

Effects of lentinan on NF- κ B activity in the liver of burn rats with sepsis

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Abstract. Effects of lentinan on nuclear factor- κ B (NF- κ B) activity in liver of burn rats with sepsis were investigated. To mimic the clinical sepsis after burn, rats were subjected to 30% full-thickness scald injury, followed by intraperitoneal (i.p.) injection of lipopolysaccharide (LPS). Seventy-two adult rats were randomly divided into six groups: the normal control group (n=12); the burn sepsis group (n=12); the burn sepsis with positive drugs; the burn sepsis with low-dose lentinan treatment group (50.0 mg/kg, n=12); the burn sepsis with middle-dose lentinan treatment group (100.0 mg/kg, n=12) and the burn sepsis with high-dose lentinan treatment group (200.0 mg/kg, n=12). Expression of NF- κ B in the liver was measured with western blot analysis. The morphology of liver was evaluated with hematoxylin and eosin staining. The expression of NF- κ B significantly increased in the liver of burn rats with sepsis. Compared with the burn sepsis group, lentinan treatment obviously reduced the damage of hepatic cell morphology, and decreased the activity of NF- κ B significantly in the medium and high concentrations of lentinan treatment groups (P<0.05). Most importantly, treatment with lentinan was able to reverse the increased concentration of IL-4, IL-6, IL-10 and TNF- α in plasma which was induced by LPS. Lentinan treatment can significantly decrease the expression of NF- κ B in the liver of burn rats with sepsis.

Introduction

Sepsis is defined as a life-threatening organ dysfunction caused by a host's dysregulation of infection. In sepsis, the immune response elicited by invading pathogens does not return to homeostasis, ultimately leading to pathological syndrome characterized by persistent excessive inflammation

and immunosuppression (1). Sepsis causes a systemic dysregulated inflammatory response characterized by excessive proinflammatory mediators (2). Therefore, it can be prevented by controlling systemic inflammation. Nuclear factor- κ B (NF- κ B) acts as a transcription factor regulating the transcription of different genes, including pro-inflammatory cytokines, chemokines, adhesion molecules and growth factors (3). Regulation of transcription factor NF- κ B activation may be a potential treatment for sepsis. Lentinus edodes polysaccharide is a cell wall glucan extracted from the fruiting body of *Lentinus edodes* with significant anticancer and antitumor and immunomodulatory activities (4). However, the anti-inflammatory mechanism of lentinan is still unclear, and the role of lentinan in sepsis is not clear. In this study, rats with scald and endotoxin challenged were used as models to simulate clinical burn sepsis. The influence of lentinan on NF- κ B activity and plasma inflammatory cytokine expression was observed. The activity of lentinan on NF- κ B *in vivo* was investigated. The influence and regulation of systemic inflammatory response in sepsis provide a theoretical basis for future research on the role of lentinan in sepsis.

Materials and methods

Main reagents and equipment. Lipopolysaccharide (LPS) (O55:B5) (L2880; Sigma-Aldrich; Merck KGaA), lentinan (Shanxi Senfu Natural Products Co., Ltd.), hematoxylin-yen dyeing solution (G1005; Wuhan Google Biotechnology), nuclear transcription factor NF- κ B antibody (bs-2695R; Beijing Boaosen Bio), secondary antibody, histological kit DAB chromogenic reagent (K5007; DAKO), interleukin-4 (IL-4), IL-6, IL-10, tumor necrosis factor- α (TNF- α) detection kit (Beijing Sizhengbai Biotechnology Co., Ltd.), dehydrator (JJ-12J; Wuhan Junjie Electronics Co., Ltd.), embedding machine (JB-P5; Wuhan Junjie Electronics Co., Ltd.), pathology slicer (RM2235; Shanghai Leika Instrument Co., Ltd.), upright fluorescence microscope (Nikon Eclipse TI-SR; Nikon).

