Int J Clin Exp Med 2016;9(4):7524-7533
www.ijcem.com /ISSN:1940-5901/IJCEM0009468

Original Article

Attenuating brain inflammation, ischemia, and oxidative damage by Xuebijing in heat stroke rats

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Received April 22, 2015; Accepted June 21, 2015; Epub April 15, 2016; Published April 30, 2016

Abstract: Xuebijing (XBJ) is a Chinese medicine compound preparation. The aim of the present study was to ascertain whether the brain inflammation, ischemia, apoptosis and oxidative damage in heat stroke (HS) rats can be attenuated by pre-treatment with XBJ. Anesthetized rats were divided into a normothermic group, a vehicle-treated HS group (4 mL phosphate-buffered saline per kg body weight twice daily for 3 days) and an XBJ-treated HS group (4 mL XBJ per kg body weight twice daily for 3 days). HS and XBJ-treated groups were exposed to an ambient temperature of 42.4°C for 1 hour to induce HS. Negative control (NCs) was exposed to room temperature (26°C). Their survival time, core temperatures, mean arterial pressures, inflammatory molecules, number of apoptotic cells in the hypothalamus and neuronal damage scores were determined. Hypothalamic neurons were treated with LPS (lipopolysaccharide) with or without XBJ and cell apoptosis was determined. The survival time for the XBJ-treated HS rats increased from the control values of 74-89 minutes to new values of 109-156 minutes. XBJ therapy caused a reduction of HS-induced cellular ischemia, hypoxia, inflammation, cell apoptosis and oxidative damage in the hypothalamus. XBJ significantly inhibited LPS-induced apoptosis of hypothalamic neurons. Our results suggest that XBJ treatment can reduce HS-induced inflammatory, ischemic, apoptotic and oxidative damage to the hypothalamus and can inhibit the apoptosis of hypothalamic neurons induced by LPS.

Keywords: Xuebijing, heat stroke, inflammation, ischemia, oxidative damage

Introduction

Based on the understanding of the pathophysiology of heat stroke (HS), it has been defined as a form of excessive hyperthermia (>40°C) associated with a systemic inflammatory response that leads to multi-organ dysfunction or failure in which central nervous system (CNS) disorders predominate such as delusion, convulsion and coma [1-3]. In an epidemiologic study, the incidence rate of stroke was 20/100000, with mortality rates of 10-70% in the United States during periods of extreme heat [4]. In Saudi Arabia, the incidence of HS varied from 22-250 cases per 100,000 population. In Saudi Arabia, the crude mortality rate is estimated to be 50% for patients with HS [3].

Recently, in an anesthetized rat model, hypothalamic ischemia, inflammation, and damage were observed after the onset of HS [5-7]. The pathologic mechanisms of HS are not very clear at present, but the direct injury by heat exposure and systemic inflammatory response syndrome (SIRS) induced by thermal injury may be the critical pathophysiological changes that lead to multiple organ dysfunction syndrome (MODS), hypotension, hypothalamic ischemia, and neuronal damage [1, 2, 4, 5, 8, 9]. Heat stress may increase intestinal permeability and induce the translocation of bacteria and endotoxin [10, 11].

Xuebijing (XBJ) is a Chinese medicine compound preparation, consisting of safflower yellow A, tetramethylpyrazine, danshensu, and ferulic acid [4]. XBJ can regulate the inflammatory response and oxidative stress and improve coagulation and immune function [4], all of which are involved in HS. All of these anti-inflammatory effects contribute to the therapeutic mechanisms of XBJ. Existing evidence

has proved the clinical efficacy and potential anti-inflammatory effects of XBJ, but the active constituents of this Chinese herbal medicine injection and their molecular mechanisms remain uninvestigated.

The aim of this study was to investigate whether HS-induced hypotension, systemic inflammatory responses, hypothalamic ischemia, damage, and multiple organ dysfunction could be attenuated by XBJ pre-treatment. Whether LPS (lipopolysaccharide)-induced apoptosis of hypothalamic neurons could be ameliorated by XBJ was also investigated.

Materials and methods

Animals

Adult Sprague-Dawley rats (weight: 250-320 g) were obtained from Vital River Company (Beijing, China). Animals were housed in standard conditions with a normal diet under an ambient temperature of 23 ± 1°C and relative humidity of 40-65%, with a 12 hour light/dark artificial light cycle. All the experiments were performed in accordance with the ethical guidelines laid down by the committee for Animal Care and Use of Guangzhou General Hospital of Guangzhou Military Command.

Surgery and physiological parameter monitoring

The right femoral artery of each rat was cannulated with a trocar (24 G) to monitor mean arterial pressure (MAP). The core temperature (Tco) was monitored continuously by a thermocouple, while heart rate (HR) was monitored continuously with a pressure transducer.

