD, USA) and quantified using FlowJo 7.6 software.

6 Western blot analysis

Western blotting. The kidney tissues or cells were lysed in protein lysis buffer (50mM Tris-HCl, 250 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 2 mM EDTA, and 1mM Na3VO4) and supplemented with 1×protease inhibitor cocktail (Roche, 04693132001) for 20 min on ice, followed by centrifugation for 15 min at 4°C at 12,000 rpm. The protein concentration was measured using the Bradford method. Western blotting analysis was performed with 30 μg of total protein, following standard methods. Antibodies against NLRP3, caspase-1 (1:1500, all from AdipoGen, San Diego, CA), SIRT1 (1:1500, Millipore, Billerica, MA, USA) , β-actin(1:3000, A2228, sigma) were diluted in 5% nonfat milk prepared in TBST (Tris-buffered saline, 0.1% Tween 20). Peroxidase-conjugated goat anti-rabbit secondary antibodies (Beyotime, A0208) (59) were used at a dilution of 1:5000. An enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA) was used to detect immunoblotted bands. Densitometric analysis was performed using ImageJ (Wayne Rasband, National Institutes of Health, USA).

7 Statistical analysis

Results are presented as means ± SEM and were analyzed using one-way ANOVA with Scheffe's test. P value <.05 was considered statistically significant for all experiments.

Results

2.1 β-sitosterol improves renal function and albuminuria in model rats

Within 8w after administration, compared with the normal control group (NC group), the renal function of the model group changed significantly, as shown in figure 1, ALB increased obviously (figure 1A, $P < 0.01$), CREA (figure 1C, $P < 0.005$) and BUN (figure 1D, $P < 0.05$) decreased significantly, these results demonstrate that a model of acute nephritis was successfully established. Meanwhile, figures 1 show that the ALB (figure 1A), CREA (figure 1C) and BUN (figure 1D) in the β-sitosterol group improved significantly compared with those in the model group. Specifically, 44.47±3.35g/L, 74.03±4.31 μmol/L and 6.88±1.17 mmol/L in high dose group (HD group) , 40.32±3.22g/L, 83.35±3.38 μmol/L and 8.19±1.09 mmol/L in low dose group(LD group). It is worth noting that the therapeutic effect of LD group is better than that of HD group. TP (figure 1B) was no significant difference among these four groups.

2.2 β-sitosterol improves renal pathology in model rats

After HE staining, There was abnormal changes among 4 groups under microscope. As shown in the figure2A, the glomerulus in the NC group has clear structure, normal size, and the arrangement of renal tubular epithelial cells is regular and orderly. In the AC group, the glomerular volume increased significantly, some renal tubular epithelial cells were vacuolar degeneration, and a small amount of inflammatory cell infiltration was observed; After β-sitosterol intervention, compared with the AC group, the glomerular volume increased, the degree of renal tubular vacuolar degeneration decreased and the inflammatory cell infiltration decreased in each dose group.

The results of Masson staining as shown in the figure 2B, the collagen fibers of glomerulus, renal tubules and interstitium in the NC group were stained normally. Compared with the NC group, the glomerular basement membrane in the AC group was thickened, the blue staining of collagen fibers was significantly increased, vacuolar degeneration was observed in some renal tubules, and the blue staining of collagen fibers in renal interstitium was increased. After β-sitosterol intervention, compared with the AC group, the thickening of glomerular basement membrane in each dose group decreased in varying degrees, and the blue staining of glomerular basement membrane and renal interstitial collagen fibers decreased.

Next, TGF in tissues was detected by immunohistochemistry, as shown in figure 2C, brown staining particles can be seen in renal tubular epithelial cells of the normal control group, indicating the expression

of TGF. Compared with the normal NC group, only a small amount of brown staining particles can be seen in renal tubular epithelial cells of the AC group, indicating that the expression of TGF is significantly weakened. After β-sitosterol intervention, compared with the AC group, the brown stained granules of renal tubular epithelial cells in HD and LD groups increased in varying degrees, suggesting that the expression of TGF recovered.