Animal grouping and model building. Seventy-two healthy male SD rats, 8 weeks old, weighing 250-270 g (purchased from Guangdong Medical Animal Center), with a feeding environment of 12 h light and 12 h darkness (animal experiments were conducted in accordance with the provisions of the National Institutes of Health guidelines for the care and

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use of experimental animals and the requirements of general recommendations, and approved by the Ethics Committee of Experimental Animal Welfare, Plastic Surgery Hospital, Chinese Academy of Medical Sciences, Beijing, China). Rats were randomly divided into 6 groups (12 in each group): Normal control group, burn sepsis group, positive drug control group, burn septic lentinan low concentration group (50 mg/kg), medium concentration group (100 mg/kg) and high concentration group (200 mg/kg). Model establishment: Preoperative fasting for 24 h, free drinking water, 10% chloral hydrate (0.3 g/kg) intraperitoneal (i.p.) injection anesthesia prior to cervical dislocation, there was no sign of peritonitis, pain or discomfort found after injection. The general indications are slow breathing, eyes are insensitive to light, the painful contraction when pinching its toes with tweezers completely disappear. After full anesthesia, the rats had regular breathing, slow eyelid reflexes and the contractile reflexes disappeared. After back hair removal, the rats given a stage III scald at 30°C for 12 sec, and then they were immediately given i.p. injection of 4 mg endotoxin (O55: B5) to simulate burn sepsis and anti-shock with 100 ml of normal saline. The rats were raised in separate cages. Each group was intraperitoneally injected with normal saline, anti-inflammatory drugs, low concentration of lentinan (50 mg/kg), medium concentration (100 mg/kg) and high concentration (200 mg/kg) 30 min before injury. The rats were monitored regularly during and after the operation. If abnormal symptoms such as convulsion or severe pain were found in the rats, the operation was stopped immediately and the neck was cut off for euthanasia. Blood collection was conducted 24 h after the model, 10% chloral hydrate (0.3 g/kg) was intraperitoneally injected for anesthesia and the toe pinching test showed that contractile reflexes disappeared. The rats were sacrificed immediately by cervical dislocation after the blood was collected from the abdominal aorta. The rats were judged dead after respiratory arrest following cervical dislocation. Some of the fresh livers were immediately fixed in 4% paraformaldehyde, and the other part of the fresh livers were preserved at -80°C for subsequent western blot protein determination.

Tissue paraffin embedded slice. The fresh tissue was fixed in 4% paraformaldehyde for 24 h, then placed in a hanging basket and dehydrated and dipped in a dewatering machine. The wax-impregnated tissue was embedded in an embedding machine, and after the wax was solidified, the wax block was taken out from the embedding frame and the wax block was trimmed. The trimmed wax block was placed on a paraffin slicer and sliced to a thickness of 4 μ m. The slices were floated on a spreader at 40°C. The tissue was flattened in warm water, the tissue was picked up with a glass slide, and placed in a 60°C oven. After the water-baked dry wax was roasted, it was taken out and stored at room temperature for later use.

Hematoxylin and eosin (H&E) staining. Paraffin sections were dewaxed in water and sliced into Harris hematoxylin for 5 min. Washed with tap water, 1% hydrochloric acid alcohol was differentiated for several seconds, rinsed in tap water, 0.6% ammonia turned water blue, and was rinsed again. The sections were stained for 3 min in Hematoxylin-Ihong dyeing solution. After dehydration, the film was placed under a

microscope for microscopic examination and image collection and analysis.

Western blot for the analysis of protein content of liver tissue. The fresh liver tissue homogenate was taken. The liver tissues of each group were dissolved in cleavage buffer and the protein concentration was measured by BCA method. The tissue lysis products were separated by 10% polyacrylamide gel electrophoresis, then transferred to PVDF membrane, and then sealed with 5% skim milk for 2 h. Then incubated with the first antibody NF- κ B (ab131546; Abcam) overnight at 4°C. The second antibody was incubated at 37°C for 1 h, rinsed with TBST buffer three times, ECL luminescent solution was reacted at room temperature for 30 sec, and pressed in the dark. GAPDH was used as an internal reference.

Plasma inflammatory factor enzyme-linked immunosorbent assay (ELISA). Twenty-four hours after the last dose, the rats were given 10% chloral hydrate i.p. injection. After full anesthesia, ~5 ml of blood was collected from the abdominal aorta by anticoagulant blood vessel. The rats were sacrificed immediately after the blood collection, the supernatant of the collected blood was centrifuged at 4°C and 1,000 x g for 15 min and stored at -20°C until use. The serum levels of IL-4, IL-6, IL-10 and TNF- α in rats were detected by ELISA. The experimental method was performed according to the kit instructions.

Statistical analysis. Data were analyzed by one-way ANOVA using SPSS 16.0, and the Tukey test was used for pairwise comparison between groups. Results are expressed as mean \pm standard deviation (mean \pm SD). $P < 0.05$ was considered statistically significant.

