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EXPERIMENTAL STUDY

Xuebijing injection alleviates liver injury by inhibiting secretory function of Kupffer cells in heat stroke rats

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Abstract

OBJECTIVE: To evaluate the effects of Xuebijing (XBJ) injection in heat stroke (HS) rats and to investigate the mechanisms underlying these effects.

METHODS: Sixty anesthetized rats were randomized into three groups and intravenously injected twice daily for 3 days with 4 mL XBJ (XBJ group) or phosphate buffered saline (HS and Sham groups) per kg body weight. HS was initiated in the HS and XBJ groups by placing rats in a simulated climate chamber (ambient temperature 40℃, humidity 60%). Rectal temperature, aterial pressure, and heart rate were monitored and recorded. Time to HS onset and survival were determined, and serum concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, alanine-aminotransferase (ALT), and aspartate-aminotransferase (AST) were measured. Hepatic tissue was harvested for pathological examination and electron microscopic examination. Kupffer cells (KCs) were separated from liver at HS initiation, and the concentrations of secreted TNF-α, IL-β and IL-6 were measured.

RESULTS: Time to HS onset and survival were significantly longer in the XBJ than in the HS group. Moreover, the concentrations of TNF-α, IL-1β, IL-6, ALT and AST were lower and liver injury was milder in the XBJ than in the HS group. Heat-stress induced structural changes in KCs and hepatic cells were more severe in the HS than in the XBJ group and the concentrations of TNF-α, IL-β and IL-6 secreted by KCs were lower in the XBJ than in the HS group.

CONCLUSION: XBJ can alleviate HS-induced systemic inflammatory response syndrome and liver injury in rats, and improve outcomes. These protective effects may be due to the ability of XBJ to inhibit cytokine secretion by KCs.

Key words: Heat stroke; Kupffer cells; Systemic inflammatory response syndrome; Xuebijing; Liver injury; Inflammatory cytokines

INTRODUCTION

Heat stroke (HS) is a serious, even life-threatening condition, characterized by fever higher than 40℃, dysfunction of the central nervous system, delirium, convulsions and/or coma. Epidemiological data have

shown the incidence rate of stroke was 20/100 000, with mortality rates of 10%-70% in the United States during periods of extreme heat. In Saudi Arabia the death rate from HS is about 50%.[1] In addition to direct injury caused by heat exposure, the pathology of HS is thought to involve systemic inflammatory response syndrome (SIRS) induced by thermal injury, with SIRS in turn inducing critical pathophysiological changes and the development of multiple organ dysfunction syndrome (MODS).[2] Heat stress may increase intestinal permeability and induce the translocation of bacteria and endotoxin.[3,4] Following early stages of heat-exposure, the pathophysiology of heat-stress induced SIRS and MODS is similar to that of sepsis, with liver injury being a frequent pathological change.

Xuebijing (XBJ) is a Chinese medicine compound preparation, consisting of safflower yellow A, tetramethylpyrazine, Danshensu, and ferulic acid. XBJ has been widely used to treat sepsis and to protect specific organs, regulating the inflammatory response and oxidative stress and improving coagulation and immune function,[5-7] all of which are involved in heatstroke. XBJ can enhance the production of hepatic gelsolin by inhibiting the effects of LPS in septic rats, thereby having beneficial effects on the liver.[8] As Kupffer cells (KCs) are key to the immune system of the liver, XBJ may protect the liver by regulating KCs.

KCs are a type of innate macrophages in the liver with many functions, including phagocytosis, immune regulation, the secretion of many bioactive substances, and the clearance of gut-derived bacteria and endotoxins. KCs can be activated by gut-derived endotoxin to secrete many types of pro-inflammatory cytokines, which induce or aggravate liver injury.[9,10] Although inhibiting cytokine secretion by KCs can reduce liver injury in sepsis, a condition with pathological mechanisms similar to those of HS,[11,12] it has not been determined whether XBJ can alleviate liver injury in HS by inhibiting cytokine secretion by KCs. We therefore tested the ability of XBJ to protect against HS-induced liver injury, as well as it mechanisms of action, in HS rats.

