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Original Article

Effect of splenectomy on attenuation of LPS-induced AKI through GTS-21-induced cholinergic anti-inflammatory pathway

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Abstract: This work was undertaken to explore the role of splenectomy on attenuation of lipopolysaccharide (LPS)-induced acute kidney injury (AKI) through GTS-21-induced cholinergic anti-inflammatory pathway. C57BL/6 mice were used to construct models of sepsis-induced renal injury. HE, Tunel and blood assays were used to determine the success of the model. The animals were examined after splenectomy with or without LPS and GTS-21+LPS treatments. The pathological changes and apoptosis in the renal tissue were detected using HE and Tunel assays. The contents of creatinine (Cr) and cystatin-C (Cys-C) were measured using ELISA. The expression of IL-6, NF-kB p65, Caspase-3, anti-apoptotic protein Bcl-2, apoptotic protein Bax and α7nAChR was quantified using qRT-PCR. The expression of Bcl-2, Bax, Caspase-3, IL-6, NF-kB p65, α7nAChR and p-STAT3 was using assessed using Western blot analysis. HE, Tunel, BUN and serum creatinine (SC) assay showed that renal injury models were successfully established. Compared with the control, the apoptosis in the LPS group was significantly increased and decreased after GTS-21 treatment. However, splenectomy combined with GTS-21 increased the apoptosis, indicating that splenectomy could partially offset the anti-apoptosis effect of GTS-21. In animals treated with LPS, the contents of Cr and Cys-C increased significantly. These contents reduced following GTS-21 treatment, but increased after splenectomy. After LPS treatment, the expression of IL-6, NF-kB p65, p-STAT3, Caspase-3 and Bax was significantly up-regulated, while the expression of α7nAChR and Bcl-2 significantly down-regulated. Compared with LPS treated mice, splenectomy reduced the expression of IL-6, NF-kB p65 and p-STAT3, suggesting that splenectomy inhibits the activation of α7nAChR pathway by the GTS-21. It is clear that GTS-21 effectively attenuates LPS-induced renal injury; splenectomy suppresses the anti-inflammatory and anti-apoptosis activity and renal protective effect of GTS-21. On other hand, splenectomy reduces the production of inflammatory cytokines in the circulation, and has certain protective effect on the kidney. Therefore, the impact of splenectomy on LPS-induced AKI depends on the strength of the two aspects.

Keywords: Splenectomy, GTS-21, AKI, LPS, inflammatory reaction, apoptosis, cholinergic anti-inflammatory pathway

Introduction

Acute kidney injury (AKI) is a common clinical syndrome, mainly manifested as the accumulation of metabolic substances and declined renal functions [1, 2]. Cholinergic anti-inflammatory pathway (CAP) is considered as one of the protective mechanisms for AKI [3, 4]. CAP is an anti-inflammatory immunomodulatory pathway that plays an anti-inflammatory effect through acetylcholine and vagus nerves [5]. GTS-21 is a selective α7 subtype N acetylcholine receptor agonist, a derivative of anisine. Studies have shown that GTS-21 reduces the

endotoxin-induced expression of TNF and IL-1β, and has a better anti-inflammatory effect than nicotine [6]. Many studies show that GTS-21 is a very effective immunomodulatory drug that can attenuate pancreatitis, improve the survival of septicemia model and reduce the endotoxin-induced TNF level in the lung tissue [7-9]. Splenectomy is shown to block the anti-inflammatory response of the vagus and cholinergic agonists [10], that is, with splenectomy, stimulation of the vagus nerve and the use of cholinergic agonists can not regulate the inflammatory response [11]. In the case of fatal endotoxemia and sepsis, splenectomy prevents the activation of CAP [12]. Therefore, a complete neural circuit from the vagus to spleen is necessary to protect CAP. On the other hand, the spleen is an important source of proinflammatory cytokines in the circulation [12, 13], and the immune cells of the spleen play a vital role in the development of sepsis. Therefore, preventive splenectomy is protective for the development of sepsis [14]. It was reported that when the spleen is intact, the inhibition of proinflammatory cytokines produced by the vagus nerve is comparable to that of preventive splenectomy [5]. In short, the effect of preventive splenectomy on organ protection in sepsis is very complicated. Therefore, in this study, we used lipopolysaccharide (LPS) to establish mouse models of sepsis renal injury, and intervened them with GTS-21 and preventive splenectomy. These animals were studied for the protective effect of GTS-21 on renal injury and the impact of preventive splenectomy on the protective effect. The findings would provide new clues for better treatment of sepsis-induced renal injury.

Materials and methods

Experimental animals

Male C57BL/6 mice were purchased from the Slackking Laboratory Animal Co., Ltd., Hunan (permit no. SCXK (Xiang) 2016-0002). Animals were housed in non-toxic plastic boxes with stainless steel wire cage on metal racks. The racks were sterilized before use. Mouse experiments were performed under a protocol approved by Harbin Medical University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All procedures were performed under anesthesia and all efforts were made to minimize pain or suffering.

