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THE ANTI-HEAT STRESS EFFECTS AND ACUTE TOXICITY TEST OF AN ANTI-HEAT STRESS PRESCRIPTION

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ABSTRACT

The study was conducted to explore the anti-heat stress efficacy of anti-heat stress prescription (AHSP) and to test its safety. Kunming (KM) mice were randomly divided into the control group, the AHSP group, the heat stress (HS) group, and the (AHSP+HS) group. Following 8 days i.g. administration, the HS group and the (AHSP+HS) group were treated with heat stress to the rectal temperature reached 41°C. Blood samples were collected from mice in all four groups to measure the activity of lactate dehydrogenase (LDH), creatine kinase (CK), superoxide dismutase(SOD), and glutathione peroxidase (GSH-Px), and the concentration of lactic acid(LD) and malondialdehyde (MDA) in the plasma. Furthermore, specimens of liver and intestines were prepared to perform paraffin-embedded sectioning and HE staining to observe the structural changes under microscopy. In another experiment, KM mice were randomly divided into the control group and the AHSP group. Animals in the AHSP group were i.g. administered with AHSP at the maximal dose, to observe the acute toxicity response, body weight change, indexes of liver, spleen, and kidneys, and pathological changes of intestines and liver of mice. It showed that compared to the control group, the HS group exhibited significantly higher activities of LDH ($P<0.05$), CK ($P<0.05$) and SOD ($P<0.01$), and concentrations of LD ($P<0.05$) and MDA ($P<0.01$), but only slightly higher activity of GSH-Px. In contrast, the (AHSP+HS) group reduced the activities of LDH and CK and the concentrations of LD and MDA, but significantly increased the activity of SOD ($P<0.05$), and further increased the activity of GSH-Px. Furthermore, HS resulted in structural damage to the liver and intestines, while AHSP reduced the degree of damage caused by HS and to some extent. In addition, during the 14 days acute toxicity test, the mice were normal at every aspect, including body weight, indexes of liver, spleen, and kidneys, and pathological changes of intestines and liver. It concluded that heat stress can enhance the anaerobic respiration to improve the antioxidant activity of the body, but can also cause enzyme release and lipid peroxidation. AHSP can alleviate the damage caused by anaerobic glycolysis during heat stress and enhance the antioxidant activity of the body, and thus can reduce the degree of damage caused by heat stress and protect livers and intestines from thermal damage. Importantly, AHSP cannot cause any obvious acute toxicity response.

Key words: heat stress; anti-heat stress prescription; radicals; mouse.

INTRODUCTION

Heat stress not only causes behavioral changes in animals, but can also influence the endocrine system, and the physiological and biochemical properties of the body. It has been suggested that high temperatures can decelerate animal growth (Walter *et al*. 2000; Cian *et al*. 2001), produce more radicals (Heise *et al*. 2003; Keller *et al*. 2004), destroy immunity, and cause blood biochemical changes (Thaxton, *et al*. 1968; Zulkifi *et al*. 2000). Furthermore, high temperatures can change the activity of some enzymes and hormone in the plasma through modulating three major stress cascade pathways: the hypothalamic-pituitary-adrenal axis, the sympathetic-adrenal-medulla axis, and the hypothalamic-pituitary-thyroid axis. Currently, one of the measures to prevent heat stress lies in the adjustment of the animal dietary protein and energy levels, of which anti-stress nutrients and drugs such as Vitamin C, E, and Zn are applied (Deyhim *et al*. 1995; Sahin *et al*. 2003). However, with the increase of global warming and urban heat island effects, the current anti-heat stress measures are no longer sufficient for controlling heat stress. Hence, it is necessary to develop new anti-heat stress measures and drugs.

Chinese herbal medicine, a unique type of medicine in China, has many advantages. Currently, the anti-stress effects of some herbs or extracts such as schizandrin (Lee *et al*. 2007) and baicalin (Chang *et al*. 2007) have been studied. On the other hand, some biochemical indexes can reflect changes in cellular energy and metabolism in the body, and thus can be used as indicators for the functional status of organs. In the present study, we studied the anti-heat stress effects of AHSP through biochemical measurements and microscopic observations, and evaluated the toxicological safety of AHSP. Our study provides scientific evidence for the clinical application of AHSP.

MATERIALS AND METHODS

Heat stress and AHSP

Animals: Mice were purchased from the Laboratory Animal Center, Sun Yet-Sen University of Medical Sciences. Equal numbers of female (body weight, 26.4±2.56 g) and male (body weight, 31.8±1.80 g) mice were raised in cages with 6 mice per cage. Mice were not constrained from food or water. The animal room had a temperature of 28.4±0.56°C, a humidity of 86.0±3.74%, and a day/night cycle of 12 h: 12 h. This experiments was conducted with ethics approval from the Hainan Normal University Animal Experimentation Ethics Committee.

Drugs and agents: AHSP, developed by the Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, was confected with honeysuckle, astragalus, ophiopogon, schisandra, diffusa, bupleurum, and γ-aminobutyric acid (GABA) according to a certain proportion (Chen *et al*. 2011). The honeysuckle, astragalus, and diffusa were produced in the Guangxi Province. The schisandra and bupleurum were produced in the Yunnan Province. The ophiopogon was produced in the Sichuan Province. GABA was purchased from Sigma (Shanghai, China).The testing kits of CK, LD, LDH, SOD, MDA, and GSH-Px were all purchased from the Jiancheng Biological Engineering Institute (Nanjing, China).