MATERIALS AND METHODS

Experimental animals

Because of the effects of estrogen on heatstroke and organ injury,[13] only adult male pathogen-free Wistar rats (Experimental Animal Center of our hospital, Animal Quality Certification No. SCXK2006-0015, Guangzhou, China), weighing 250-320 g, were used. All experiments adhered to our hospital's guidelines for animal care and were approved by the Animal Care and Use Committee of our hospital.

Preparation of heat stroke model

Rats were housed for 6 h at ambient temperature (25℃±0.5℃) and humidity (35%±5%) and were randomly designated into three groups: a vehicle-treated HS group (HS group; n=24), an XBJ-treated HS group (XBJ group; n=24) and a normothermic group (Sham group; n=12). Rats in the Sham and HS groups were injected twice daily for 3 days with 4 mL phosphate buffered saline per kg body weight through the tail vein,[8,14] whereas rats in the XBJ group were intravenously injected twice daily for 3 days with 4 mL XBJ (The Chinese medicine accurate character Z20040033, Tianjin, China) per kg body weight.

Rats were anesthetized with intraperitoneally injected sodium pentobarbital (50 mg/kg), which also abolished the corneal and pain reflexes. The right femoral artery of each rat was cannulated with a trocar (24G) to monitor mean arterial pressure (MAP). Rats in the HS and XBJ groups were placed in a pre-warmed incubator maintained at 40.0℃±0.5℃ and a relative humidity of 60%±5%. The animals in the Sham group were sham-heated at a temperature of 25℃±0.5℃ and humidity of 35%±5%. Rectal temperature (Tc) was monitored at 10 min intervals using a Multi-parameter Physiological Monitor (Infinity Delta XL, Drager, Germany). Animals were withdrawn from the chamber when MAP dropped to 25 mm Hg and core temperature was over 42℃.[15]

Experimental design

To assess survival time, pathological injury to the liver and structural changes in KCs, the rats were withdrawn from heat-stress and exposed to a room temperature of 25℃, and their physiological parameters were monitored continuously. Physiological parameters and times of death were recorded.

In addition, 5 mL blood samples were withdrawn from the right femoral artery of each rat immediately after removal from heat stress to measure the concentrations of TNF-α, IL-1β, IL-6, ALT and AST. The rats were subsequently sacrificed by overdoses of sodium pentobarbital to obtain KCs.

Blood and tissue sampling

Blood samples were left to stand for 60 min and were centrifuged at 300 ×g for 15 min and at 5000 ×g for 5 min to remove cellular components and prepare serum. Aliquots of serum were stored at -80℃ in polypropylene microcentrifuge tubes. Liver tissues obtained at autopsy were fixed in 10% formalin, embedded in paraffin blocks, and sectioned; and the sections were stained hematoxylin and eosin (HE). Tissue samples for transmission electron microscopy were approximately 1-2 mm^3 in size; these samples were prefixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated in an ethoxide/acetone gradient, and embedded in epoxy resin Epon812.

