Research on Protective Effect and Mechanism of Idazoxan on Lps Attacked Acute Hepatic Injury

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Abstract. Objective: To observe the protection effect of Idazoxan (IDA) on LPS induced acute hepatic injury, and to explore its action mechanism. Methods: 60 adult C57BL/6 mice were divided into a control group (20 mice, intraperitoneal injection of phosphate buffer), a model group (20 mice, intraperitoneal injection of LPS 10 mg/kg) and a agmatine group (20 mice, intraperitoneal injection of LPS 10 mg/kg and agmatine 200 mg/kg) according to random number table method. Blood and liver tissue were collected for preparation of tissue homogenate. Enzyme-linked immunosorbent assay (ELISA) was adopted for detecting tumor necrosis factor-α (TNF-α) and interleukin (IL-1β and IL-6) contents in the serum and liver tissue at 24h after molding. Automatic biochemical analyzer is used for determining alanine transaminase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) level at 24h after molding; The liver tissue pathology changes were observed at 24h after molding. Macrophage RAW264.7 cells were stimulated by 10 µg/mL LPS and with or without IDA (100 µmol/L). 2',7'-dichlorofluorescin diacetate (DCFH-DA) was used as a fluorescent probe for detection of intracellular reactive oxygen species (ROS) level; qRT - PCR method was used for detecting antioxidant enzymes HO-1 and NQO-1 mRNA expression level at 2h, 4h and 8 h. Results: mice in the model group suffered from depression, curling and food water forbidding at 6h after molding. Mice in the Idazoxan group have obviously better spirit and activity than that of model group. The serum ALT, AST and LDH level of LPS attacked acute hepatic injury mice can be effectively alleviated after Idazoxan treatment. The expression of proinflammatory factor TNF-α and IL-6 in the liver can be reduced. The liver showed obvious pathological changes at 24 h after injection, such as liver cell swelling, necrosis, congestion, inflammatory cell infiltration, etc.; The liver cell injury was...
prominently alleviated in IDA treatment group. Compared with the control group, LPS significantly increased ROS level in RAW264.7 cells. The ROS level was decreased with concentration dependence after IDA intervention. IDA increased HO-1 mRNA expression of RAW264.7 cells. It had no influence on NQO-1mRNA. Conclusion: IDA significantly reduces the serum liver injury indexes and contents of TNF-α, IL-6 and other inflammatory mediators in liver tissues. It can alleviate the liver pathology change, thereby it can generate protection function on LPS attacked acute hepatic injury. Its action mechanism may be related to IDA-enhanced liver macrophage antioxidant function.

1. Introduction
Inflammation refers to the defense response to injury factors. The body generates excessive inflammation for external stimuli such as LPS during occurrence and development of serious illnesses, such as severe trauma, burn, infection, etc., proinflammatory and anti-inflammatory balance is broken, MODS even can be caused, and patients die eventually [1, 2]. It is generally believed that the liver is an important place to remove enterogenous bacteria and its LPS on the one hand, it is also one of the organs most vulnerable to the pathologic process on the other hand [3, 4]. Therefore, effective suppression of excessive inflammatory response and improvement of pathological changes in vital organs can be beneficial for alleviating or reversing the progress of such systemic infectious diseases. Idazoxan (IDA) is regarded as selective α2 adrenergic receptor antagonist and imidazoline receptor antagonist [5]. Some people also think that it is imidazoline II receptor (I2R) ligand and α2 adrenergic receptor antagonist [6]. Meanwhile, it is also NMDA receptor antagonist. It is also non-competitive aspartic acid (NMDA) receptor antagonist. Wang, etc. Wang, etc. [6] discovered the follows in experimental autoimmune encephalomyelitis (EAE) model: IDA suppresses the spinal inflammatory response by regulating the balance between pro-inflammatory and anti-inflammatory factors. Rusu et al. [7] utilizes rat forced swimming model to find out that IDA can enhance the motor function and tolerance of rats. In addition, some scholars have found that IDA has anti-tumor effect [8]. However, it is not clear whether it has protection function on function injury and pathologic changes of important viscera of severe infectious inflammatory organs or not. In the research, LPS is acted on mice and cell preparation inflammation model, thereby observing whether IDA can improve mice liver injury and cell oxidative injury or not, exploring the possible mechanism of action, and providing new strategy for clinical treatment of severe infectious diseases.

2. Materials and methods

2.1. Experimental animals and reagents
C57BL6 mice with body mass of 18 to 21g were purchased from Experimental Animal Center of Daping Hospital. LPS, IDA and DCFH-DA probe were purchased from American Sigma; Fetal bovine serum and RPMI1640 medium were purchased from American Gibco; TNF-α and IL-6 ELISA kits were purchased from Wuhan Boster, and reverse transcription and qPCR kits were purchased from TaKaRa Company.

