

Original Article

GTS-21 attenuates LPS-induced renal injury via the cholinergic anti-inflammatory pathway in mice

Yang Gao^{1*}, Kai Kang^{2*}, Haitao Liu³, Weilan Kong², Qiuyuan Han¹, Xing Zhang¹, Rui Huang¹, Jingdong Qu¹, Hongliang Wang¹, Sicong Wang³, Ruijin Liu³, Yansong Liu³, Kaijiang Yu^{3,4}

¹Department of Critical Care Medicine, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, China; ²Department of Critical Care Medicine, The First Affiliated Hospital of Harbin Medical University, Harbin 150001, China; ³Department of Critical Care Medicine, The Cancer Hospital of Harbin Medical University, Harbin 150081, China; ⁴Institute of Critical Care Medicine in Sino Russian Medical Research Center of Harbin Medical University, Harbin 150081, China. *Equal contributors.

Received July 7, 2017; Accepted September 30, 2017; Epub October 15, 2017; Published October 30, 2017

Abstract: This study aimed to investigate the role of GTS-21 in cholinergic anti-inflammatory pathway-mediated protection of LPS-induced septic renal injury in mice. C57BL/6 mice were used to construct septic injury models. The optimal duration of lipopolysaccharide (LPS) treatment was determined using HE staining and TUNEL assay. Mice injected with saline were used as blank control and with LPS (10 mg/kg) as model, which were further treated with α -bungarotoxin (BT-LPS), GTS-21 (GTS-21-LPS) and BT and GTS-21 (BT-GTS-21-LPS). The pathological examinations were performed on HE stained renal tissues, apoptosis was determined using TUNEL assay, mRNA expression of NF- κ B p65, Caspase-3, Caspase-8, Bcl-2, Bax, p53 and α 7nACh was quantified using qRT-PCR, protein levels of IL-6, IL-1 β , TNF- α and phosphorylated STAT3 (p-STAT3) were analyzed using Western blots. HE staining and TUNEL assays showed that the optimal LPS treatment time for renal injury induction was 16 h. Compared with the blank control, mice in LPS group had significantly higher levels of NF- κ B p65, Caspase-3, Caspase-8, Bax, p53, IL-6, IL-1 β , TNF- α and p-STAT3, while α 7nAChR and Bcl-2 levels were decreased significantly ($P < 0.01$); GTS-21 and BT significantly increased the expression of NF- κ B p65, Caspase-3, Caspase-8, Bax, p53, IL-6, IL-1 β , TNF- α and p-STAT3, while α 7nAChR and Bcl-2 levels were decreased significantly ($P < 0.01$). It is concluded that GTS-21 can effectively alleviate the renal injury, while α 7nAChR-specific blocker BT is antagonistic against the anti-inflammatory effect of GTS-21 on sepsis in mice.

Keywords: Cholinergic anti-inflammatory pathway, GTS-21, renal injury, sepsis, gene expression

Introduction

Acute kidney injury (AKI) is a common clinical syndrome, which is characterized by the accumulation of metabolic substances and the decline of renal function [1, 2]. The incidence of AKI has been very high over the years and no effective medicines are available for the disease. AKI has poor prognosis with high mortality. Studies have shown that for some patients AKI may progress to chronic kidney disease, and even to end-stage renal disease. Cholinergic anti-inflammatory pathway (CAP) may be one of the protective mechanisms for AKI [3, 4]. As an anti-inflammatory immune regulatory pathway, it participates in the immune modulation through acetylcholine and vagus nerve to achieve

anti-inflammatory effect [5]. CAP can regulate the inflammatory response and reduce the inflammatory damage through the regulation of endogenous neural feedback mechanism. It is a fast, effective and controllable response. Studies have shown that CAP inhibits a variety of inflammatory factors, and is anti-inflammatory in multiple organs such as liver, kidney, lung, intestine and heart [6]. GTS-21, a derivative of anabasine, and is a selective α 7 subunit N-acetylcholine receptor agonist. GTS-21 can down-regulate the expression of endotoxin-induced TNF and IL-1 β and is better than nicotine as an anti-inflammatory agent [7]. Many studies have shown that GTS-21 is a very effective immunomodulatory drug and can attenuate pancreatitis disease, improve the survival rate of sepsis

Table 1. Primers for qRT-PCR

Gene	Primer
Caspase-8	F: CCGATTCTCTGGGCAACTGT R: ATCCACATGTGTCCCGTTCC
$\alpha 7$ -nAChR	F: ACCTCGTGTGATCCAAAGCC R: GGTTTCCTCTTGCTCAGGGT
Caspase-3	F: CCACAGCACCTGGTTATT R: ATTCTATCGCCACCTTCC
NF- κ B p65	F: CGACGTATTGCTGTGCCTTC R: TGAGATCTGCCAGGTGGTAA
Bax	F: CCAGGATGCGTCCACCAA R: AAAGTAGAAGAGGGCAACCAC
BCL-2	F: GTGGCCTTCTTTGAGTTCT R: ACCCAGCCTCCGTTATCC
P53	F: TGACGGAGGTCGTGAGAC R: CCAGATACTCGGGATACAAA
β -actin	F: CCTCTATGCCAACACAGTGC R: GTACTCCTGCTTGCTGATCC

model, and reduce the level of TNF induced by endotoxin in the lung tissues [8-10]. GTS-21 significantly decreased the levels of high mobility proteins in serum of severe sepsis mice induced by cecal ligation and improves their survival [7]. α -bungarotoxin (BT) is a neurotoxin, which binds specifically and irreversibly N-type acetylcholine receptor with high affinity. Therefore, in this study mouse kidney sepsis models induced by LPS were intervened with GTS-21 and BT to investigate the protection of GTS-21 on renal injury and role of BT in blocking the anti-inflammatory effect. The findings would provide new insights into clinical treatment of septic renal injury.