Results

Lentinan treatment can significantly reduce liver damage in burns and sepsis. H&E staining results show that the liver cells of the control group have clear boundaries and complete morphology. Normal control group liver tissue was basically normal (Fig. 1A). The burnt sepsis model group had blurred liver cell boundaries, severe vacuolar degeneration, and hepatic cord disorder. There were a large number of inflammatory cells (Fig. 1B), mild inflammatory cell infiltration in the low-concentration lentinan-treated group, mild vacuolar degeneration (Fig. 1D), and medium- and high-concentration lentinan-treated group compared with the model group. The inflammatory cell infiltration was obviously relieved, the hepatic cord was clear, and the morphology was intact, and there was no obvious difference from the positive drug control group (Figs. 1C, E and F). This result suggests that lentinan can reduce the damage of LPS on rat liver in the burn sepsis model.

Lentinan treatment can significantly reduce the expression of NF- κ B in liver tissue. Western blot results showed that the expression of NF- κ B in liver tissue of burn sepsis group was significantly enhanced (Fig. 2b) compared with normal control group (Fig. 2a), while in burn septic lentinan treatment group, the expression of NF- κ B in liver tissue was weakened compared with the burn sepsis group (Figs. 2d-f). High concentrations

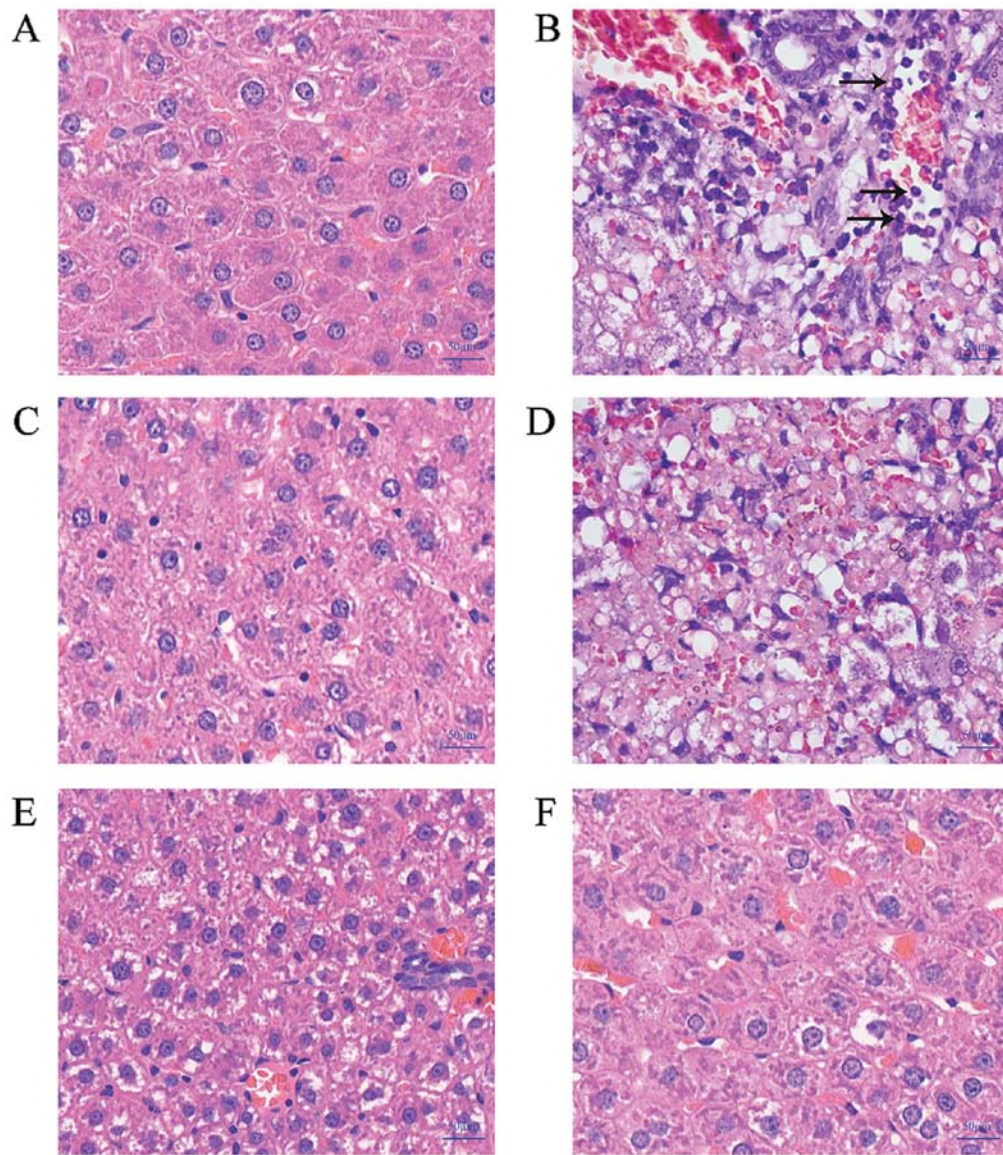


Figure 1. H&E staining of liver in each group. (A) Normal control group. (B) Burn sepsis group, the arrow represents inflammatory cell infiltration. (C) Positive drug control group. (D) Lentinan low concentration groups (50 mg/kg). (E) Medium concentration groups (100 mg/kg). (F) High concentration groups (200 mg/kg). H&E, hematoxylin and eosin.