Reagents and instruments

Pentobarbital sodium (P3761) and GTS-21 (SML0326) were purchased from Sigma, USA; LPS (607i031) was from Solarbio, USA; mouse cystatin-C (Cys-C) (m1001886) and creatinine (Cr) (m1037726) ELISA kits were purchased from Mlbio, Shanghai, China; ultrapure RNA Extraction Kit (CW0581M), HiFiScript cDNA first chain synthesis kit (CW2569M), UltraSYBR Mixture (CW0957M) were products of CWBIO, Beijing, China; TUNEL assay kit (C1086) was from Beyotime Biotechnology, Beijing, China; antibodies against p-TAT3 (ab32143, 1/5000), Bcl-2 (ab692, 1/500), Bax (ab32503, 1/5000), and NF-kB p65 (ab32536, 1/50000) were from Abcam, UK; antibodies against α7nAChR (bs-1049R, 1/1500), Caspase-3 (sm-33284M, 1/1000) and IL-6 (bs-0379R, 1/1000) were purchased from Bioss, USA. Fluorescent PCR instrument (CFX Connect™), fluorescence microscope (742BR1154) and Gel imaging system (ChemiDocTM XRS) were product of Bio-Rad, USA.

Modelling and splenectomy

Models of sepsis-induced renal injury were established by intraperitoneally injection with LPS at 10 mg/kg. The injected animals were examined using HE and Tunel assays for success of modelling. The models were weighed and anesthetized by intraperitoneally injection of 0.6% pentobarbital sodium at a dose of 70 mg/kg, shaved and sterilized with iodophor on the surgical area and its surrounding area. The abdominal cavity was opened at the position of the spleen and the spleen was removed. After the removal, the wound was sutured. After splenectomy, the mice were raised for 7 days before being used for drug treatments.

Experimental grouping

Control was normally reared male C57BL/6. Mice injected intraperitoneally with the same amount of normal saline was used as sham; for LPS and GTS-21+LPS groups, mice were intraperitoneally injected with LPS at 10 mg/kg and GTS-21 (8 mg/kg) 1 h before LPS injection. The same schemes of drugs treatments were ap-

Table 1. Primers for qRT-PCR

Primer	Sequence
IL-6 F	TGCCTTCTTGGGACTGATG
IL-6 R	ACTCTGGCTTTGTCTTTCTTGT
α7nACHR F	TACGCTGGTTCCCTTTTGA
α7nACHR R	GTGACATCTGGGTATGGCTCT
Caspase-3 F	GACTGGAAAGCCGAAACTCT
Caspase-3 R	CCACTGTCTGTCTCAATGCC
Bax F	CAGGATGCGTCCACCAA
Bax R	AAAGTAGAAGAGGGCAACCAC
NF-kB p65 F	TCTGTTTCCCCTCATCTTTCC
NF-kB p65 R	TGTCTTGGTGGTATCTGTGCTT
BCL-2 F	CCCTGGCATCTTCTCCTTC
BCL-2 R	AGAGTTCCTCCACCACCGT
α7nACHR F	TACGCTGGTTCCCTTTTGA
α7nACHR R	GTGACATCTGGGTATGGCTCT
GAPDH F	AAGAAGGTGGTGAAGCAGG
GAPDH R	GAAGGTGGAAGAGTGGGAGT

plied to mice receiving preventive splenectomy to generate Splenectomy, Splenectomy+LPS and Splenectomy+GTS-21+LPS groups.

HE staining

Kidney tissue of appropriate size was harvested, rinsed with PBS, fixed in 10% neutral formaldehyde solution and embedding in paraffin. The tissue was sliced, stained with HE as previously described [15] and observed under an optical microscope.

Tunel assay

The Tunel assay was used to detect apoptosis as reported previously [16]. The tissue slices were stained with Tunel solution and examined for apoptosis according to the supplier's instructions.

ELISA

ELISA assays were performed using commercial ELISA kits (mouse SC kit, MM-09221M1, mouse BUN kit, MM-0692M1, mouse UP kit, MM-44287M1) according to the supplier's instructions.

Fluorescence quantitative PCR

RNA was extracted from the kidney tissues using the ultrapure RNA Extraction Kit according to the manufacturer's instructions. cDNA was synthesized using HiFiScript cDNA first chain synthesis kit according to the manufacturer's instructions and used as template for fluorescence quantitative PCR on CFX Connect™ instrument. The PCR was carried out in a total volume of 15 μl containing 1.5 μl of diluted and pre-amplified cDNA, 10 μl of UltraSYBR Mixture and 1 μl of each fluorescence probe using the primers listed in **Table 1**. The cycling conditions were 50°C for 2 min, 94°C for 10 min followed by 40 cycles, each one consisting of 15 s at 94°C and 1 min at 61°C. Samples were run in triplicate and the mean value was calculated for each case and β-actin was used as internal reference.