Materials and methods

Animals

Six-week-old specific-pathogen-free, C57BL/6 male mice weighing 23-27 g were purchased from Slack Experimental Animal Co, Hunan, China, and all mice were housed under pathogen-free conditions and had access to standard mouse food and water *ad libitum*. The animal studies were approved by Harbin Medical University Animal Care and Use Committee.

Animal model and drug treatment

Septic renal models were constructed by injection of LPS (10 mg/kg, Sigma, USA) via tail vein.

The duration of LPS treatment for successful modelling was determined using HE staining and TUNEL assay based on the severity of sepsis and apoptosis. For drug treatment, the animals were randomly grouped into control, model (LPS) (injected with LPS (10 mg/kg) only), BT-LPS (injected with α -bungarotoxin (1 μ g/kg, Sigma, USA) and LPS), GTS-21-LPS (injected with GTS-21 (8 mg/kg, Sigma, USA) and LPS) and BT-GTS-21-LPS (injected with α -bungarotoxin, GTS-21). For BT-LPS and GTS-21-LPS, BT and GTS-21 were injected 1 h before LPS, and for BT-GTS-21-LPS, BT and GTS-21 were injected 1 h before GTS-21 or before LPS, respectively.

HE staining and TUNEL assay

Renal tissue was washed with PBS, fixed in 10% neutral formaldehyde solution, embedded in paraffin and sectioned. The slides were stained with HE stain as described [11] and viewed under light microscope. The sections were also used to detect apoptosis using the TUNEL method as reported [12].

qRT-PCR

Total RNA was isolated from renal tissue using the TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quantity was measured by a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). RNA purity was evaluated by the A260/A280 ratio. Reverse transcription was performed with 200 ng of RNA in a total volume of 10 μ l using the High Capacity cDNA Transcriptase Reverse kit (Applied Biosystems by Life Technologies, Carlsbad, California, USA) according to manufacturer's recommendations. A total of 2.5 μ l of the resulting cDNA was subjected to pre-amplification using the SYBR Green qPCR SuperMix (Invitrogen, USA) in a total volume of 12 μ l. Non-fluorescent probes were used at 1X. Pre-amplification cycling conditions were 10 min at 95°C followed by 14 cycles, each one consisting of 15 s at 95°C and 4 min at 60°C. RT-qPCR was performed on the 7900HT Fast Real-Time PCR system using TaqMan gene expression assays probes (Applied Biosystems). Human β -actin was used as an internal control. The PCR was carried out in a total volume of 10 μ l containing 1.5 μ l of diluted and pre-amplified cDNA, 10 μ l of TaqMan Gene Expression Master Mix and 1 μ l

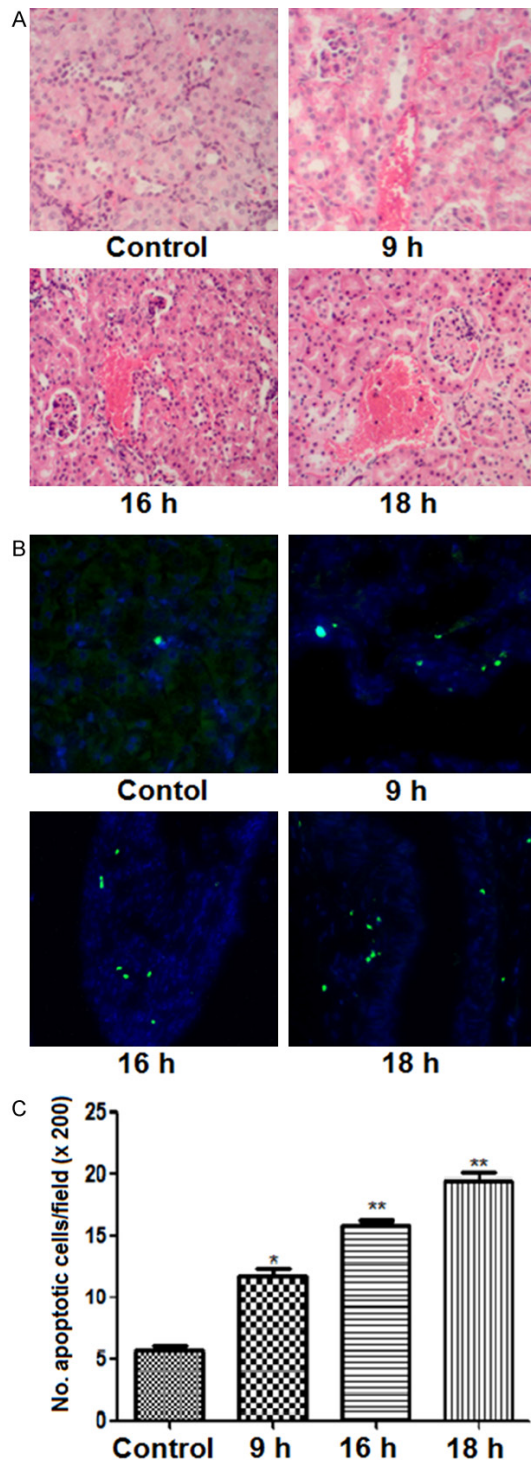


Figure 1. HE staining and TUNEL results of renal tissues following LPS treatments for different times (200 \times). A. HE staining, B. Microphotos of TUNEL assay, C. Number of apoptotic cells.

of each fluorescence TaqMan probe using primers listed in **Table 1**. The cycling conditions were 50°C for 2 min, 95°C for 10 min follow-

ed by 40 cycles, each one consisting of 15 s at 95°C and 1 min at 60°C. Samples were run in triplicate and the mean value was calculated for each case.