Experimental groups

Rats were housed for 6 h at ambient temperature (25°C ± 0.5°C) and humidity (35 ± 5%). Three groups of animals were randomly designated for the experiment, a normothermic group (Sham group; n=32), a vehicle-treated HS group (HS group; n=32) and an XBJ-treated HS group (XBJ group; n=32). In the Sham and HS groups, the animals were treated with a dose of vehicle (4 mL phosphate-buffered saline per kg body weight) twice daily for 3 days through the tail vein [12]. In contrast, in the XBJ group, rats were intravenously injected a dose of XBJ (4 mL XBJ per kg body weight twice daily for 3 days, The Chinese medicine accurate character Z20040033, Tianjin, China) [4].

Experiment 1 (effect of XBJ on percent survival and Tco after heat stress treatment): Animals (n=8 for each group) were exposed to 42.4°C for 1 hour and then allowed to recover at room temperature (26°C). To assess survival time, the rats were withdrawn from heat-stress and exposed to a room temperature of 26°C, and their physiological parameters were monitored continuously.

Experiment 2 (effect of XBJ on hypothalamic apoptosis): Animals (n=8 for each group) were exposed to 42.4°C for 1 hour and then allowed to recover at room temperature (26°C). The effects of XBJ on apoptosis in the hypothalamus were assessed 4 hours post-WBH (Whole Body Hyperthermia).

Experiment 3 (effect of XBJ on hypothalamic ischemia and neuronal damage): Animals (n=8 for each group) were exposed to 42.4°C for 1 hour and then allowed to recover at room temperature (26°C). The effects of XBJ on nitric oxide and cellular ischemia (glutamate and lactate-to-pyruvate ratio) and damage (glycerol and nitrite) markers of the hypothalamus were assessed 4 hours post-WBH.

Experiment 4 (effect of XBJ on hypothalamic levels of cytokines): Animals (n=8 for each group) were exposed to 42.4°C for 1 hour and then allowed to recover at room temperature (26°C). The effects of XBJ on hypothalamic levels of TNF-α and interleukin (IL)-10 were assessed 4 hours post-WBH.

Murine model of HS

The Tco of the anesthetized animals was maintained at about 37°C with an infrared light lamp, except during the heat stress experiments. The animals were exposed to heat stress treatment (42.4°C; relative humidity, 50-55%; 1 hour) in a controlled environmental chamber [13]. The heat-stressed mice were returned to room temperature (26°C) at the end of the heat exposure. Survival time (the interval between the start of heat stress and the death of the animal) was determined. Core temperatures were measured every 5 minutes with a copper constant thermocouple inserted into the rectum and connected to a thermometer (HR1300; Yokogawa, Tokyo, Japan). Before the thermal experiments, rats were housed at an ambient temperature (26°C) below the thermoneutral zone for this species [14]. After the 1-hour heating period, animals were fed properly and hydrated.

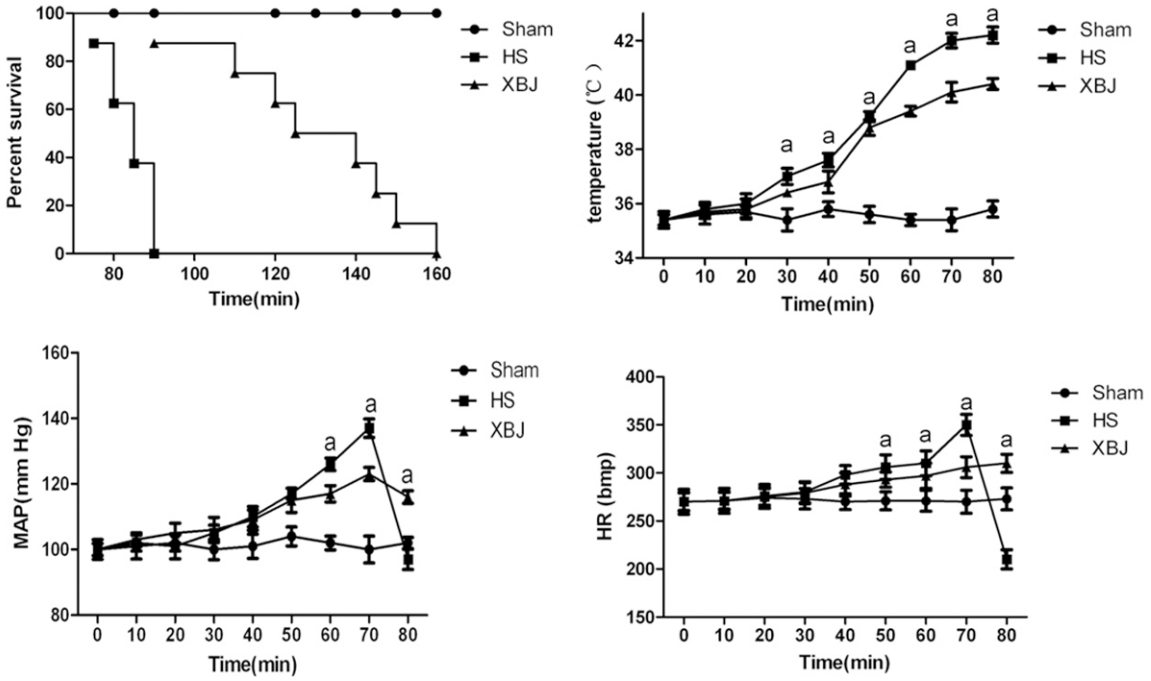

Figure 1. Survival time, Tco, MAP and HR of rats in the XBJ, HS, and Sham groups. Tco: core temperature; MAP: mean arterial pressure; HR: heart rate. Pairwise comparisons of the three groups, $P<0.05$.