The data were managed using the Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated by using comparative Ct method and obtaining the fold change value ($2^{-\Delta\Delta Ct}$) according to previously described protocol [17].

Western blot analysis

Proteins were extracted from the renal tissue in RIPA buffer that containing protease and phosphotase inhibitors cocktail (Roche, UK). The supernatants were collected after centrifugation at 12000 rpm for 20 min. The protein was applied to polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and then detected by the proper primary and secondary antibodies before visualization with a chemiluminescence kit. The intensity of blot signals was quantitated using Quantity one software (General Electric, UK).

Statistical analysis

All data were expressed as means ± standard error of the mean (SEM) obtained from at least three independent experiments. Statistical comparisons between experimental and control groups were assessed by using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Renal injury models

Sepsis-induced renal injury models of mouse were established by intraperitoneally injecting LPS. HE staining of renal tissue showed that

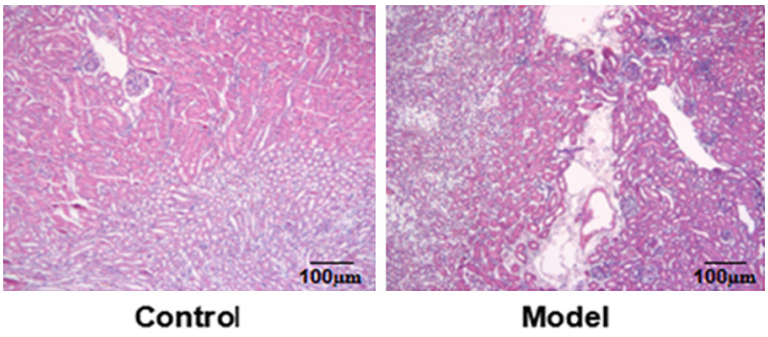
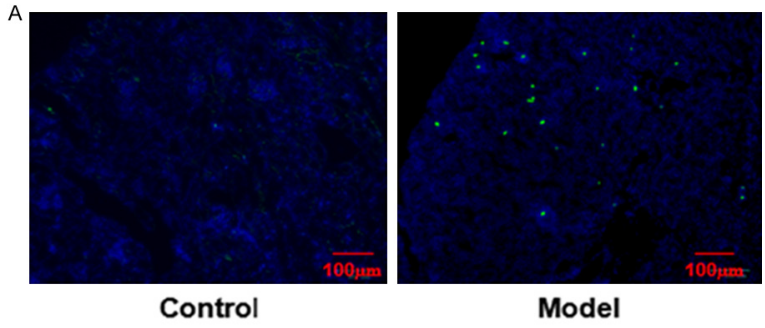


Figure 1. HE staining of renal tissue following LPS injection.



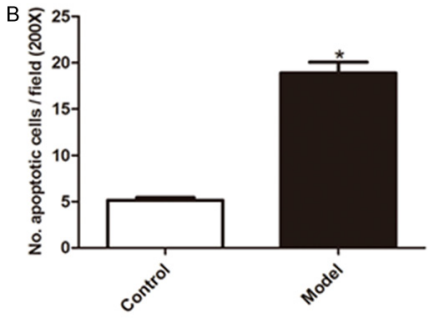


Figure 2. Tunel assay of renal tissue following LPS injection. A. Microphoto of real tissue; B. No. of apoptotic cells. *Denotes significant difference vs control.

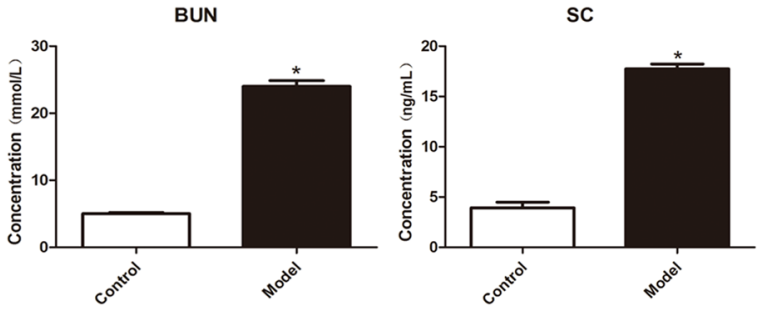


Figure 3. Blood urea nitrogen (BUN) and serum creatinine (SC) contents in mice following LPS injection. *Denotes significant difference vs control.

normal mice had uniform staining and normal morphology without leakage of red cells and infiltration of inflammatory cells, while the model had increased size of kidney with pale cortex. The renal medulla had blood stasis and was dark in color and the renal tubules were degraded and dead due to severe ischemia (**Figure 1**). Tunel assay showed significantly more apoptosis in the models than in control (**Figure 2**). Furthermore, the blood urea nitrogen (BUN) and serum creatinine (SC) were significantly increased in the models (**Figure 3**). These data suggested that the modelling is successful.