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 [13].

Western blot analysis

Tissue was washed twice with cold PBS and lysed with RIPA buffer that contains protease and phosphatase inhibitors (Roche, UK) for 30 min at 4°C. 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 (against IL-6, IL-1 β , TNF- α , NF- κ B p65, p-STAT3 and α 7nAChR, Abcam, USA) before visualization with a chemiluminescence kit. The intensity of blot signals was quantitated using ImageQuant TL analysis software (General Electric, UK).

Statistical analysis

All data were expressed as means \pm standard derivation (s.d.) 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

Optimal time for renal injury modelling

We treated the mice with LPS for different times and then examined their renal injury. The results showed that the renal tissues of normal mice were evenly stained with normal morphology, regularly arranged fibers and without leakage of red blood cells on the muscle and had no infiltration of inflammatory cells. 9 h after LPS treatment, the glomerulus was normal with slightly swollen renal tubular epithelial cells and degradation of fat and vacuole. 16 h after LPS treatment, the kidney was seen enlarged with pale renal cortex and congested, dark-colored medulla. At 18 h, tubular epitheli-

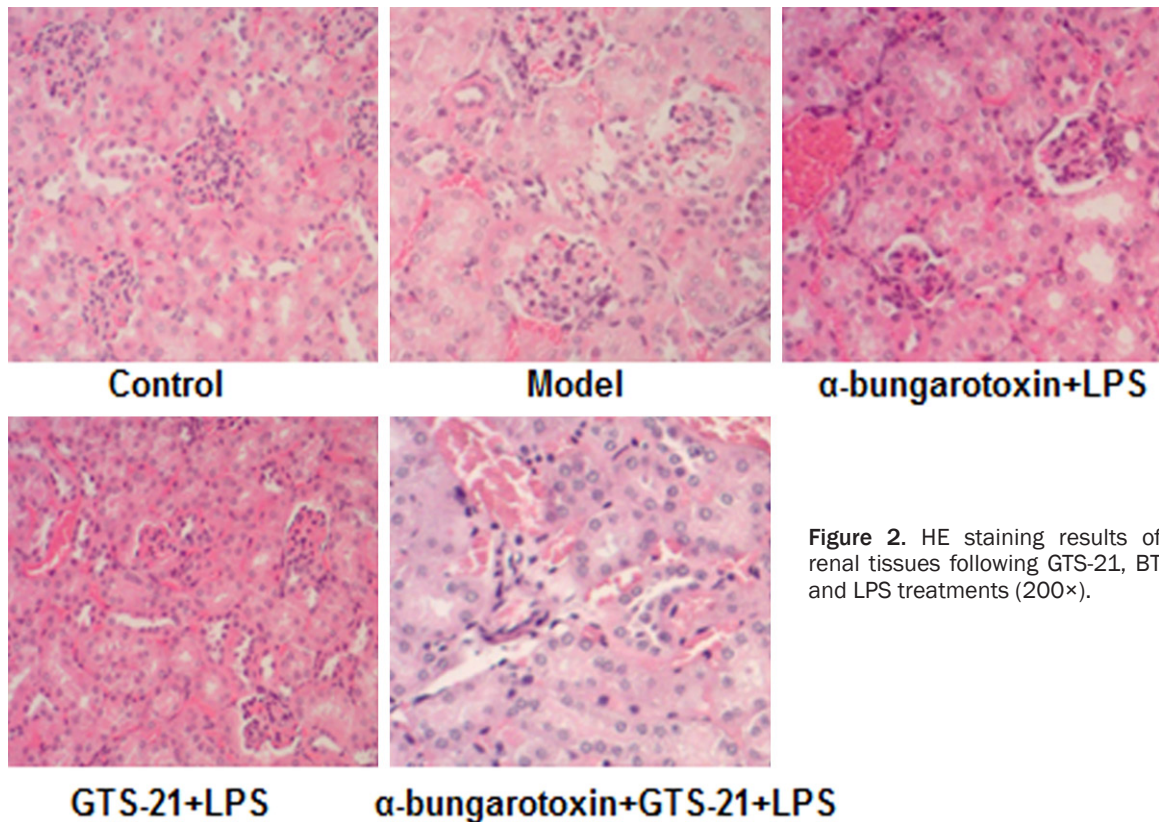


Figure 2. HE staining results of renal tissues following GTS-21, BT and LPS treatments (200 \times).

al cells showed various degree of necrosis, nuclear condensation, dissolution and disappearance (**Figure 1A**). The necrotic epithelial cells were dropped into the lumen as fuzzy granular structure. Cell casts were observed in the distal tubules and collecting ducts with congested and swollen mesenchyme around the tubules. As shown in **Figure 1B, 1C**, the control and 9 h LPS-treatment had less apoptosis, which increased after 16 h and reached the maximum at 18 h. However, at 18 h, the animals had a sharply reduced body temperature with very poor mental state (limp instability and near death). Therefore, mice treated with LPS for 16 h were used for subsequent experiments in this study.