Table 1. Neuronal damage scores for rats

Treatment groups	Neuronal damage score (0-3)
Sham	0
HS+vehicle	2 ± 0.5680*
HS+XBJ	1 ± 0.6222#

*$P<0.05$ compared with NC; #$P<0.05$ compared with vehicle-treated heatstroke rats.

Neuronal damage score

4 hours after the 1-hour heating period, animals were killed by an overdose of urethane. The brains were fixed in 10% neutral-buffered formalin and then, they were removed and embedded in paraffin blocks. Hypothalamus sections were stained with hematoxylin and eosin for microscopic evaluation. The extent of neuronal damage was scored on a scale of 0-3, according to the grading system of Pulsinelli et al [15]. 0 is normal and indicates damage of approximately 30% of neurons; 2 indicates damage of approximately 60% of neurons; and 3 indicates damage of 100% of neurons.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

The TUNEL assay was performed to determine the number of apoptotic cells in the hypothalamus. Sections of the hypothalamus were obtained and treated with the reaction mixture of TUNEL (terminal deoxynucleotidyl transferase nucleotide mixture; Roche, Mannheim, Germany) at 37°C for 1 hour. The sections were washed with distilled water. They were then incubated with anti-fluorescein antibody-conjugated with horseradish peroxidase at room temperature for 30 minutes, rewashed, and then visualized using the avidin-biotin-peroxidase complex technique and 0.05% 3, 3-diaminobenzidine tetrachloride as a chromogen. The numbers of TUNEL-positive cells were counted by a pathologist at 200× magnification, in 30 fields per section. Blinding was performed for the pathologist's grading of results.

Extracellular levels of glutamate, lactate-to-pyruvate ratio, glycerol, and NO metabolite in the hypothalamus

The hypothalamus samples were prepared as described in previous reports [16]. NO concentrations were measured with the Eicom ENO-20 NOx-analysis system (Eicom, Kyoto, Japan) [17]. The dialysates were injected onto a CMA600 microdialysis analyzer (Carnegie, Medicine, Stockholm, Sweden) to measure lactate, pyruvate, glycerol, and glutamate.

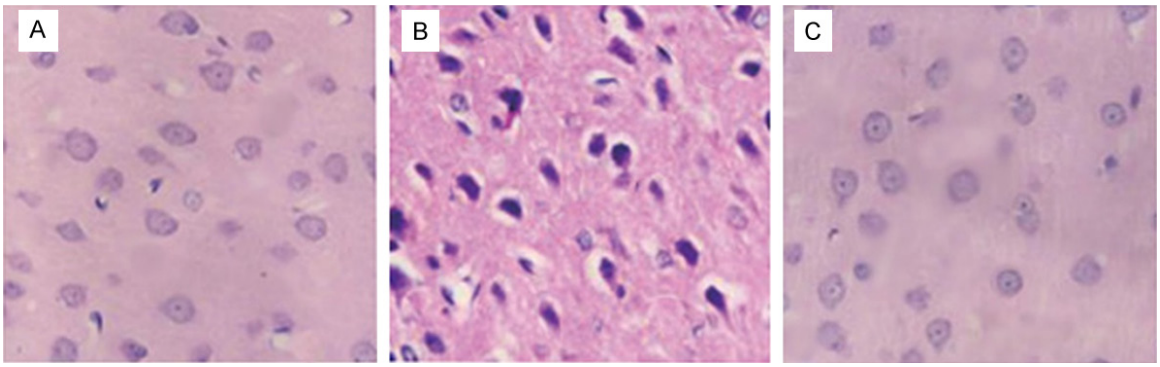

Figure 2. Histological examination of neuronal damage. A. Photomicrograph of the hypothalamus for Sham rat; B. Photomicrograph of heatstroke rat treated with vehicle; C. Photomicrograph of a HS rat treated with XBJ. Magnification ×400.