2.2. In vitro culture of RAW264.7 cells
RAW264.7 cell line was provided by Institute of Field Surgery under The Third Military Medical University, which was cultivated in medium containing 10% fetal bovine serum and 1% double-antibody RPMI1640. It was placed in 37 °C 5% CO2 incubator, and the medium was regularly replaced and passed according to the state of cells.
2.3. Experimental grouping
60 mice were divided into three groups according to random number table: control group, model group and agmatine group. Acute hepatic injury mice model was prepared by intraperitoneal injection of LPS 10mg/kg. Mice in the control group were injected with equivalent amount of PBS intraperitoneally. Mice in the agmatine group were injected with LPS 10mg/kg and agmatine 200mg/kg intraperitoneally. 10 mice were respectively killed in each group at 6h and 24h after molding, and blood and liver tissue were obtained for examination.

The disposal method of experimental animals conforms to animal ethics standard.

2.4. Determination of serum liver function level
Mouse eye was removed for bleeding at 24h after molding. It stands statically for 1h, which is centrifuged for examining supernatant. Automatic biochemical analyzer is used for testing mice alanine transaminase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) contents.

2.5. Liver pathological observation
Mouse liver was taken at 24h after molding, which was fixed with polyformaldehyde for 24h. It underwent gradient ethanol dehydration, embedding, tissue slicing, xylene dewaxing and dyeing with HE, and then it was observed under the light microscope.

2.6. Determination of liver tissue homogenate TNF-α and IL-6
The liver tissue was taken at 6h after molding, the protein cracking fluid was added, it was homogened on ice, which was centrifuged for examining supernatant. The cytokine content was determined by ELISA kit instruction.

2.7. ROS detection
The RAW264.7 cells at logarithmic phase was obtained. 1×10^6 cells were inoculated in each hole of 6-orifice plate. The culture was discarded after the above grouping, and DCFH-DA with concentration of 20μmol/L was dripped in each hole (excitation wavelength of 485 nm, emission wavelength of 525 nm, and green fluorescence intensity is proportional to the production of ROS in cells). It was incubated at 37 ℃ for 20min at shady place. Cells were washed once with asepsis PBS. The fluorescence intensity was detected by flow cytometry.

2.8. Anti-oxidase HO-1 detection with qRT-PCR and NQO-1 expression
RAW264.7 cells at logarithmic growth phase were obtained, which were inoculated in a 6-orifice plate according to 1×10^6/hole. After cells were washed with PBS, 1mL TRIzol was added in each hole. Total RNA was obtained according to TaKaRa RR047A kit. qPCR quantitative analysis was performed according to the operation of TaKaRa RR820A kit. Amplification conditions: 95 ℃ for 3 min. 95 ℃ for 0s, 60 ℃ for 30s, 40 cycles. 2^ΔΔCt method was adopted for calculating the gene expression according to the obtained Ct value.

2.9. Statistical analysis
All experiments were repeated for three times. SPSS17.0 statistical software was used for analysis. Measurement data is expressed, and one-way analysis of variance was performed.

3. Results
3.1. In vivo experimental results
3.1.1. General condition of animal, Mice in the control group were characterized by fine fur, bright eyes, even breathing, active actions as well as brown and black bowels. In the model group, mice were
characterized by curling and less action, vertical and dark hair, slightly closing eyes, tight breath, diarrhe, less and thick blood during blooding. Mice of IDA groups were also in poor condition generally, but they were significantly better than that in the model group.

3.1.2. Comparison of liver function index level of mice in each group, The serum live function index AST, ALT and LDH in mice was significantly higher than that in the control group after intraperitoneal injection of LPS. The liver function index level was significantly lower than that in the model group after IDA intervention (all $P < 0.01$).

![Figure 1](image)

**Figure 1.** Influence on serum liver function level of IDA LPS induced acute hepatic injury mice (reduction of liver injury index)

3.1.3. Pathological changes of liver tissues, the model group were characterized by massive necrosis of liver cells, unclear structure of hepatic lobule and prominent hyperemia in portal area according to observation under light microscope. The remnant liver cells were in ballooning degeneration to different degrees. Cytoplasm was transfered. Neutrophils and lymphocytes infiltrated to necrosis area and portal area massively. Compared with the model group. Liver tissue necrosis lesions in IDA intervention group were significantly reduced. Liver cell edema and acidophilic change WERE REDUCED. The inflammatory cell infiltration was decreased.

The control group (A) had normal live tissue structure and no pathological changes. The LPS induced acute hepatic injury model group (B) had obvious hepatic cell ballooning degeneration and necrosis, congestion in the portal area and inflammatory cell infiltration. The pathological changes of Idazoxan treatment group (C) were significantly reduced compared with the model group.