Pathological results following drug treatments

We first examined the pathological changes following drug treatments. No red cells and infiltration of inflammatory cells was observed in control mice (**Figure 2**), which also had evenly stained tissue and morphologically normal kidney. However, the models and mice in BT-GTS-21-LPS group had obviously swollen kidney cells with pale cortex and congested, dark-col-

ored medulla. The renal tubules were degenerated and necrotic due to severe ischemia. Mice in GTS-21-LPS group had normal glomeruli, but the epithelial cells were swollen with degradation of fat and vacuole. After BT-LPS treatment, the tubular epithelial cells had various degree of necrosis, nuclear condensation, dissolution and disappearance. The necrotic epithelial cells were dropped into the lumen as fuzzy granular structure (**Figure 2**).

Apoptosis following drug treatments

As shown in **Figure 3**, compared with the control, the model group had significantly increased apoptosis. GTS-21 reduced the apoptosis, which increased again when BT was used in combination with GTS-21, suggesting that BT may partly block the anti-inflammatory effects of GTS-21.

Expression of NF- κ B and α 7nAChR mRNA

In the renal tissue, the expression of NF- κ B p65 and α 7nAChR was significantly up- and down-regulated in the model group, respectively ($P < 0.01$, **Figure 4A**), as compared with the blank

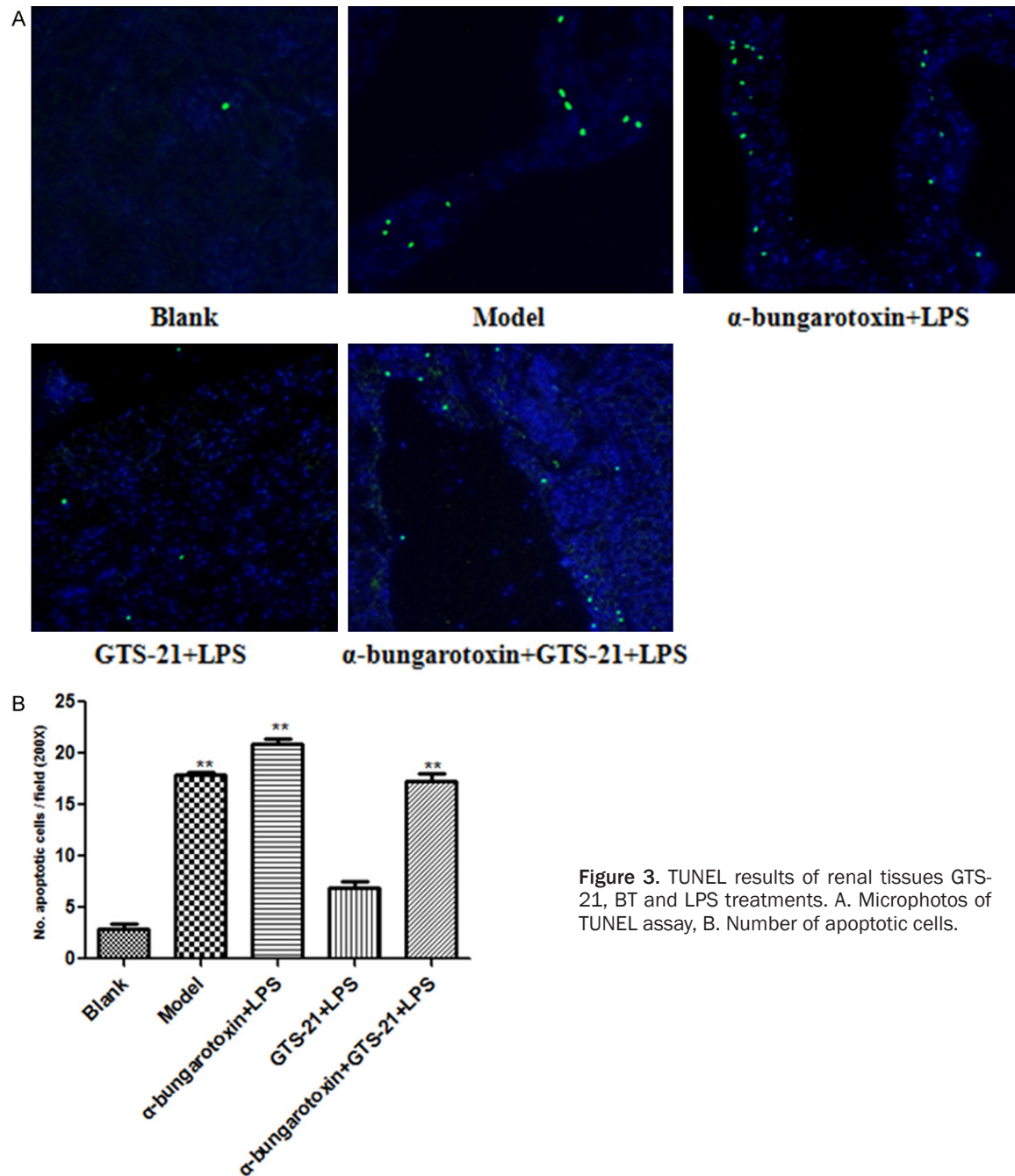


Figure 3. TUNEL results of renal tissues GTS-21, BT and LPS treatments. A. Microphotos of TUNEL assay, B. Number of apoptotic cells.

control. GTS-21 alone did not increased the expression further in the model ($P > 0.05$), but significantly increased NF-Kb p65 expression and decreased $\alpha 7$ nAChR expression when applied together with BT ($P < 0.01$, **Figure 4A**).