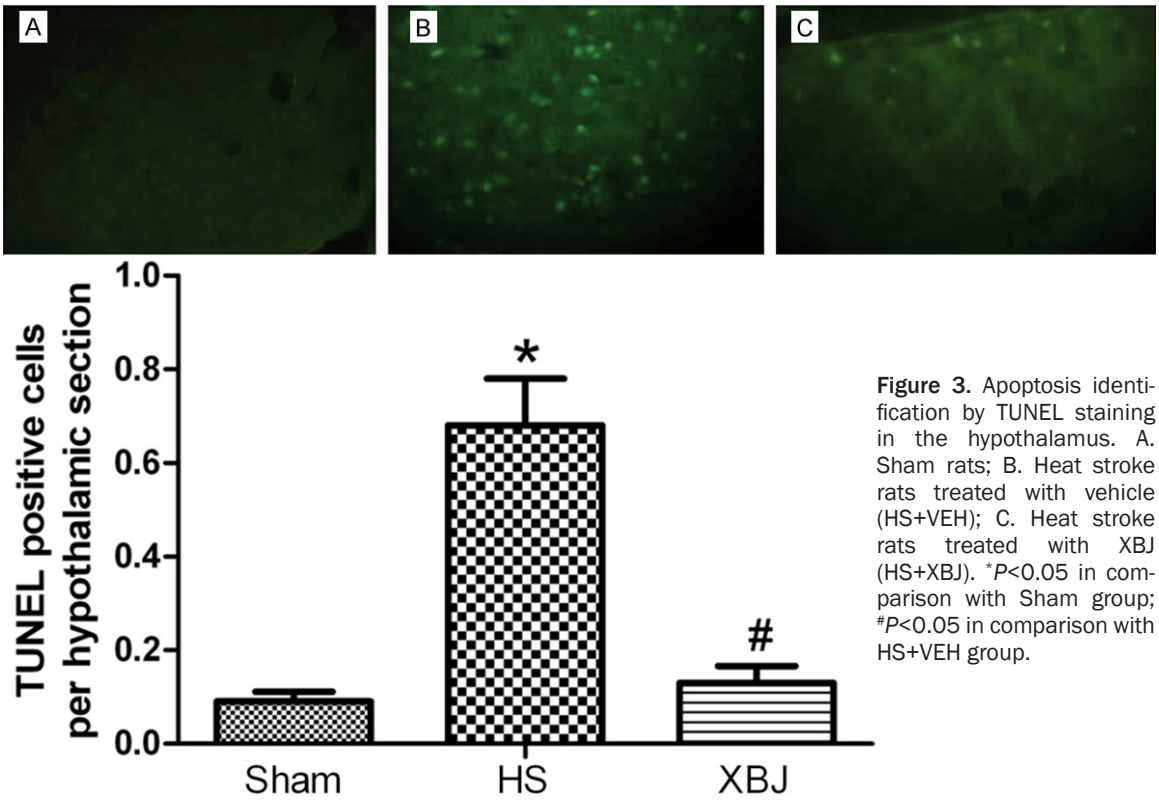

Figure 3. Apoptosis identification by TUNEL staining in the hypothalamus. A. Sham rats; B. Heat stroke rats treated with vehicle (HS+VEH); C. Heat stroke rats treated with XBJ (HS+XBJ). $^{*}P<0.05$ in comparison with Sham group; $^{\#}P<0.05$ in comparison with HS+VEH group.

Measurement of serum TNF-α and IL-10 levels

Blood samples were collected, immediately separated, and stored at -80°C until they could be assayed. We used commercially available ELISA kits for the determination of serum TNF-α and IL-10 levels (Quantikine, R&D Systems Inc. Minneapolis, MN, USA) according to the manufacturer's instructions.

Cell culture

Hypothalamic neurons were separated as described by Poon et al [18]. Hypothalami from embryos of SD rats were microdissected in Mg^{2+}/Ca^{2+}-free Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) and placed in 0.05% trypsin-EDTA for 30 minutes at 37°C (Invitrogen, Carlsbad, CA, USA), as previously described [19]. The cells were triturated with 0.01% deoxyribonuclease in Neurobasal Media using a 1000 μL pipette tip, passed through a 70 μm followed by a 40 μm cell strainer (Fisher Scientific, Waltham, MA, USA), and spun down. The cells (1 million/mL) were resuspended in Neurobasal Media containing B27 supplement (Invitrogen, Carlsbad, CA, USA) and cultured at