Pathological changes

Examination showed that the size of kidney from LPS and splenectomy+GTS-21+LPS was larger and HE staining revealed that the real tissues in the two groups had pale cortex tissue with medullary congestion and dead renal tubules due to severe ischemia (**Figure 4**). In GTS-21+LPS treated mice, the size of glomerulus was normal but the tubular epithelial cells were swollen, fatty and vacuolarized. After splenectomy and LPS treatment, the epithelial cells of renal tubule showed various degree of necrosis, leading to nuclear pyknosis, dissolution and disappearance.

Apoptosis

Tunel results showed higher apoptosis in the renal tissue following LPS injection, while GTS-21 reduced apoptosis (**Figure 5**). However, use of GTS-21 following splenectomy significantly increased apoptosis, suggesting that splenectomy could partially block the anti-apoptosis effect of GTS-21.

Cr and Cys-C content

As shown in **Figure 6**, compared with the control, the content of Cr and Cys-C in the LPS and

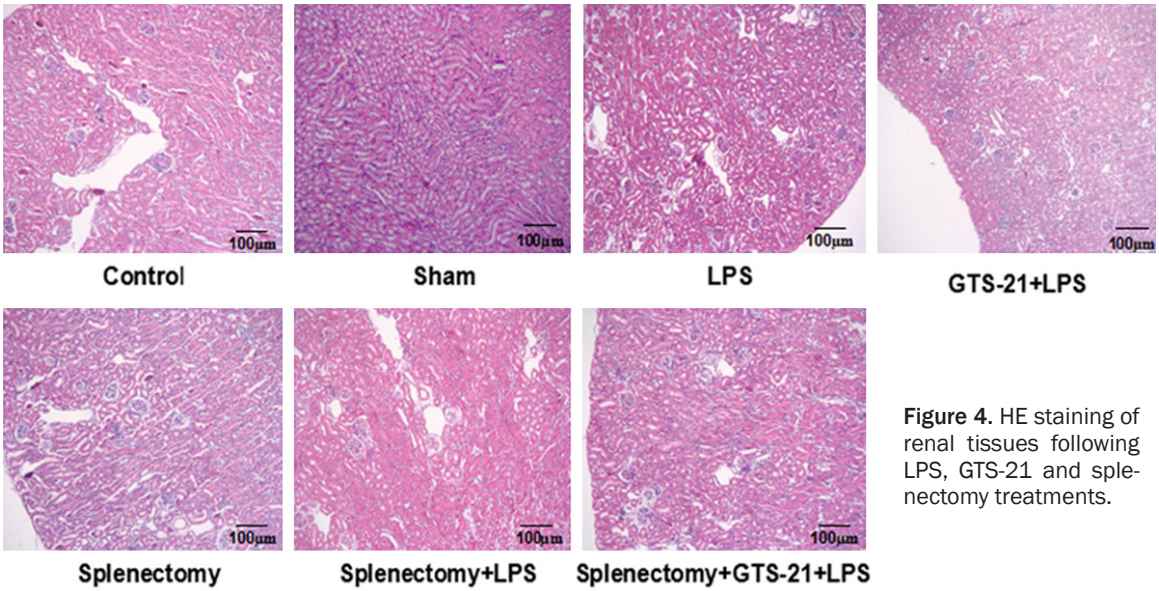


Figure 4. HE staining of renal tissues following LPS, GTS-21 and splenectomy treatments.