Expression of Caspase-3, Caspase-8, Bcl-2, Bax and p53 mRNA

We then assayed the mRNA levels of Caspase-3, Caspase-8, anti-apoptotic protein Bcl-2, pro

apoptotic proteins Bax and p53 in the renal tissue. The results showed that compared with the blank control, LPS significantly increased the mRNA levels of all these genes except Bcl-2, which was decreased significantly ($P < 0.01$, **Figure 4B**). Again, GTS-21 alone did not change the expression ($P > 0.05$), but enhanced the expression of these genes significantly except Bcl-2, which was decreased significantly ($P < 0.01$, **Figure 4B**) when it was used with BT.

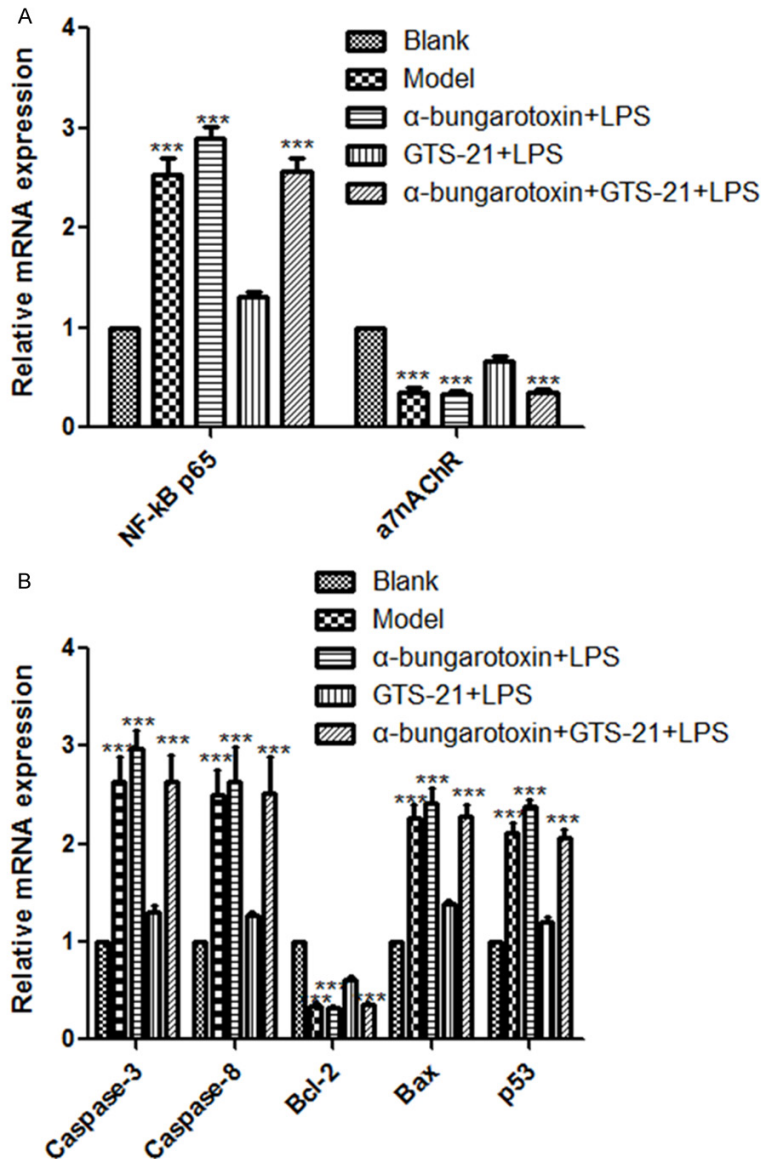


Figure 4. Relative mRNA level of NF-kB, α7nAChR, Caspase-3, Caspase-8, Bcl-2, Bax and p53 mRNA in renal tissue following GTS-21, BT and LPS treatments. A. NF-kB and α7nAChR expression; B. Caspase-3, Caspase-8, Bcl-2, Bax and p53 expression. *** denote $P < 0.001$ vs control.

Expression of IL-6, IL-1β, TNF-α and p-STAT3

Western blot analyses showed that the levels of IL-6, IL-1β, TNF-α and p-STAT3 were significantly up-regulated following LPS treatment. Similarly, GTS-21 alone did not change the expression ($P > 0.05$), but enhanced the expression of these proteins significantly ($P < 0.01$, **Figure 5, Supplementary Figure 1**) when it was used with BT.

Discussion

LPS is commonly used to construct model of sepsis. As a lipopolysaccharide and main com-

ponent of the cell wall in gram negative bacteria, LPS elevates the levels of a number of inflammatory factors, causing septic shock [14]. After injection of LPS, different types of cells are stimulated to release inflammatory mediators that trigger the process of sepsis, leading to systemic sepsis that results in multiple organ dysfunction syndrome (MODS) [15]. Therefore, the injection of LPS can be used to reproduce model of sepsis with similar clinical characteristics of human sepsis. However, the optimal time after LPS is variable and we found that 16 h after LPS injection the models had sufficient but not excessive sepsis and were suitable for our experiments.