Measurement of proinflammatory cytokines in serum

The concentrations of TNF-α, IL-1β and IL-6 were

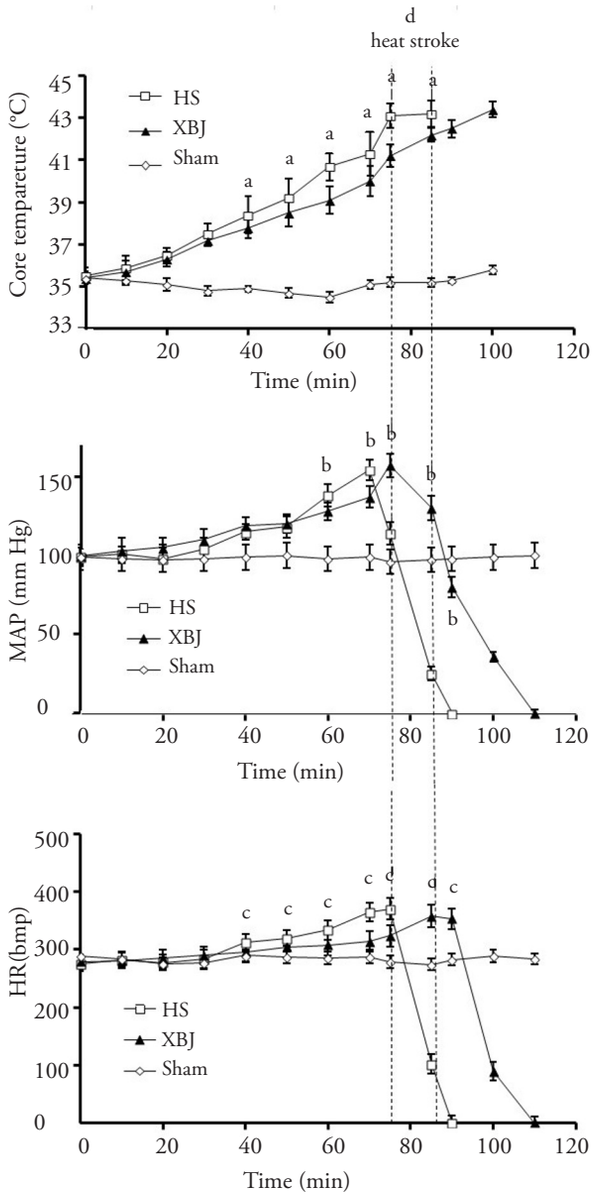

Figure 1 Tc, MAP and HR of rats in the XBJ, HS, and Sham groups

XBJ: Xuebijing; HS: heat stroke; Tc: rectral temperature; MAP: mean arterial pressure; HR: heart rate. Pairwise comparisons of the three groups, [a]*P*<0.05; Pairwise comparisons of the three groups, [b]*P*<0.05; Pairwise comparisons of the three groups, [c]*P*<0.05; HS vs XBJ, [d]*P*<0.05.

measured by ELISA (R&D Systems, Minneapolis, Minn, USA) according to the manufacturer's instructions.

AST and ALT analyses

Serum ALT and AST activities were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

Separation and identification of KCs

An in vitro perfusion method was used to obtain KCs from rat livers.[16,17] KC activation was verified by staining of the tropochrome with 0.4% benzo blue (Sigma, St. Louis, MO, USA) and by the ability of these cells to phagocytose sterile carbon pellets. KCs were transferred to six well plates and cultured for 24 h in an incubator maintained at 37℃ and 5% carbon dioxide.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Data are presented as means ± *SD*. Dunn's test was used for post-hoc multiple comparisons among means, and unpaired Student's *t*-tests were performed to test the significance of differences between means. Survival was analyzed by the Kaplan-Meier method and compared by the log rank test. Between-group comparisons were assessed by one-way analysis of variance (ANOVA). Pearson correlation analysis was performed to test the correlation between two variables. A two-tailed $P<0.05$ was considered statistically significant.

RESULTS

XBJ improves physiological parameters in heat stroke

The kinetics of MAP, HR and Tc during rat exposure to high temperature and humidity are shown in Figure 1. The core temperatures rose more slowly in the XBJ than in the HS group, being significantly different after heat exposure for 40 min ($P<0.05$). MAPs were significantly lower in the XBJ than in the HS group 60 to 70 min after heat-stress ($P<0.05$), but were significantly higher in the XBJ group after 75 min ($P<0.05$). HRs were significantly lower in the XBJ than in the HS group after 40 min ($P<0.05$), but were significantly lower in the HS group after 75 min ($P<0.05$). Time to heatstroke onset [(85.2±2.9) vs (73.6±1.8) min, $P<0.05$] and overall survival time [(108.6±2.6) vs (84.0±1.6) min, $P<0.05$] were significantly longer in the XBJ than in the HS group (Figure 2).