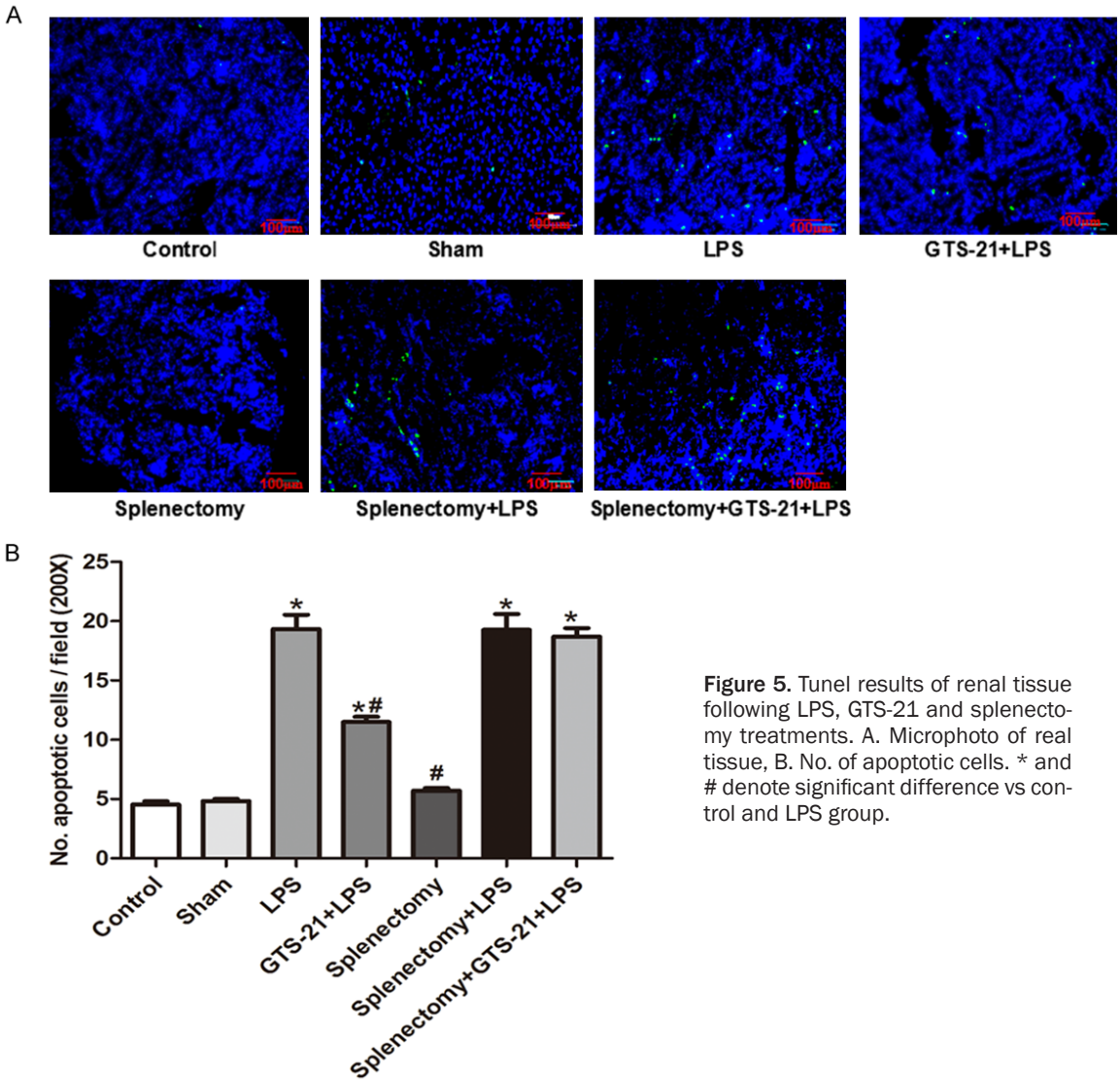


Figure 5. Tunel results of renal tissue following LPS, GTS-21 and splenectomy treatments. A. Microphoto of real tissue, B. No. of apoptotic cells. * and # denote significant difference vs control and LPS group.

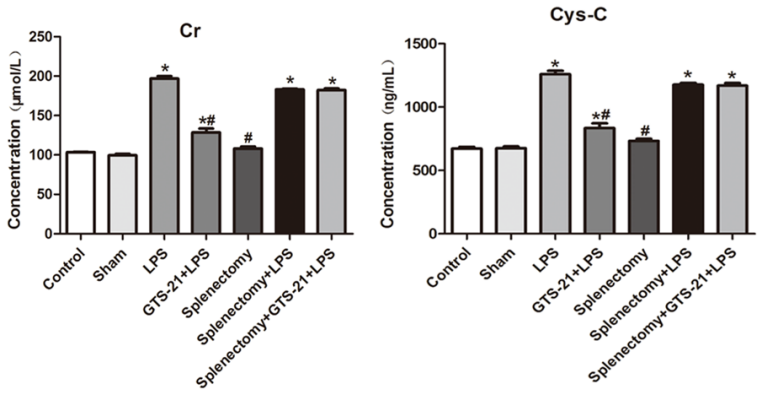


Figure 6. Cr and Cys-C contents in renal tissue following LPS, GTS-21 and splenectomy treatments. * and # denote significant difference vs control and LPS group.

GTS-21 groups increased significantly. After preventive splenectomy, GTS-21 increased the contents.

Leukocytes, neutrophils, SC, BUN, proteinuria

Compared with control, the counts of leukocytes and neutrophils and contents of SC, BUN and proteinuria were significantly increased after LPS treatment. GTS-21 treatment reduced the increase, while splenectomy increased the counts of leukocytes and neutrophils (**Figure 7**).