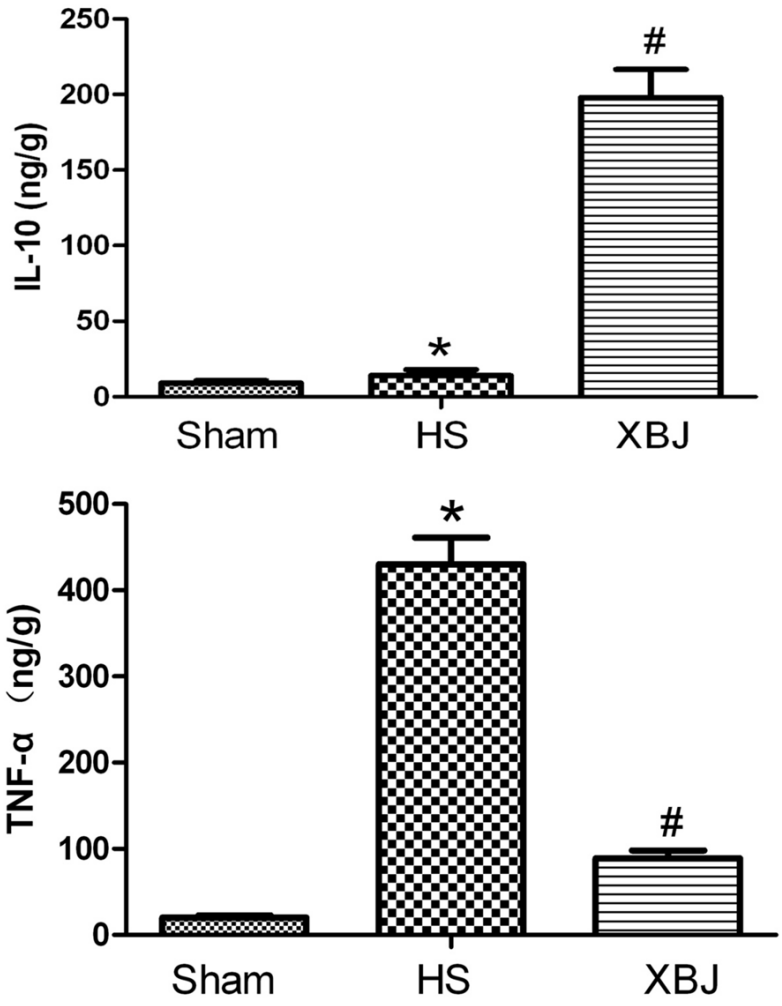

Figure 4. Effects of heat exposure (42.4°C for 1 hour) on extracellular levels of TNF-α and IL-10 in the hypothalamic levels. Bars are mean ± SD 8 rats per group. $^{*}P<0.05$ in comparison with Sham group; $^{\#}P<0.05$ in comparison with HS+ vehicle group.

1×10^6 cells per well in a six-well plate (BD Biosciences, Sparks, MD, USA). Cells were then placed in a humidified, 5% CO_2 incubator at 35°C.

Apoptosis assay

Cell apoptosis was assayed by flow cytometry with the Annexin V-FITC Apoptosis Detection Kit (Sigma, MO, USA). Hypothalamic neurons were pre-treated with XBJ (50 mg/mL) for 2 hours then treated with LPS (100 μg/mL) for 12 hours. They were washed twice with buffer; Annexin V/FITC was then added. After incubation for 10 min at room temperature in the dark, the cells were washed and resuspended; propidium iodide was then added to a final concentration of 1 mg/L. Stained cells were analyzed using a FACScalibur (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

All statistical analyses were performed using SPSS 10.0 statistical software (SPSS Inc., Chicago, IL, USA). All data are expressed as means ± standard deviations. Oneway analysis of variance with Tukey's multiple comparisons test was used for serum markers, Tco, MAP, survival time, and apoptotic cell numbers. The Wilcoxon test was used for histological assessment. $P<0.05$ was considered statistically significant.

Results

XBJ attenuates heat-induced lethality and hyperthermia

As seen in **Figure 1**, the survival time was 74-89 minutes (n=8) for of vehicle-treated HS rats and 109-156 minutes (n=8) for XBJ-treated rats. The XBJ treated HS rats had significantly a longer survival time compared with vehicle-treated HS rats ($P<0.05$). **Figure 1** shows the effect of heat exposure on Tco, MAP and HR in rats treated with vehicle and in rats treated with XBJ. The Tco and HRs were higher in the vehicle-treated HS rats than in the Sham group, Tco was significantly lower in the XBJ than in the HS group after 30 min ($P<0.05$) and HRs was significantly lower in the XBJ than in the HS group after 50 min ($P<0.05$). On the other hand, MAP was significantly higher in the XBJ than in the HS group after 70 min ($P<0.05$). Heatstroke-induced bradycardia, hyperthermia and hypotension were significantly reduced by XBJ treatment.

XBJ attenuates hypothalamic neuronal degeneration and apoptosis during HS

As shown in **Table 1**, the hypothalamic neuronal damage scores were higher in the HS group