2.3 β-sitosterol improves renal Inflammatory level in model rats

For detection the effects of β-sitosterol in vivo, the inflammatory factors MCP-1, iNOS and TNF-α, IL-1 β and IL-6 was detected. At week 8, MCP-1, iNOS levels in AC group were significantly higher than those in NC group (figure 3A and B, $P < 0.001$), IL-1 β, IL-6 and TNF-α levels were higher than those in NC group (figure 3C, D and E, $P < 0.005$). The levels of inflammatory factors in LD group and HD group were significantly lower than those in AC group, and those in HD group were significantly lower than those in LD group (figure 3). The difference of some indexes was statistically significant. Specifically, 3.35±0.25ng/L, 0.31±0.01 U/mg□51.25±5.42 pg/mL, 61.67±4.62 pg/mL and 39.32±4.71 pg/mL in high dose group (HD group) , 5.49±0.15ng/L, 0.35±0.04 U/mg, 68.66±8.49 pg/mL, 73.67±6.49 pg/mL and 51.88±5.93 pg/mL in in low dose group(LD group). It is worth noting that the therapeutic effect of LD group is better than that of HD group. There was no significant difference in the therapeutic effect between the high-dose group and the low-dose group, indicating that low-dose β-sitosterol can achieve the effect of reducing inflammation.

2.4 β- Sitosterol reduces the level of apoptosis

Annexin V-FITC/PI staining and flow cytometric analysis were performed to determine the degree of cell apoptosis in all groups. As shown in figure 4A and B, the apoptosis rate in AC group (Q2, 16.6%, Q3,14.8%) increased significantly compare with NC group (Q2, 2.01%, Q3,2.61%). This apoptosis caused by inflammation can be improved by β-sitosterol. The apoptosis rates in HD and LD group both decreased significantly, Specifically, Q2, 5.57%, Q3, 5.56% in HD group and Q2, 8.04%, Q3, 7.70% in HD group (figure 4C and D). It is worth noting that the therapeutic effect of HD group is better than that of LD group.

2.5 β-sitosterol enhances SIRT1-induced autophagy and inhibits NLRP3 inflammasome

We further tested whether β-sitosterol inhibits the activation of NLRP3 inflammasome through the activation of Sirt1. Western blot results showed that compared with the NC group, the expression of sirt in the AC group was significantly decreased, and the LD group and HD group could increase the expression

of sirt to a certain extent in renal tissue (figure 5A). Meanwhile, we found that the expressions of NLRP3 and caspase-1 were significantly increased in the AC group, while their expressions were decreased in both the LD and HD groups(figure 5A). Quantitative analysis of blot showed significant differences between the LD and HD groups compared to the AC group (figure 5B, C and D) These findings suggest that β-sitosterol inhibits NLRP3 inflammasome by activating the expression of SIRT1-mediate autophagy.

Discussion

Inflammation is the early stage of a number of human key diseases, and its improper control may lead to serious diseases. Nephritis is caused by a variety of pathogens, lead to allergic damage to glomerular capillary basement membrane, edema, proteinuria and other symptoms [11]. Traditional treatment methods mainly focus on drugs which can anti-infection, lowering blood pressure or other effects, these treatments always not ideal, and there are toxic and negative effects. At present, many studies are looking for new drugs with better characteristics than drugs on the market. In these studies, plants with anti-inflammatory properties have potential value. The medicinal effect of plant extracts is due to the synergy between the identified compounds, including flavonoids, tannins, acetylketone derivatives and sterols [12, 13]. In addition, due to the existence of a variety of useful chemicals in plants, drug plants can act on a variety of diseases, which complicates the correlation between drug and disease. Therefore, another effective research way is to determine the pharmacological effects induced by specific compounds.

Phytosterola are plant derived steroids and part of plant cell membrane. They have similar structure with cholesterol. In this study, the effects of β-sitosterol in acute nephritis was evaluated by establishing a rat model. The results show that, β-sitosterol has significant anti-inflammatory potential in inflammatory model. β-sitosterol improved renal function, proteinuria and renal pathology in model rats, clears inflammasome through SIRT mediated autophagy at the meantime.