Gene expression

Compared with the control, the mRNA levels of IL-6, NF-kB p65, p-STAT3, Caspase-3 and Bax were significantly up-regulated, while the mRNA levels of α7nAChR and Bcl-2 were significantly down-regulated in LPS-treated mice (**Figure 8A**). Compared with LPS group, mice treated with GTS-21 had significantly lower mRNA levels of IL-6, NF-kB p65, p-STAT3, Caspase-3 and Bax, and significantly higher levels of α7nAChR and Bcl-2 (**Figure 8A**).

Compared with LPS or GTS-21+LPS, splenectomy reduced the mRNA levels of IL-6, NF-kB p65 and p-STAT3, or α7nAChR (**Figure 8A**), suggesting that splenectomy inhibits the activation of the α7nAChR pathway by GTS-21. Similar results were observed at protein levels for these genes (**Figure 8B**).

Discussion

LPS is a commonly used agent to model sepsis, which increases the expression of various inflammatory factors and triggers septic shock [18]. Intravenous injection of LPS stimulates the release of inflammatory mediators in different types of cells, initiate sepsis process, and then cause systemic sepsis that leads to multiple organ dysfunction syndrome (MODS) [18]. Sepsis plays a very important role in the occurrence and development of sepsis, and can cause renal tissue damage and apoptosis. GTS-21 is a α7nAChR-specific agonist. Studies have shown that GTS-21 can prevent from renal injury induced by mechanical ventilation [19]. Preventive splenectomy is shown to offset the protective effect of GTS-21 on the kidney [14].

In this study, LPS was used to establish a model of sepsis-induced renal injury. After treatment with GTS-21, the number of injured renal cells and apoptosis was reduced. However, the damage to renal tissue and apoptosis were not effectively alleviated when GTS-21 was used after splenic resection. In order to further confirm the results of the study at molecular level, the expression of apoptosis-related proteins Caspase-3, Bcl-2 and Bax was measured. Caspase-3 and Bax were found increased and anti-apoptotic Bcl-2 protein decreased after LPS treatment. On other hand, When LPS was given after splenic removal, the expression of Caspase-3 and Bax increased significantly, while the expression of Bcl-2 decreased significantly although the expression of Caspase-3, Bax and Bcl-2 remained unchanged as compared with LPS treatment. The expression of apoptosis-related proteins and Bax was significantly decreased when the mice were treated with GTS-21, suggesting that GTS-21 can reduce the expression of apoptotic proteins and thus protect the kidneys, while preventive splenectomy can offset the protective effect of GTS-21 on the kidneys.

Once inflammation occurs, the levels of leukocytes and neutrophils increase significantly. Our results also showed that the levels of leukocytes and neutrophils were significantly increased after AKI model was established, and GTS-21 was helpful to alleviate the inflamm-