AKI plays very important role in the occurrence and development of sepsis, which can lead to renal injury and apoptosis. We found that after LPS injection, there was several injury and apoptosis in the renal tissues. Analysis also showed that the expression of apoptosis-related proteins Caspase-3, Caspase-8, Bax and p53 was also increased, while the expression of anti apoptosis protein Bcl-2 was decreased. GTS-21, on the other hand, alleviated the injury and reduced the number of apoptotic cells.

Use of BT together with GTS-21 offset the alleviation, suggesting BT and GTS-21 are antagonistic. GTS-21 also reduced renal apoptosis and the expression of Caspase-3, Caspase-8, Bax and p53 significantly, while the expression of anti apoptosis protein Bcl-2 was significantly increased. When used together with BT, GTS-21 was not able reduce apoptosis and expression of apoptosis-related proteins, suggesting that the renal protection of GTS-21 is mediated through the α7nAChR signal pathway. Early study showed that GTS-21 can prevent renal injury induced by

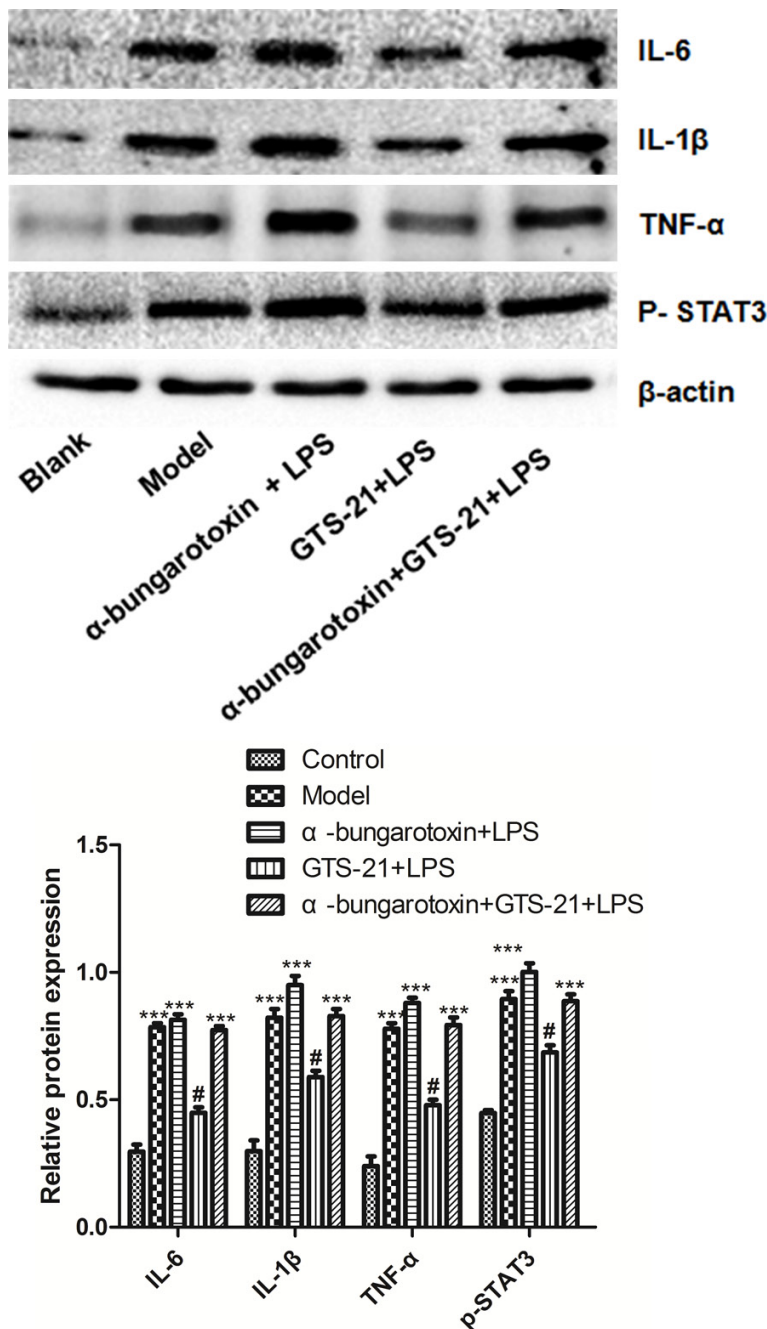


Figure 5. Expression of IL-6, IL-1 β , TNF- α and p-STAT3 proteins in renal tissue following GTS-21, BT and LPS treatments. Upper panel: representative Western blots, lower panel: relative protein levels. *** denote $P < 0.01$ vs control.