Among the activation signals of NLRP3 inflammasome, the activation of NLRP3 inflammasome leads to the secretion of IL-1β [14]. In this study, we proved that β-sitosterol inhibits the secretion of IL-1β. NF-κB can activate inflammatory signaling pathway, promote the release of inflammatory related factors, the assembly of inflammasome, activate NF-κB p65 transfers to the nucleus and promotes the expression of NLRP3 inflammasome related modules, such as NLRP3, proIL − 1β and proIL − 18. The study found that the protective effect of β-sitosterol on inflammation related diseases may be due to its regulating effect of NF-κB pathway [15, 16]. Our results showed that, β-sitosterol significantly inhibited the phosphorylation of NF-κB p65 and thus reducing the activation of NLRP3 inflammasome. NLRP3 inflammasome, as a component of systemic immunity, plays an important role in cell homeostasis and can be activated by various intracellular and extracellular stimuli [17]. Human diseases are often the result of the destruction of cell homeostasis. When cell homeostasis is not restored, it will lead to continuous dysfunctions. Significantly, autophagy depends on the autophagy-lysosomal pathway and plays a crucial role in maintaining and reversing abnormal cell homeostasis [18]. In addition, the autophagy deficiency in human aortic endothelial cells can promote the activation of NLRP3 inflammasome [19]. Another

research also showed that autophagy was negatively correlated with neuritis [20]. This study shows that β-sitosterol promotes autophagy mediated by SIRT and promotes the clearance of NLRP3 inflammatory body. Therefore, the immune regulation of NF-κB/NLRP3 inflammasome and SIRT1/autophagy axis together constitute the mechanism of β-sitosterol in acute nephritis.

In summary, this study found that β-sitosterol showed therapeutic effects on acute nephritis in rats, including promote renal function, reduce proteinuria and improve renal pathology. Mechanically, β-sitosterol inhibit the formation of inflammasome through NF-κB and autophagy. Our results provide a theoretical reference for β-sitosterol use as a treatment of acute nephritis.

Declarations

Ethics approval and consent to participate

Animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals at Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine and approved by the ethics committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine. Informed consent is not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published

article

Competing interests

The authors declare that they have no competing interests

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none.

Authors’ contributions

Jianrao Lu conceived and designed the study. Jing Hu designed the experiments. Jing Hu performed the experiments and analyzed the data. Jianrao Lu wrote the manuscript. All authors commented on the manuscript.

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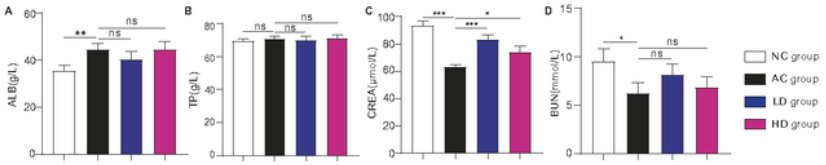
Figures

Figure 1



Renal function index	Experimental groups			
	NC group	AC group	LD group	HD group
ALB(g/L)	35.23 ± 2.46	44.53 ± 2.61	40.32 ± 3.22	44.47 ± 3.35
TP(g/L)	69.34 ± 1.47	70.77 ± 1.62	70.12 ± 2.17	70.88 ± 2.31
CREA (μmol/L)	93.22 ± 3.39	63.35 ± 1.25	83.35 ± 3.38	74.03 ± 4.31
BUN (mmol/L)	9.52 ± 1.31	6.27 ± 1.04	8.19 ± 1.09	6.88 ± 1.17

Figure 1

Effects of β-sitosterol on renal function in diabetic nephropathy rats

Albuminuria and renal function. A, ALB. B, TP. C, CREA. D, BUN. Data are expressed as means ± SEM for ten rats per group. ALB, Urine albumin; TP, total protein; CREA, creatinine; BUN, blood urea nitrogen; *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, ns, no significant difference.