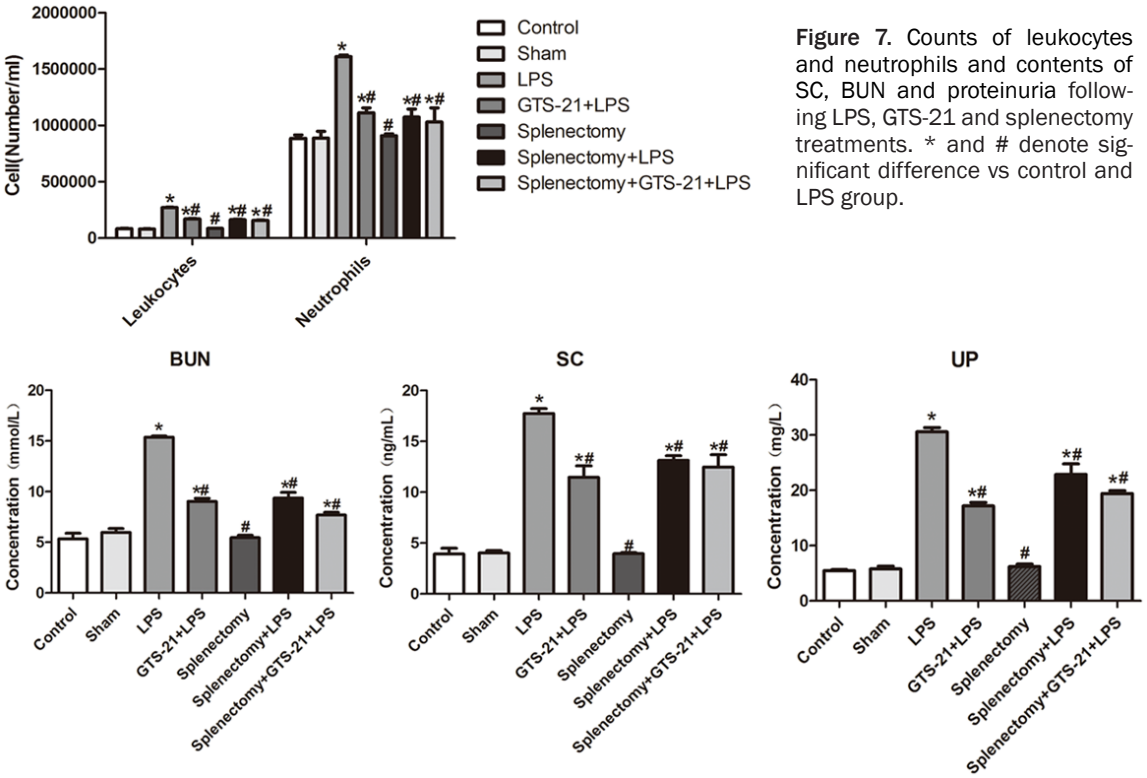


Figure 7. Counts of leukocytes and neutrophils and contents of SC, BUN and proteinuria following LPS, GTS-21 and splenectomy treatments. * and # denote significant difference vs control and LPS group.

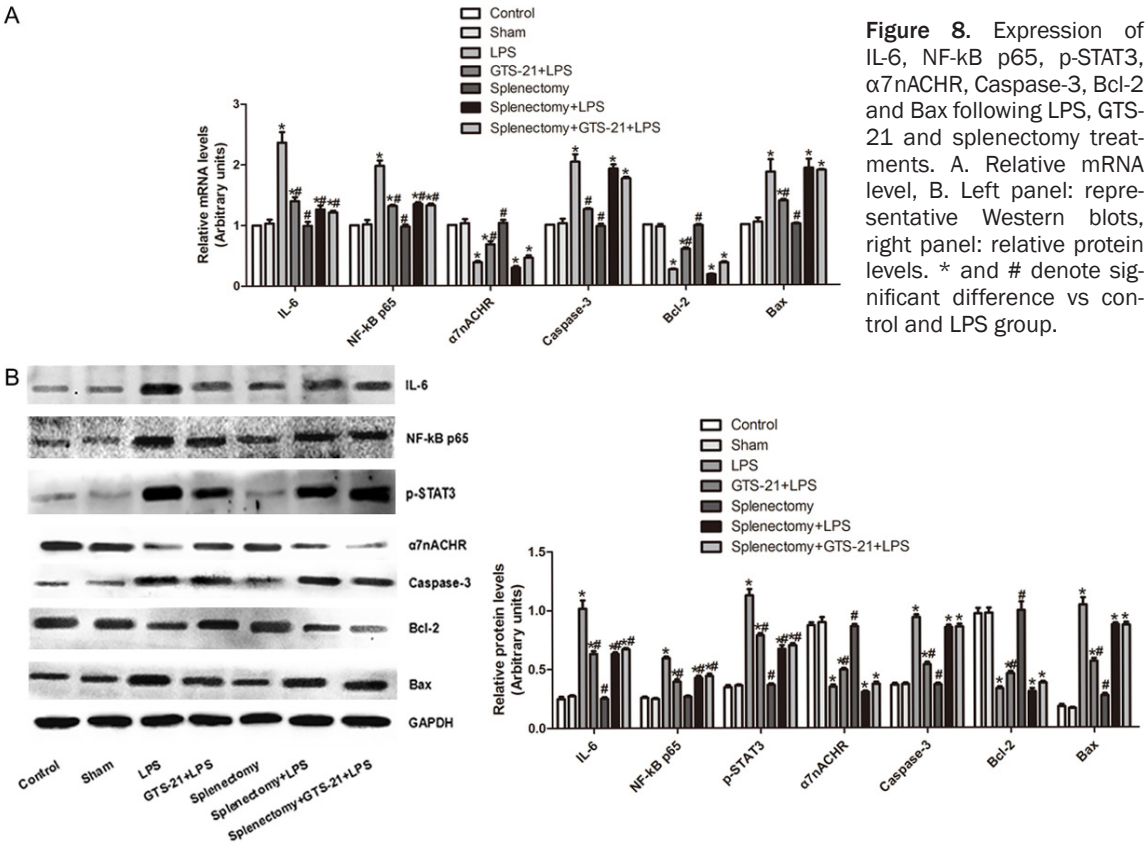


Figure 8. Expression of IL-6, NF-kB p65, p-STAT3, α7nACHR, Caspase-3, Bcl-2 and Bax following LPS, GTS-21 and splenectomy treatments. A. Relative mRNA level, B. Left panel: representative Western blots, right panel: relative protein levels. * and # denote significant difference vs control and LPS group.

ation effect, leading to reduced leukocyte and neutrophil counts. SC as the final product of creatine metabolism is almost not reabsorbed in renal tubules after glomerular filtration and proposed to be an AKI marker [20]. Renal parenchymal lesions, such as glomerulonephritis, interstitial nephritis, acute and chronic renal failure, intrarenal space occupying and destructive lesions, can increase BUN. Extra renal factors can also cause elevated BUN. Therefore, BUN is one of the diagnostic indicators of uremia [21]. When proteinuria occurs, the amount of proteins entering renal tubular epithelial cells increases and the activity of lysosomes increases, suggesting that proteins cause lysosomes to overflow into tubular cytoplasm, and subsequent cell damage can stimulate inflammation and scar formation. In our study, SC and BUN increased significantly after AKI model was established, while GTS-21 could alleviate inflammation and decrease SC and BUN.