mechanical ventilation [16] and the prevention of renal injury is mediated via the $\alpha 7$ nAChR signal pathway [17], which is consistent with the results of this study. CAP is a recently discovered systemic inflammatory response pathway with complicated regulatory mechanisms. $\alpha 7$ nAChR is one of the most important molecules with wide distribution and is a key anti-

inflammatory agent. The expression of $\alpha 7$ nAChR in the model group was significantly down-regulated, while the expression of inflammatory factors IL-6, IL-1 β and TNF- α were up-regulated. Studies have shown that inflammatory injury and inhibition of vagus transmission may result in CAP dysfunction [18]. When sepsis occurs, the peripheral signal transmission from vagus nerve is disrupted, leading to weakened CAP anti-inflammatory function. If sepsis is severe, the anti-inflammatory function of CAP is compromised, resulting in signal transmission disruption, excessive activation of alkali esterase, desensitization of $\alpha 7$ nAChR and disappearance of anti-inflammatory activity. These would lead to excessive production of inflammatory factors and ultimately cell death [19]. We found that the expression of inflammatory factors IL-6, IL-1 β and TNF- α increased with the decrease in the expression of $\alpha 7$ nAChR, suggesting that $\alpha 7$ nAChR plays an important role in biogenesis of inflammatory factors during sepsis. GTS-21 is a derivative of the natural product anabaseine that acts as a partial agonist at $\alpha 7$ nAChR. After application of GTS-21, the renal injury was significantly reduced with less apoptosis. Meanwhile, the expression of inflammatory cytokines IL-6, IL-1 β and TNF- α was significantly down-regulated,

while the expression of the $\alpha 7$ nAChR gene was significantly up-regulated. These effects were, however, vanished when BT was used together, further suggesting that the anti-inflammatory effect of GTS-21 is related to $\alpha 7$ nAChR. Studies have shown that GTS-21 significantly reduces the production of inflammatory factors and proinflammatory cytokines,

and improves the survival rate of endotoxemia [20]. Also, it has been shown that GTS-21 significantly reduces the synthesis of inflammatory factor TNF- α induced by lipopolysaccharide [21]. GTS-21 is shown to sensitize desensitized $\alpha 7$ nAChR to exert anti-inflammatory effect [22]. Taken together, our results suggest that GTS-21 can effectively alleviate renal injury and have anti-inflammatory activity against septic renal injury. These activities are associated with increased $\alpha 7$ nAChR expression.

Acknowledgements

The work was supported by National Natural Science Foundation of China (grant no. 815-71871) and Postdoctoral Funding of Heilongjiang Province, China (grant no. LBH-Z16147) and Talent Fund of Harbin Science and Technology Bureau (2017RAQXJ177).

Disclosure of conflict of interest

None.

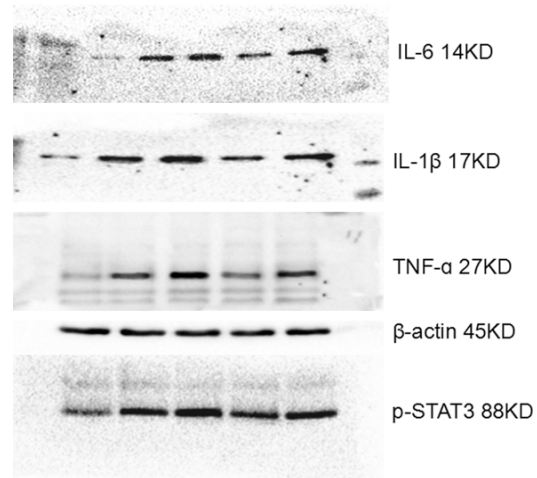
Address correspondence to: Dr. Kaijiang Yu, Department of Critical Care Medicine, The Cancer Hospital of Harbin Medical University, 150 Haping Road, Harbin 150081, China; Institute of Critical Care Medicine and Institute of Sino Russian Medical Research Center of Harbin Medical University, 150 Haping Road, Harbin 150081, China. Tel: 861330-3608899; Fax: 8645186298999; E-mail: drkaijiang1@126.com