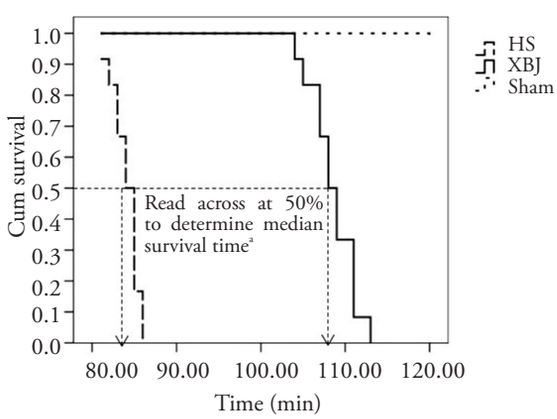

Figure 2 Survival times of rats in the XBJ, HS, and Sham groups

XBJ: Xuebijing; HS: heat stroke. Survival was assessed by the Kaplan-Meier method. [a]Median survival time in the HS and XBJ groups compared by the log rank test; *Chi*-square= 58.57, [a]*P*=0.00.

XBJ attenuates systemic inflammation

Assays of serum TNF-α, IL-1β and IL-6 concentrations showed that all were significantly higher in HS than in Sham rats ($P<0.05$ each; Figure 3). XBJ treatment, however, significantly reduced the serum concen-

trations of TNF-α, IL-1β and IL-6 compared with HS rats (P<0.05 each).

XBJ alleviates liver injury

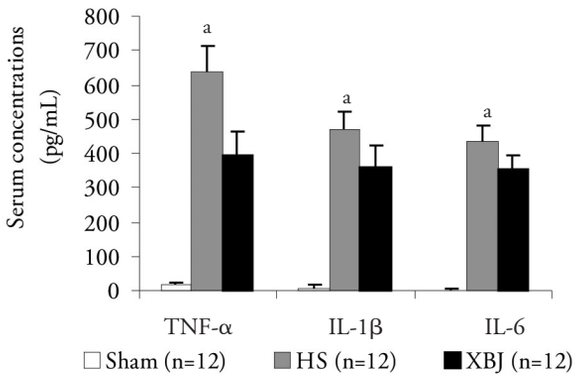

Figure 3 Serum concentrations of TNF-α, IL-1β and IL-6 in HS, XBJ and Sham rats

XBJ: Xuebijing; HS: heat stroke; TNF-α: tumor necrosis factor; IL-1β: interleukin-1β; and IL-6: interleukin-6. Pairwise comparisons of the three groups, [a]P<0.05.

AST and ALT concentrations were significantly higher in HS than in Sham rats (P<0.05 each), with both being significantly lower in the XBJ than in the HS group (P<0.05 each; Figure 4).

These serological findings were confirmed by histological changes in rat livers. Pathological changes in the XBJ group were milder than those in the HS group (Figure 5). In addition, rats in the HS group showed more severe liver injury than those in the XBJ and Sham groups, as determined by histologic injury scores (Figure 6).

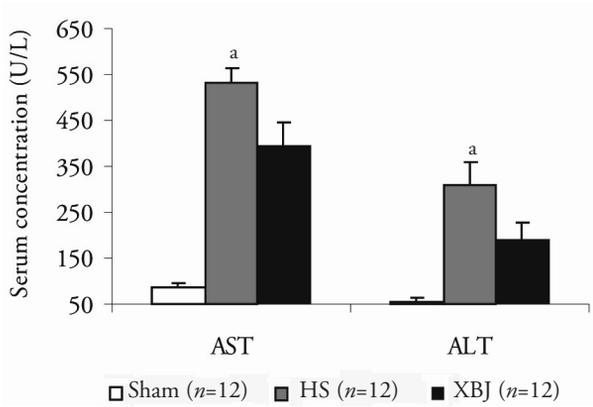

Figure 4 Serum concentrations of AST and ALT in HS, XBJ and Sham rats

XBJ: Xuebijing; HS: heat stroke; AST: aspartate aminotransferase; ALT: alanine aminotransferase. Pairwise comparisons of the three groups, [a]P<0.05.