As the final product of creatine metabolism, Cr and Cys-C can be used as a marker of AKI [21] which is hardly reabsorbed in renal tubules after glomerular filtration. Cys-C is a cysteine protease inhibitor [22]. The content of Cr and Cys-C increased significantly after the injection of LPS in the experimental animals, indicating that LPS could cause renal injury. The content of Cr and Cys-C decreased after the use of GTS-21, further demonstrating that GTS-21 has a protective effect on the kidney. On other hand, after preventive splenectomy, GTS-21 injection resulted in increased Cr and Cys-C contents, suggesting that splenectomy eliminates the protective effect of GTS-21 on the kidney.

CAP is a recently discovered systemic anti-inflammatory response pathway. In this pathway, α7nAChR is one of the most critical molecules and is the basis for its anti-inflammatory effect. The expression of α7nAChR was significantly lower in models than that in the control, while the expression of inflammatory-related factors IL-6 and NF-kB p65 increased. Studies have shown that there are inflammatory reaction and vagus inhibition, which can cause CAP dysfunction [7]. During severe sepsis, it can result in different levels of CAP dysfunction such as the abnormality of the vagus nerve, the impairment of nerve function, the excessive activation of the alkaline esterase, and the desensitization of the α7nAchR. As a result, the anti-inflammatory effect is weakened, and the excessive inflammatory factors lead to the final death of cells [23]. STAT as an important member of the JAK/STAT signaling pathway, which is activated by various cytokines. When the cell is stimulated, STAT binds to tyrosine residue to form p-STAT3, which transconducts the signal to the nucleus to regulate the expression of the corresponding genes, and then mediate the activation of cells and other important physiological functions. p-STAT3 increases the expression of inflammatory factors [24]. Abnormal activation of STAT3 signaling pathway can cause cell dysfunction, resulting in the occurrence and amplification of systemic inflammatory response. Studies have shown that various cytokines play an important role in the development of the inflammatory response by activating the JAK/STAT signaling pathway. These inflammatory factors increase significantly in AKI and aggravate the damage to glomerular endothelial cells and mesangial cells [25]. Our results showed that the expression of inflammation-related factors IL-6, NF-kB p65 and p-STAT3 increased while the expression of α7nAChR decreased, suggesting that α7nAChR plays an important role in the formation of inflammatory factors in the process of sepsis.

The results of GTS-21 intervention showed that, compared with the model group, the expression of IL-6, NF-kB p65 and p-STAT3 decreased significantly after GTS-21 intervention, and the expression of the α7nAChR gene increased significantly. This further suggests that the anti-inflammatory effect of GTS-21 is related to α7nAChR. Studies have shown that GTS-21 significantly reduces the production of inflammatory and proinflammatory factors, and improves the survival rate of endotoxemia [14]. Similarly, studies have shown that GTS-21 significantly reduces LPS-induced synthesis of inflammatory cytokines TNF-α [26]. GTS-21 is shown to restore the sensitivity to α7nAChR after desensitization and play an anti-inflammatory role [27]. We found no significant difference in the expression of IL-6, NF-kB p65 and p-STAT3 and α7nAChR between splenectomy+LPS and splenectomy+GTS-21+LPS groups, and reduced IL-6, NF-kB p65 as compared with the LPS group. These results suggest that splenectomy can block the anti-inflammatory effect of GTS-21. In addition, the study also showed that compared with GTS-

21+LPS, in mice treated with splenectomy+GTS-21+LPS the expression of α7nAChR decreased significantly, suggesting that splenectomy can block GTS-21 from alleviating LPS-induced AKI through the activation of CAP by αn AChR. As a result, the protective effect of GTS-21 on the kidneys is weakened. However, splenectomy can reduce the formation of proinflammatory cytokines in the circulation and reduce the content of proinflammatory cytokines in the kidney, which is protective for the kidneys.

In summary, we have shown that GTS-21 can effectively attenuate the damage of LPS to the kidney; splenectomy can block the anti-inflammatory, anti-apoptosis and protective effects of GTS-21 on the kidney. Furthermore, splenectomy can reduce the formation of inflammatory cytokines in the circulation and protect the kidneys. The impact of splenectomy on LPS-induced AKI depends on the above two aspects.

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Disclosure of conflict of interest

None.

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