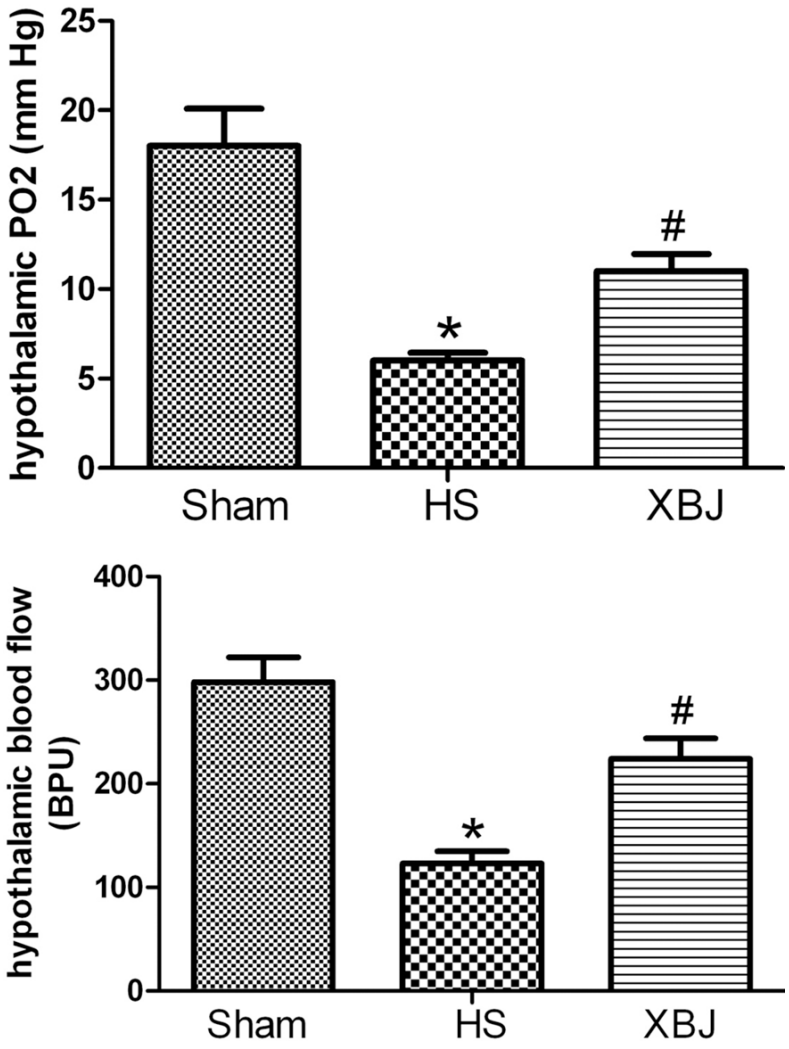

Figure 5. Values of hypothalamic PO2 and blood flow for Sham rat, heatstroke rats treated with vehicle (HS+VEH) and heatstroke rat treated with XBJ (HS+XBJ). *P<0.05 in comparison with NC group; #P<0.05 in comparison with HS+VEH group. Data are means ± SD 8 rats per group.

compared with Sham group and can be attenuated by XBJ. Histopathologic verification revealed that heat-induced hypothalamic neuronal degeneration was greatly reduced in XBJ-treated heatstroke rats (**Figure 2**).

Figure 3 shows the number of TUNEL-positive cells in the hypothalamus. The numbers of TUNEL positive cells in the hypothalamus of vehicle-treated HS rats was significantly higher than that of Sham rats and was significantly reduced by XBJ treatment.

XBJ attenuates hypothalamic inflammation in HS rats

Figure 4 shows the hypothalamic levels of TNF-α and IL-10. The levels of hypothalamic TNF-α of vehicle-treated HS rats were significantly higher than those of NCs. In XBJ-treated HS rats, the increase in levels of hypothalamic TNF-α was significantly suppressed (P<0.05). However, compared with NCs, vehicle-treated HS rats had lower levels of IL-10 and XBJ-treated HS rats had higher (P<0.05) levels of IL-10.

XBJ improves hypothalamic ischemia and hypoxia in HS rats

Figure 5 shows the hypothalamic levels of PO_2 and cerebral blood flow. PO_2 and cerebral blood flow were significantly lower in the vehicle-treated HS rats than in Sham rats, and heat-induced hypothalamic hypoxia and ischemia were significantly reduced by treatment with XBJ.

XBJ reduces cellular levels of damage markers and the toxic oxidizing indicators in the hypothalamus in HS rats

Figure 6 depicts the effects of heat exposure on the cellular levels of glutamate, glycerol, lactate-to-pyruvate ratio and NO in the hypothalamus of Sham rats, vehicle-treated HS rats, and XBJ-treated HS rats. The levels of glutamate, glycerol, lactate-to-pyruvate ratio, and NO in the hypothalamus of vehicle-treated HS groups were all significantly higher than those of NCs and were reduced by treatment with XBJ.

XBJ inhibits LPS-induced neuronal apoptosis

Flow cytometry was utilized to quantify LPS-induced neuronal apoptosis. As shown in **Figure 7**, under normal conditions, there was a very low level (9.8%) of neuronal apoptosis, but the percentage of apoptosis was significantly increased to 49% (P<0.01) after heat exposure. The level was reduced to 29.1% when rats were treated with XBJ (P<0.05).