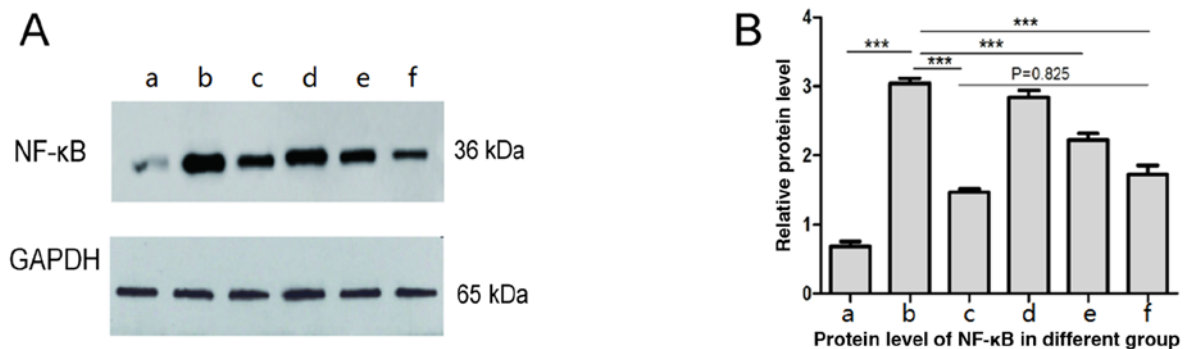


Figure 2. Western blot results of liver in each group. (A) Western blot results. (B) The protein levels of NF-κB in different groups. a, Normal control group; b, burn sepsis group; c, positive drug control group; d, lentinan low concentration groups (50 mg/kg); e, medium concentration groups (100 mg/kg); f, high concentration group (200 mg/kg). *** $P < 0.001$. NF-κB, nuclear factor-κB.

of lentinan treatment significantly reduced NF-κB expression (Fig. 2f), and there was no statistical significance

compared with the positive control group ($P > 0.05$) (Fig. 2c). The results suggest that the protective effect of lentinan on the

Table I. Concentration of plasma IL-4, IL-6, IL-10 and TNF- α in different groups.

Group	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TNF- α (pg/ml)
Normal control group	60.07 \pm 11.90 ^a	41.32 \pm 11.62 ^a	17.19 \pm 4.34 ^a	114.50 \pm 16.43 ^a
Burn sepsis group	106.97 \pm 13.93	68.99 \pm 10.06	36.62 \pm 4.78	200.61 \pm 20.78
Positive drug control group	83.27 \pm 14.11 ^a	41.55 \pm 6.34 ^a	24.31 \pm 4.68	117.85 \pm 13.08 ^a
Low concentration groups	82.05 \pm 18.17 ^a	67.20 \pm 8.34	30.40 \pm 2.74	165.15 \pm 14.19 ^a
Medium concentration group	75.17 \pm 10.74 ^a	62.13 \pm 12.81	26.48 \pm 5.12	140.78 \pm 17.54 ^a
High concentration group	60.64 \pm 8.84 ^a	44.86 \pm 8.63 ^a	24.71 \pm 4.01 ^a	117.36 \pm 14.34 ^a

n=12, ^aP<0.05 compared with the model group. IL, interleukin; TNF- α , tumor necrosis factor- α .

liver of burned sepsis model rats is achieved by inhibiting the NF- κ B inflammatory pathway, so the levels of inflammatory factors in plasma of each group were further tested.