XBJ stabilizes KCs and protects HCs

Changes in the structure and cellular organs of KCs and hepatic cells (HCs) were monitored by transmission electron microscopy (Figure 7). HCs in the HS group showed obvious degeneration, with shrinking nuclei and condensed chromatin. Many electron-dense lysosomes and moderately dilated rough endoplasmic reticula were observed in KCs. In contrast, KCs in the XBJ group were spindle-shaped, with many ecphyma on their surface and hyperchromatin. The three-layer structure of local cell membranes was unclear, but there was little damage to organelles was not very clear. HC nuclei were spherical, with vacuole degeneration visible in the cytoplasm.

XBJ down-regulates the secretion of inflammatory cytokines by KCs

KCs extracted from the three groups of rats were cultured, and the concentrations of TNF-α, IL-1β and IL-6 secreted into the supernatant were measured. The concentrations of all three cytokines were significantly higher in the supernatants of HS KCs than in the supernatants of KCs from the XBJ and Sham groups (P< 0.05 each). Furthermore, there were high correlations between serum and supernatant concentrations. The Pearson correlation coefficients of TNF-α, IL-1β and IL-6 were 0.60, 0.76, and 0.62, respectively, in the HS group and 0.79, 0.88, and 0.73, respectively, in the XBJ group.

DISCUSSION

HS often leads to MODS despite adequate whole body cooling and organ supporting therapy.[18,19] HS can attack organs through immediate damage induced by heat-stress and secondary injury caused by gut-derived endotoxin.[20] We previously assessed the molecular

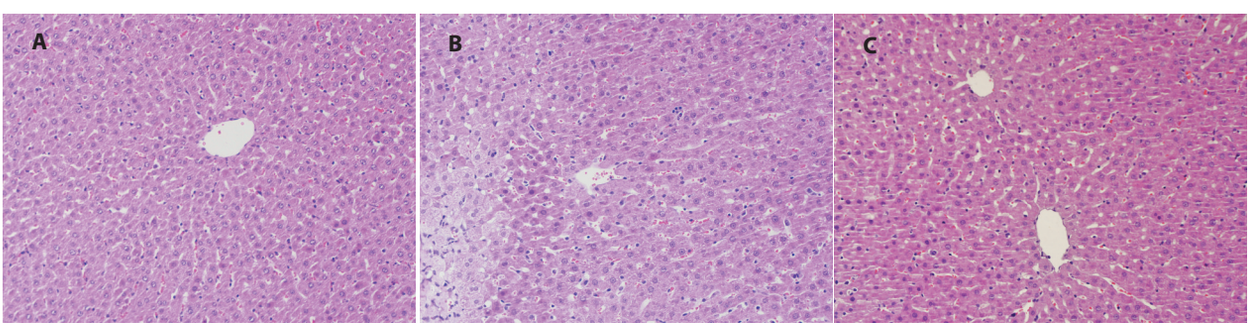

Figure 5 Histology of rat livers (HE staining, ×200)

A: Sham group. The structure and shape of the hepatic lobule were normal, with no evidence of degeneration or necrosis of hepatocytes and no congestion in the sinus hepaticus and vena centralis. B: HS group. Hepatic tissue was infiltrated by inflammatory corpuscles. Congestion in the hepatic sinus and hepatocyte edema were evident, with ballooning degeneration of some hepatocytes (arrow). C: XBJ group. Congestion in the hepatic sinus and hepatocyte edema were mild, with little degeneration of hepatocytes. HE: hematoxylin eosin.

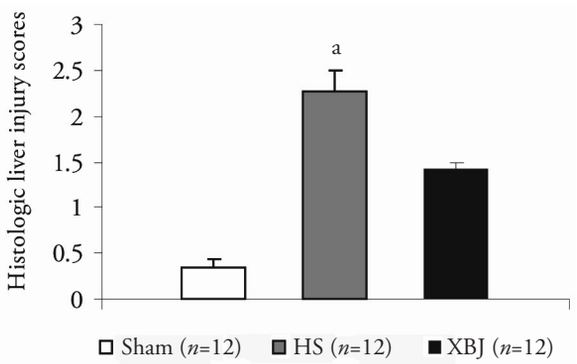

Figure 6 Histologic liver injury scores

XBJ: Xuebijing; HS: heat stroke. Pairwise comparisons of the three groups, $^{a}P<0.05$.