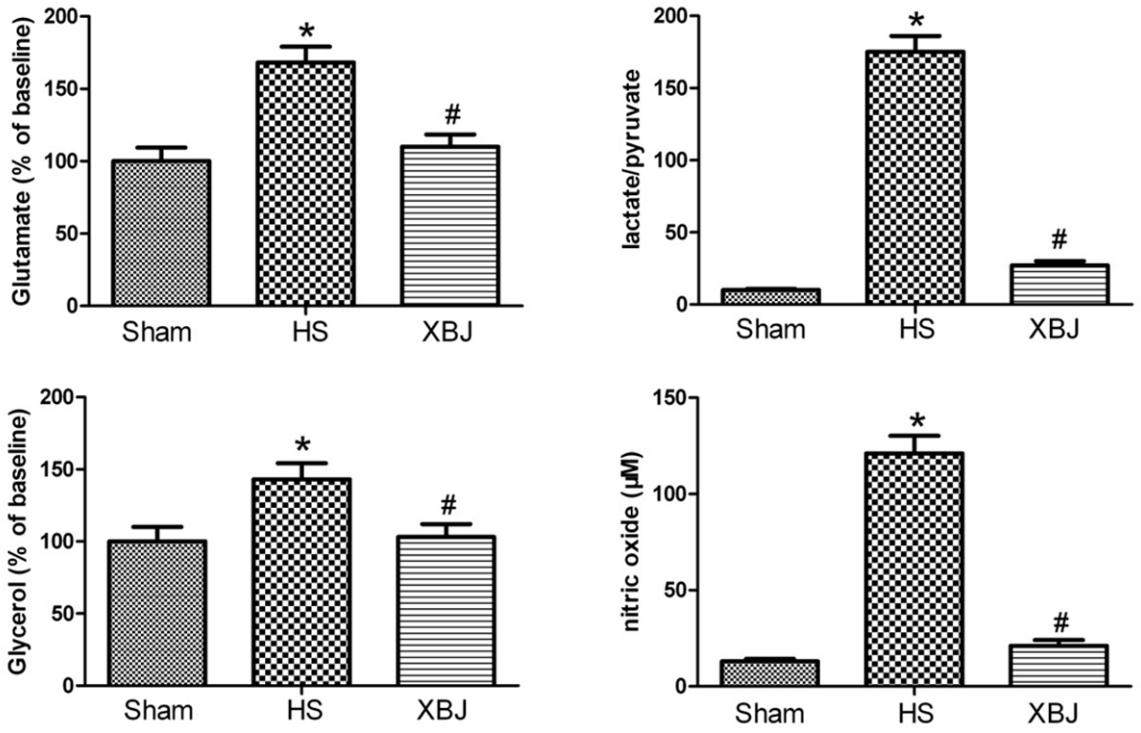

Figure 6. Effects of heat exposure (42.4°C for 1 hour) on cellular levels of glutamate, glycerol, lactate/pyruvate ratio and nitric oxide in the hypothalamus in NC rats, vehicle-treated heatstroke rats, and XBJ-treated heatstroke rats. Data are means ± SD 8 rats per group. $^{*}P<0.05$ in comparison with Sham group; $^{\#}P<0.05$ in comparison with HS+VEH group.

Discussion

HS is defined as a condition in which the Tco is elevated to a critical level, and often leads to MODS despite adequate whole-body cooling and organ supporting therapy [2, 20]. HS can attack organs through immediate damage induced by heat stress and secondary injury caused by gut-derived endotoxin [10]. In 25% of patients with HS, tissue damage continues to develop after whole-body cooling to normal body temperature [21]. In a normal person, enduring a core temperature of about 42°C may cause no minimal tissue injury [7]. It has been suggested that tissue ischemia and hypoxia, rather than hyperthermia itself, are the main cause of the occurrence of HS [2].

XBJ has been used in China for 8 years to treat SIRS and MODS induced by infection or ischemia/reperfusion. It has been reported that XBJ can significantly decrease serum concentrations of proinflammatory cytokines such as IL-1 and IL-8 in ICU patients [4]. XBJ can regulate the inflammatory response and oxidative stress and improve coagulation and immune function, all of which are involved in HS [4]. Moreover, XBJ can reduce the secretion of TNF-α and IL-6 and inhibit SIRS during cardiopulmonary bypass [4]. In our research, we investigated the effects of XBJ on heat stress-induced damage.

We utilized a HS model in which rats were exposed to high temperature (42.4°C) and humidity (50055%) for 1 hour. We found that XBJ treatment significantly attenuated the bradycardia, hyperthermia, and hypotension induced by heat stress and enhanced survival time, which suggests that XBJ may protect tissues and organs from the deleterious effects of heat stress.