Lentinan treatment can reverse the increase of plasma inflammatory factors in burn sepsis model. By measuring the levels of plasma inflammatory factors in each group, plasma IL-4, IL-6, IL-10 were detected in burned sepsis model rats. The level of TNF- α was significantly increased (P<0.05), while the high concentration of lentinan treatment significantly reduced plasma IL-4 (P<0.05), and IL-6 (P<0.05), TNF in burned sepsis model rats, TNF- α (P<0.05) and IL-10 (P<0.05) levels, while low-middle concentration also showed a downward trend, showing concentration-dependent, high concentration of lentinan treatment had good anti-inflammatory effect (Table I).

Discussion

Sepsis is a serious clinical syndrome with a complicated path mechanism. Systemic inflammation and immune response are important factors in the formation of sepsis (5). The function of different organ systems of the body is impaired when the infection develops beyond the compensatory capacity of the body. In general, the respiratory system is the first system to malfunction, followed by the liver, kidneys, heart, and so on. Organ failure is closely related to mortality in patients with sepsis. The results of this study show that lentinan can reduce the damage of sepsis to the liver to a certain extent, and provide a theoretical basis for the application of lentinan in sepsis.

The inflammatory response is the main cause of sepsis. Therefore, controlling inflammation within an effective range is a key method for the treatment of sepsis. The inflammatory response to sepsis is mainly caused by overproduction of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6. In addition, inhibition of secretion of these cytokines can delay and reduce the incidence of sepsis and mortality in patients or animal models of sepsis (6). Our results are consistent with previous studies. The inflammatory factors such as TNF- α and IL-6 in the plasma of the model group were significantly increased. The results show that the treatment of lentinan can greatly reduce the expression levels of these inflammatory factors in plasma. This result suggests that lentinan can reduce the damage of liver caused by sepsis, which may be achieved by inhibiting the expression level of

inflammatory factors. It points the way for further study of its specific mechanism.

NF- κ B is involved in the transcriptional expression of a variety of genes, including pro-inflammatory cytokines, chemokines, adhesion molecules and other inducing factors, and is a very common nuclear transcription factor in cells (7). Normally located in the cytoplasm and inhibited by I κ B- α , I κ B- α binds to NF- κ B to form a polymer when the cells are not stimulated. At this time, NF- κ B is inactive and stimulated by TNF- α , I κ B- α is degraded by the ubiquitin system, and NF- κ B is activated into the nucleus to participate in the expression of inflammatory factors, forming a vicious circle. Williams *et al* (8) found that when NF- κ B was activated early, sepsis mortality would be positively correlated. Controlling NF- κ B is obviously helpful for the treatment of sepsis (9). The results of this experiment indicate that lentinan reduces the damage of sepsis to the liver by inhibiting the nuclear transcription factor NF- κ B.

Lentinus edodes polysaccharide is a kind of β -glucan, which has antitumor, anti-bacterial, anti-viral and immunomodulatory effects, and is extracted from the fruiting body of the mushroom (10-12), and the effect of lentinan on inflammatory reaction has also been reported (13). A study found that Lentinus edodes polysaccharide can downregulate TLR4-mediated NF- κ B signaling pathway and IL-13, CD30L and expression of other cytokines under conditions of lipopolysaccharide-induced inflammatory response (14). TLR4 is distributed in tissues such as spleen, liver and lung, and is an important molecule for cell recognition of LPS. The expression of TLR4 is closely related to the response of cells to LPS. Inhibition of TLR4 and its downstream signaling pathways, including overexpression of inflammatory factors, is considered as a treatment for sepsis (15). Our experimental evidence also suggests that lentinan can downregulate the expression of NF- κ B and further confirmed that the mushroom polysaccharide treatment can inhibit the excessive increase of IL-4, IL-6, IL-10 and TNF- α levels in plasma.

In conclusion, the results of this study suggest that lentinan may reduce the content of serum IL-4, IL-6, IL-10 and TNF- α by downregulating the expression of NF- κ B in liver tissue, thereby reducing burn sepsis and causing acute damage to the liver. The results of this study suggest that lentinan administration may be an adjuvant treatment for sepsis treatment, laying the foundation for further research.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QL made substantial contributions to the design of the study and wrote the manuscript. YG and YQ were responsible for immunohistochemistry and ELISA. ZL and GEM contributed to observation indexes analysis. The final version was read and adopted by all the authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Plastic Surgery Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Animal experiments were conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines (16).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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