mechanisms underlying heat-stress induced changes in the small intestines of septic mice, and found that damage to the jejunum was caused by intestinal epithelial cell necrosis and loss and by villous desquamation. Furthermore, intestinal fructose 1, 6-bisphosphatase may function as a marker predicting gut dysfunction in heatstroke.[21] Owing to their special anatomic structure, KCs in the liver can regulate SIRS. During early stages of heat-stress, lipopolysaccharide (LPS) is significantly increased in the portal vein, and the concentrations of inflammatory cytokines, which may be secreted primarily by KCs, tend to increase in liver and plasma.[22] These findings suggested that regulating KCs is important in alleviating SIRS and organ damage induced by heat-stress.

In China, XBJ has been injected into patients for 8 years to treat SIRS and MODS induced by infection or ischemia/reperfusion. XBJ has been reported to significantly decrease the serum concentrations of IL-1 and IL-8 in ICU patients.[23] Moreover, XBJ injection was found to reduce the secretion of TNF-α and IL-6 and to inhibit SIRS during cardiopulmonary bypass.[24] A meta-analysis evaluating the efficacy and safety of XBJ injection in the treatment of sepsis showed that XBJ injection may decrease 28-day mortality rates, complication rates, average length of hospital stay, and APACHE II scores.[25] The clinical efficacy of XBJ in sepsis suggested that this agent can reduce the secretion of inflammatory cytokines by LPS-activated mononuclear cells/macrophages.[26] We found that ulinastatin inhibited the inflammatory activity of KCs by regulating the α2A-AR system, with ulinastatin pretreatment of septic rats significantly reducing the concentrations of TNF-α in KC supernatants. We therefore we investigated the effects of XBJ pretreatment on liver injury induced by heat-stress in rats, and whether the mechanisms underlying these effects were correlated with pro-inflammatory cytokines secreted by KCs.

We utilized a heatstroke model, in which rats were exposed to high temperature (40℃) and humidity (60%), monitoring arterial pressure, heart rate and core temperature during this process. We found that pre-exposure to XBJ delayed the onset of heatstroke and enhanced survival time, suggesting that XBJ may protect tissues and organs from the deleterious effects of heat-stress.

We also measured the serum concentrations of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in these animals, finding that the concentrations of all three were significantly lower in XBJ than in HS rats, suggesting that XBJ injection may moderate SIRS induced by heat-stress.

In assaying the effects of XBJ injection on liver injury in heatstroke rats, we found that the concentrations of aminotransferases were lower in XBJ than in HS rats, but were significantly higher in XBJ than in Sham rats. Pathologic analysis showed that hepatic injury was milder, and liver injury scores were lower, in XBJ than in HS rats, indicating that XBJ protected rat livers against heat-stress.

Changes in structure and cellular organelles in KCs and HCs were assessed by transmission electron microscopy. Structural changes and injury to cellular organelles were mild in KCs and HCs of XBJ rats. In HS rats, however, we observed dysfunctional lysosomes, indicative of apoptosis and autophagy in KCs, as well as nuclear shrinking, indicative of HC degeneration. These findings suggest that XBJ may regulate the abnormal activation of KCs induced by heat-stress and endogenous LPS, alleviating liver injury in HS rats.