The increase in plasma levels of proinflammatory cytokines is associated with the severity of HS. It has been suggested that IL-10 may have a therapeutic potential in acute and chronic inflammatory diseases. Exogenous administration of recombinant IL-10 protects mice from lethal endotoxemia by reducing TNF-α release [22]. Therefore, we measured the concentrations of TNF-α and IL-10. We found that XBJ

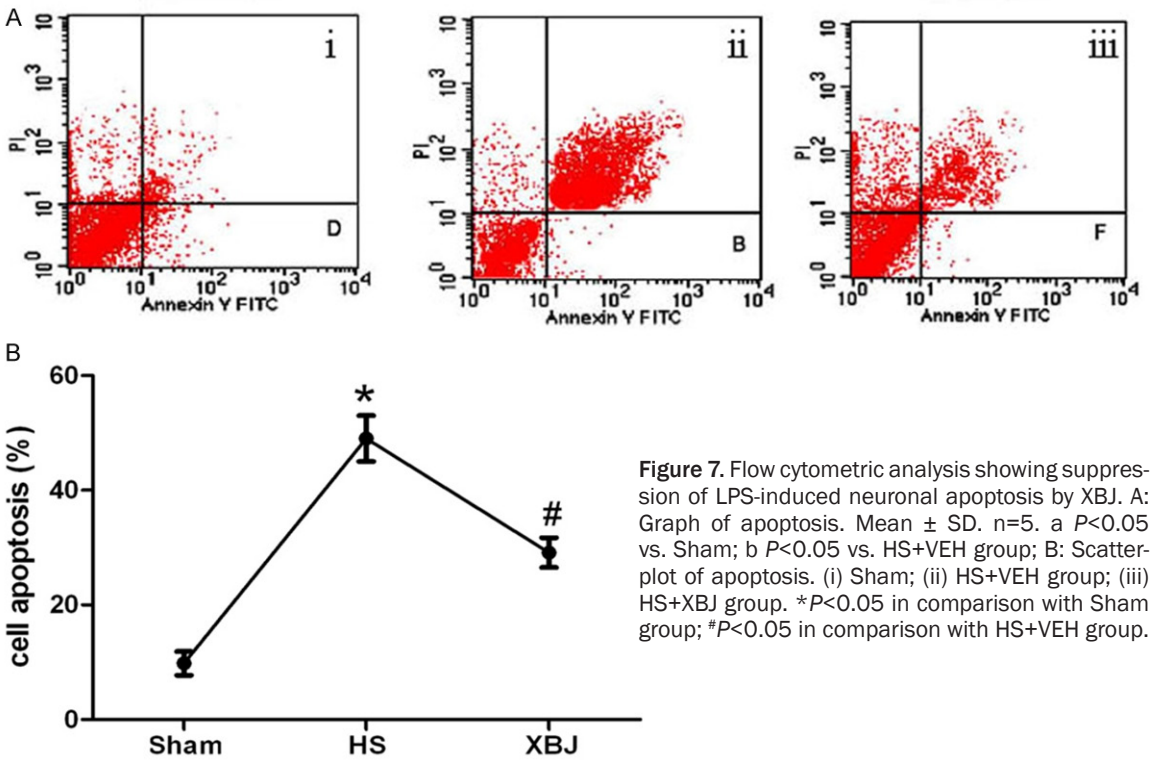

Figure 7. Flow cytometric analysis showing suppression of LPS-induced neuronal apoptosis by XBJ. A: Graph of apoptosis. Mean ± SD. n=5. a $P<0.05$ vs. Sham; b $P<0.05$ vs. HS+VEH group; B: Scatterplot of apoptosis. (i) Sham; (ii) HS+VEH group; (iii) HS+XBJ group. *$P<0.05$ in comparison with Sham group; #$P<0.05$ in comparison with HS+VEH group.

could significantly down-regulate the expression of proinflammatory cytokine TNF-α and upregulate the expression of anti-inflammatory cytokine IL-10, which suggests that XBJ injection may moderate SIRS induced by heat stress.

Recent findings have documented that unanesthetized, unrestrained mice with HS displayed hypothermia when exposed to room temperature. The HS-induced thermoregulatory deficits may have resulted from neuronal apoptosis and cell degeneration in the hypothalamus [8, 13, 23, 24]. In our research, we also demonstrated the protective effects of XBJ in reducing HS-induced hypothalamic neuronal apoptosis and degeneration. Also, we found that XBJ could reduce the hypothalamic neuronal apoptosis induced by LPS.

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are related to the pathogenesis of CNS injury, such as spinal cord injury, traumatic brain injury, chronic neurodegenerative diseases, and HS [23-25]. In the CNS, ischemia or damage, overproduction of glutamate, ROS, and RNS all contribute to a series of events that may precede neuronal death. In addition, our current findings show that XBJ can be used to limit cerebral ischemia and neuronal damage in the hypothalamus during HS, by reducing overproduction of both RNS and ROS in the hypothalamus.

In summary, the current findings show that XBJ treatment significantly prevents rats from HS-induced hypothalamic thermoregulatory deficits and mortality. Also, XBJ treatment can reduce HS-induced inflammatory, ischemic, apoptotic, and oxidative damage to the hypothalamus. Moreover, we found that XBJ could reduce the hypothalamic neuronal apoptosis induced by LPS. Therefore, these observations suggest that XBJ may be important in treating HS.

Acknowledgements

This study was supported by National Natural Science Foundation of Guangdong province (NO. 2014A030313601) and Science and technology planning project of Guangdong province (NO. 2013B031800010).

Disclosure of conflict of interest

None.

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