Figure 2

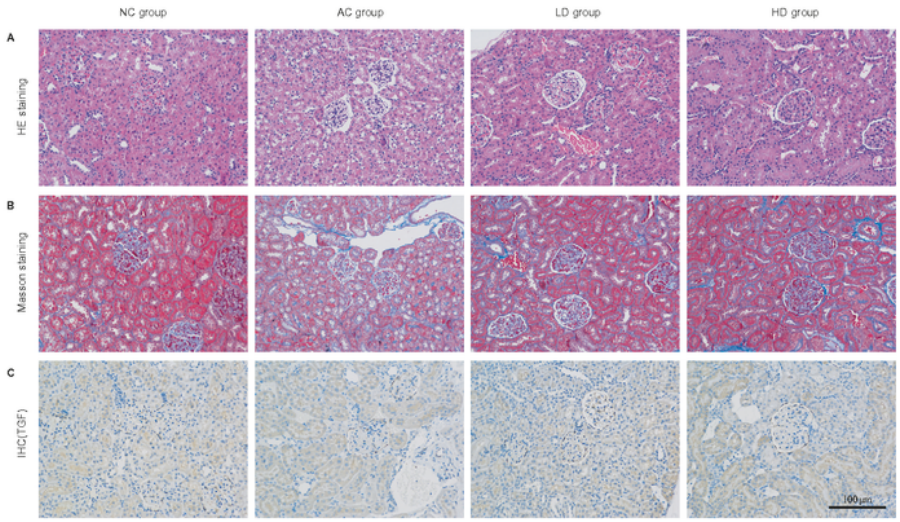
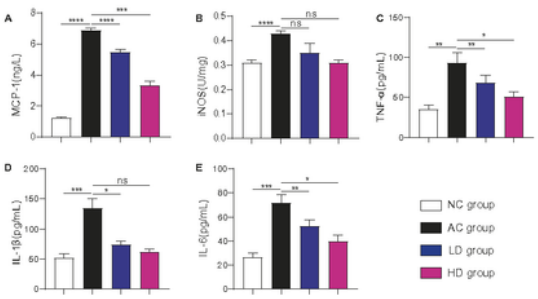


Figure 2

Pathological observation

A, H&E staining. B Masson staining. C Anti-TGF immunohistochemistry. Scale bars = 100 μm.

Figure 3



Inflammatory factors	Experimental groups			
	NC group	AC group	LD group	HD group
MCP-1 (ng/L)	6.91 ± 0.11	1.25 ± 0.05	5.49 ± 0.15	3.35 ± 0.25
iNOS (U/mg)	0.43 ± 0.01	0.31 ± 0.01	0.35 ± 0.04	0.31 ± 0.01
TNF-α (pg/mL)	93.39 ± 12.47	34.97 ± 5.47	68.66 ± 8.49	51.25 ± 5.42
IL-1β (pg/mL)	135.14 ± 15.55	51.47 ± 7.44	73.67 ± 6.49	61.67 ± 4.62
IL-6 (pg/mL)	71.63 ± 6.87	26.17 ± 3.73	51.88 ± 5.93	39.32 ± 4.71

Figure 3

Effect of β-sitosterol on kidney inflammation in vivo

nflammatory factors level analysis. A, MCP-1. B, iNOS. C, TNF-α. D, IL-1β. E, IL-6. Data are expressed as means ± SEM for ten rats per group. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, ns, no significant difference.

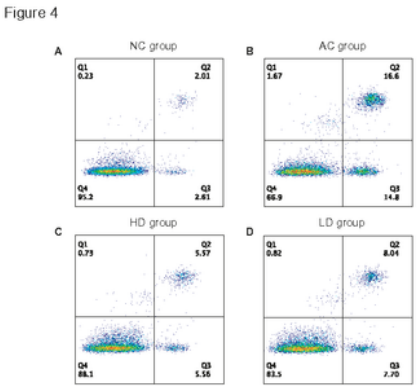


Figure 4

Flow cytometry of renal cells shows decreased percentage of apoptotic and necrotic cells in cisplatin-treated renal cells. A, NC group. B, AC group. C, HD group. D, LD group.

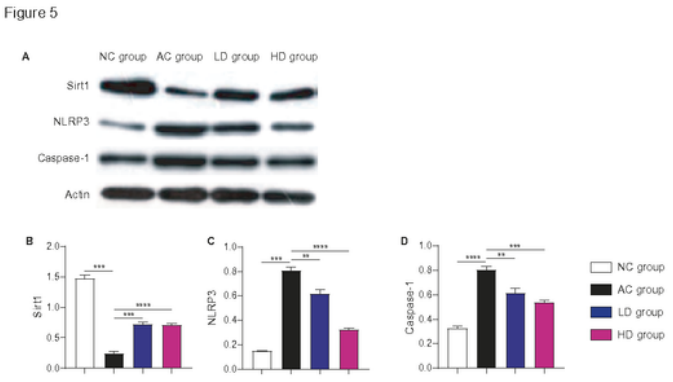


Figure 5

Effect of β-sitosterol on renal mesangial cell inflammation in vitro

A, Renal Sirt1, NLRP3 and Caspase-1 expression by Western blot analysis and semiquantitative analysis. Data are expressed as means ± SEM for ten rats per group. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, ns, no significant difference.