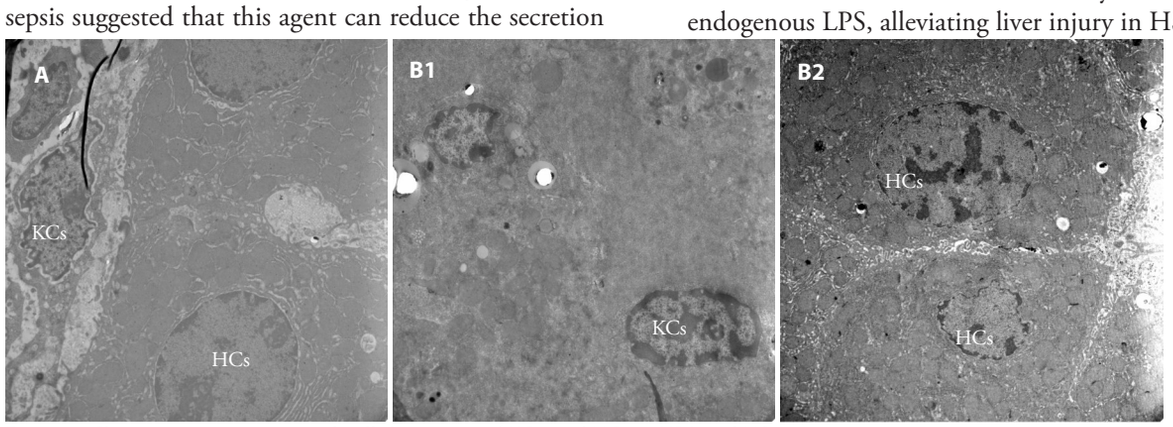

Figure 7 Changes in structure and cellular organ of KCs and HCs in XBJ and HS rats

KCs: Kupffer; HCs: cellshepatic cells. A: XBJ rats. KCs were spindle-shaped, with many ecphyma on their surface and hyperchromatin. The three-layer structure of local cell membranes was unclear, but there was little damage to organelles. HC nuclei were spherical, with vacuole degeneration visible in the cytoplasm. B1 and B2: HS rats. HCs had degenerated, with shrunken nuclei and chromatin condensation. KCs contained many electron dense lysosomes and moderately dilated rough endoplasmic reticulum.

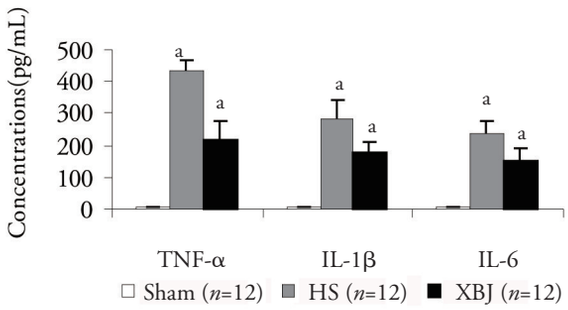

Figure 8 Concentrations of TNF-α, IL-1β and IL-6 in supernatants of KCs from the three groups of rats

XBJ: Xuebijing; HS: heat stroke; Pairwise comparisons of the three groups, $^{a}P<0.05$.

Liver KCs were incubated for 24 h and the concentrations of TNF-α, IL-1β, and IL-6 in their supernatants were assayed quantitatively. Secretion of all three cytokines was significantly greater in KCs from the HS than from the Sham rats, whereas XBJ pretreatment reduced the concentration of these pro-inflammatory cytokines. We also observed strong and positive correlations between the cytokine concentrations in KC supernatants and in serum. Thus, XBJ may protect rats against heatstroke rats by inhibiting cytokine secretion by KCs.

This study had several limitations in the present study. First, we did not test the effects of the inhibitor gadolinium chloride because it is a highly toxic chemical reagent. Second, although we investigated the effects of XBJ on KC secretory function, we did not systematically test its effect on phagotrophic function due to the absence of an accepted, quantitative assay method. Third, XBJ was administered before exposure to heat-stress. An additional study is required to test the effects of XBJ administered following heat-stress.

Many studies have analyzed methods to treat heatstroke. Whole body cooling was used to attenuate acute lung inflammation and injury in a heatstroke rat model,[27] and kynurenic acid, hyperbaric oxygen and activated protein C were tested for their ability to protect the biosystem against heatstroke-induced MODS.[28,29] he ability of Shengmai San, a compound herbal agent, to alleviate the overproduction of inducible nitric oxide synthase-dependent nitric oxide in the brain and inflammatory cytokines in the peripheral blood in rats has also been assayed.[30] Our findings indicate that XBJ injection can alleviate SIRS and liver injury induced by heatstroke in rats, as well as improving the prognosis of heatstroke animals. These protective effects of XBJ may be due to its ability to inhibit the secretion by KCs of pro-inflammatory cytokines. Regulating the functions of KCs, by, for example, XBJ injection, may therefore be important in treating heatstroke.

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