References

- [1] Angeli P, Gines P, Wong F, Bernardi M, Boyer TD, Gerbes A, Moreau R, Jalan R, Sarin SK, Piano S, Moore K, Lee SS, Durand F, Salerno F, Caraceni P, Kim WR, Arroyo V, Garcia-Tsao G; International Club of Ascites. Diagnosis and management of acute kidney injury in patients with cirrhosis: revised consensus recommendations of the International Club of Ascites. *Gut* 2015; 64: 531-537.
- [2] Hoste EA, Bagshaw SM, Bellomo R, Cely CM, Colman R, Cruz DN, Edipidis K, Forni LG, Gomersall CD, Govil D, Honore PM, Joannes-Boyau O, Joannidis M, Korhonen AM, Lavrentieva A, Mehta RL, Palevsky P, Roessler E, Ronco C, Uchino S, Vazquez JA, Vidal Andrade E, Webb S and Kellum JA. Epidemiology of acute kidney injury in critically ill patients: the multinational AKI-EPI study. *Intensive Care Med* 2015; 41: 1411-1423.
- [3] Mao Y, Tokudome T, Kishimoto I, Otani K, Nishimura H, Yamaguchi O, Otsu K, Miyazato M and Kangawa K. Endogenous ghrelin attenuates pressure overload-induced cardiac hypertrophy via a cholinergic anti-inflammatory pathway. *Hypertension* 2015; 65: 1238-1244.
- [4] Payolla TB, Lemes SF, de Fante T, Reginato A, Mendes da Silva C, de Oliveira Micheletti T, Rodrigues HG, Torsoni AS, Milanski M and Torsoni MA. High-fat diet during pregnancy and lactation impairs the cholinergic anti-inflammatory pathway in the liver and white adipose tissue of mouse offspring. *Mol Cell Endocrinol* 2016; 422: 192-202.
- [5] Liu F, Li Y, Jiang R, Nie C, Zeng Z, Zhao N, Huang C, Shao Q, Ding C, Qing C, Xia L, Zeng E and Qian K. miR-132 inhibits lipopolysaccharide-induced inflammation in alveolar macrophages by the cholinergic anti-inflammatory pathway. *Exp Lung Res* 2015; 41: 261-269.
- [6] Lanza P, Torsoni M, Souza C. Modulation of JAK2/STAT3, CREB proteins and cholinergic anti-inflammatory pathway in offspring obese dams: implications for the control antiinflammatory response in the liver. *Xxiv Congresso De Iniciação Científica Da Unicamp* 2016.
- [7] Kashiwagi S, Khan MA, Yasuhara S, Goto T, Kem WR, Tompkins RG, Kaneki M and Martyn JA. Prevention of burn-induced inflammatory responses and muscle wasting by GTS-21, a specific agonist for $\alpha 7$ nicotinic acetylcholine receptors. *Shock* 2017; 47: 61-69.
- [8] Khan MA, Khan MF, Kashiwagi S, Kem WR, Yasuhara S, Kaneki M, Tompkins RG and Martyn JA. An $\alpha 7$ nicotinic acetylcholine receptor agonist (GTS-21) promotes C2C12 myonuclear accretion in association with release of interleukin-6 (IL-6) and improves survival in burned mice. *Shock* 2017; 48: 227-235.
- [9] Kong FJ, Ma LL, Zhang HH and Zhou JQ. Alpha 7 nicotinic acetylcholine receptor agonist GTS-21 mitigates isoflurane-induced cognitive impairment in aged rats. *J Surg Res* 2015; 194: 255-261.
- [10] Yue Y, Liu R, Cheng W, Hu Y, Li J, Pan X, Peng J and Zhang P. GTS-21 attenuates lipopolysaccharide-induced inflammatory cytokine production in vitro by modulating the Akt and NF-kappaB signaling pathway through the $\alpha 7$ nicotinic acetylcholine receptor. *Int Immunopharmacol* 2015; 29: 504-512.
- [11] Lillie RD, Pizzolato P and Donaldson PT. Nuclear stains with soluble metachrome metal mordant dye lakes. The effect of chemical end-group blocking reactions and the artificial introduction of acid groups into tissues. *Histochemistry* 1976; 49: 23-35.
- [12] Kasagi N, Gomyo Y, Shirai H, Tsujitani S and Ito H. Apoptotic cell death in human gastric carcinoma: analysis by terminal deoxynucleotidyl

- transferase-mediated dUTP-biotin nick end labeling. *Jpn J Cancer Res* 1994; 85: 939-945.
- [13] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 2001; 25: 402-408.
- [14] Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, Kolde R, Vlamakis H, Arthur TD, Hamalainen AM, Peet A, Tillmann V, Uibo R, Mokurov S, Dorshakova N, Ilonen J, Virtanen SM, Szabo SJ, Porter JA, Lahdesmaki H, Huttenhower C, Gevers D, Cullen TW, Knip M, Group DS and Xavier RJ. Variation in microbiome LPS immunogenicity contributes to autoimmunity in humans. *Cell* 2016; 165: 842-853.
- [15] Knox DB, Lanspa MJ, Kuttler KG, Brewer SC and Brown SM. Phenotypic clusters within sepsis-associated multiple organ dysfunction syndrome. *Intensive Care Medicine* 2015; 41: 814-822.
- [16] Cloke JM and Winters BD. alpha4beta2 Nicotinic receptor stimulation of the GABAergic system within the orbitofrontal cortex ameliorates the severe crossmodal object recognition impairment in ketamine-treated rats: implications for cognitive dysfunction in schizophrenia. *Neuropharmacology* 2015; 90: 42-52.
- [17] Lewis AS, Mineur YS, Smith PH, Cahuzac EL and Picciotto MR. Modulation of aggressive behavior in mice by nicotinic receptor subtypes. *Biochem Pharmacol* 2015; 97: 488-497.
- [18] Knott V, Impey D and Choueiry J. An acute dose, randomized trial of the effects of CDP-Choline on Mismatch Negativity (MMN) in healthy volunteers stratified by deviance detection level. *Neuropsychiatric Electrophysiology* 2015; 1: 1.
- [19] Wang YL, Liu LX, Wang SH and Qi WX. [Temporal and spatial changes of alpha7nAChR and nNOS in cerebral cortex and hippocampus of Abeta-induced cognitive dysfunction rats]. *Sheng Li Xue Bao* 2016; 68: 775-782.
- [20] Nullens S, Staessens M, Peleman C, Schrijvers DM, Malhotra-Kumar S, Francque S, Matteoli G, Boeckxstaens GE, De Man JG and De Winter BY. Effect of Gts-21, an alpha7 nicotinic acetylcholine receptor agonist, on Clp-induced inflammatory, gastrointestinal motility, and colonic permeability changes in mice. *Shock* 2016; 45: 450-459.
- [21] Batsikadze G, Paulus W, Grundey J, Kuo MF and Nitsche MA. Effect of the nicotinic alpha-4beta2-receptor partial agonist varenicline on non-invasive brain stimulation-induced neuroplasticity in the human motor cortex. *Cereb Cortex* 2015; 25: 3249-3259.
- [22] Zhou W, Wang J, Li Z, Li J and Sang M. MicroRNA-2055b inhibits HMGB1 expression in LPS-induced sepsis. *Int J Mol Med* 2016; 38: 312-318.

GTS-21 and renal injury



Supplementary Figure 1. Original Western Blot analysis of IL-6, IL-1 β , TNF- α and p-STAT3.