![Figure 2](image)

**Figure 2.** Influence of IDA on LPS-induced acute hepatic injury mice liver histopathology (reduction of liver injury)

3.1.4. Comparison of liver tissue homogenate TNF-α and IL-6 levels in different groups, Compared with the control group, the liver tissue homogenate TNF-α and IL-6 levels of mice in the LPS-induced acute hepatic injury model group were prominently increased. However, the TNF-α and IL-6 of IDA intervention group were significantly decreased (all $P < 0.05$).
3.1.5. Comparison of in vitro experiment result RAW264.7 oxidative injury indexes, ROS production was significantly increased in LPS stimulation group compared with the control group. The generation of ROS in IDA treatment groups with different doses was reduced compared with that of LPS group, which was dose-dependent (all P < 0.01) (figure 4A). HO-1mRNA expression of LPS stimulation group was prominently increased in the IDA. It was time-dependent (all P < 0.01) (FIG. 4B), and IDA had no significant impact on expression of NQO-1mRNA (figure 4C).

4. Conclusion
Idazoxan is regarded as a new drug which is studied more widely in recent years. Many functions are constantly discovered. It can combine with I2R and α2-adrenalin receptor. IDA has nerve protection [9], opioid function regulation [10], movement function improvement [7] and other roles, but also has anti-inflammatory action. Wang, etc.[6] demonstrated that IDA could activate astrocytes and inhibit microglia in EAE model. The expression of pro-inflammatory factor IL-12p40 and γ-interferon (IFN-γ) is decreased in rat spinal cord model. The expression of anti-inflammatory cytokine IL-10 and transforming growth factor-β1(TGF-β1) was increased. In addition, the research on in vitro blood-brain barrier inflammation model shows that IDA can alleviate blood-brain barrier injury. It can effectively suppress the TNF-α induced tight junction protein ZO-1 reduction, and improve the distribution thereof. Meanwhile, the expression of extracellular matrix metalloproteinase 9 (MMP 9) and its specific value thereof with tissue inhibitor TIMP-1 ratio were reduced, thereby reversing the abnormally increased permeability [11].

Liver is an important organ for body synthesis, metabolism, detoxification and energy supply, which plays a crucial role in maintaining the steady state of the body. However, liver is vulnerable to injury of various factors [12, 13]. After LPS attacks the liver, the surface transmembrane receptor TLR4 of liver Kupffer cell is combined with LPS for activating NF-kB signaling pathways, thereby promoting IkB degradation, causing NF-kB phosphorylation. The downstream signal proinflammatory factor TNF-α are upregulated [14, 15]. TNF-α and other inflammatory factors induce neutrophils to accumulate in the liver for leading to inflammatory liver injury. On the one hand, it promotes the
activation of liver coagulation and further aggravates liver injury [16]. He Tao and Zhang Jianhui [18] implemented continuous lavage simvastatin therapy one week before LPS was used for inducing sepsis rats. It is found that liver tissue malondialdehyde (MDA) is decreased obviously. Meanwhile, serum IL - 6 is prominently reduced. It is obvious that simvastatin can achieve the function of protecting livers through inhibiting the inflammatory factor and reducing oxidative stress reaction. Therefore, effective inhibition of cell activation in the liver and release of inflammatory cytokines are important strategies to protect endotoxin-induced liver injury.

LPS induced liver injury has been extensively studied. After Kang Jie and Zhang Bili [19] gave curcumin treatment to rats with sepsis, they found that IL-18 and TNF-α were significantly reduced, excessive inflammation was inhibited, liver and kidney functions were improved significantly. Fang Shijing, etc. [21] studied liver injury mice models. It was discovered that liver tissue TNF-α and IL-1β contents were reduced, superoxide dismutase (SOD) activity was increased, and liver injury was significantly reduced after advance administration of cordyceps polysaccharide. All the above studies showed that if LPS-induced inflammatory cytokine release and hepatocyte injury can be inhibited, the liver injury thereof can be effectively protected.

In the experiment, mice were attacked by LPS for 6 h. It was found that hepatic injury indexes ALT, AST and LDH were increased in serum. The liver inflammation indexes IL-6 and TNF-α were also increased significantly. Meanwhile, the liver showed obvious pathological changes. However, the above phenomenon of liver injury was significantly improved in the IDA intervention group. It is obvious that IDA stimulates RAW264.7 cell LPS cultivated in vivo. Meanwhile, IDA with different concentration is given for intervention. The ROS generated in the cell was prominently reduced. When 100μg/mL IDA was given for invention, the HO-1 mRNA expression was increased with time dependence mode. However, NQO-1 mRNA was not changed prominently. It shows that IDA has protection function on LPS-induced macrophage oxidative injury mainly through increasing HO-1 expression rather than NQO-1 possibly.

In summary, IDA generates protection role on LPS attacked acute hepatic injury through significantly reducing serum liver injury indexes and the contents of TNF-α, IL-6 and other inflammatory mediators in liver tissues, and alleviating the liver pathology change in the LPS-induced mice acute hepatic injury model. The action mechanism may be related to IDA strengthening of liver macrophage antioxidant function. Therefore, IDA has obvious anti-inflammatory and liver protection function, and the drug is safe and economic with good prospect of clinical application.

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