Animal groups and sample collecting: After 3 days adaptive feeding, mice were randomly divided into four groups: the control group, the AHSP group, the HS group, and the (AHSP+HS) group. The AHSP group and the (AHSP+HS) group were i.g. administered with AHSP at a dose of 0.2 ml, and the control group and the HS group were i.g. administered with an equal amount of saline. Drug administration was performed at 8:00 am each day for 8 days. 30 min after the last administration, mice were anaesthetized with 0.2 ml 20% urethane injection. The HS group and the (AHSP+HS) group were stimulated with heat stress in a cabin with a temperature of 39±1°C and a humidity of 65±5%. The rectal temperature was measured using a CW100 temperature transducer and a BL-420S biological function system (Taimeng Ltd., Chengdu, China). The heat stress stimulation was terminated when the rectal temperature reached 41°C (Sharma, 2007; Yin *et al*. 2011), and blood was collected from the eyes of the mice. For the control group and the AHSP group, blood was collected without prior heat stress stimulation. Blood samples were treated with anticoagulant heparin, centrifuged at 3000 rpm for 10 min. Supernatants were collected and frozen at -20°C for use. In the meantime, the livers and intestines were dissected out and fixed in Bouin's solution or 10% formaldehyde, respectively, for 12 h, and stocked in 70% alcohol for use.

Measurements of plasma biochemical indicators: To evaluate the changes in metabolism, we measured the activity of CK and LDH and the concentration of LD. To evaluate the changes in the antioxidant activity, we measured the activity of SOD and GSH-Px, and the concentration of MDA. All experiments were performed according to the manufacturer's instructions.

Microscopic changes in the structure of liver and intestines: The samples of liver and intestines were performed with paraffin-embedded sectioning and HE staining, and observed under an Olympus CX-21 microscope (Shanghai, China) to examine the structural changes of the specimens.

AHSP acute toxicity test: The AHSP acute toxicity test was performed as described previously (Xu, 2001). Briefly, 28 mice (14 male and 14 female, body weight 25-30 g) were randomly divided into the control group and the AHSP group. Following an 8-12 h constraint of food but not water, mice were i.g. administered with AHSP at a dose of 0.3 ml/10g (30 g/kg) twice after 8 h intervals. The mice were carefully monitored for 2-3 h following the AHSP administration, and continuously observed for 14 days. We observed the mouse indexes including appearance, behavior, activity, respiration, secretion, defecation, and death, and measured the body weight every 3 days. After 14 days, the mice were killed, dissected, and examined for changes in major organs. The livers, spleens, and kidneys were weighed to calculate the organ coefficient (the ratio of organ weight to the total body weight). The liver and intestine samples of four randomly selected mice were fixed in Bouin's solution or 10% formaldehyde solution, respectively, for 12 h, and stocked in 70% alcohol for use. Samples were performed with paraffin-embedded sectioning and HE staining and observed under a microscope to examine the structural changes of the specimens.

Statistical analysis: All data were analyzed with the SPSS 17.0 Statistical Software Package. The independent-samples t-test was used and the data were shown as $\bar{x}$±SD. Effects were considered significant or highly significant in all statistical calculations if P-values were < 0.05 or <0.01 respectively.

RESULTS

Heat stress and AHSP anti-heat stress experiments

The metabolism and antioxidant ability in the plasma of mice: As shown in Table 1, compared to the control group, the HS group exhibited significantly higher LDH and CK activities ($P<0.05$) and LD concentration ($P<0.05$). In contrast, AHSP administration reduced the activities of LDH and CK and the concentration of LD in the plasma of heat stress treated mice, which, however,

were still higher than in the control group and the AHSP group.

As shown in Table 2, compared to the control group, the HS group exhibited significantly higher SOD activity (P<0.01) and MDA concentration (P<0.01), and slightly higher GSH-Px activity in the plasma. In the meanwhile, the AHSP administration can significantly increase the SOD activity (P<0.05), further increase GSH-Px activity, and reduce MDA concentration.

Microscopic changes in the structure of mice livers and intestines: As shown in Fig.1, Under a microscope, we found that compared to the control group and the AHSP group, heat stress caused a series of changes in the structure of the livers and intestines. These changes included: scattered arrangement of liver cells, increased intercellular cleft, swollen nuclei, blurred cell boundary, central venous congestion, villi edema and fracture, the central lacteal dispersion (which can hurt villous microcirculation), and loosened arrangement of columnar epithelial cells (which can affect the immune activity of intestinal mucosa). Furthermore, AHSP administration can reduce the damage to the livers and intestines by heat stress. In detail, these effects included: neat arrangement of liver cells from the central vein towards the surrounding region as the control, reduced intercellular cleft, clear cell boundary, dense arrangement of columnar cells in the intestinal villi, reduced villi fracture, and central lacteal dispersion. These observations indicated that AHSP can take protective effects on the livers and intestines of heat stress treated mice.

AHSP acute toxicity test: After drug administration, the AHSP group exhibited normal feeding, behavior, respiration, secretion, and response. During the following 14 days, all mice were living normally without obvious toxic response, and the increase of body weight was also normal (Table 3). After 14 days, we dissected the mice, and failed to observe any obvious change in the color and morphology of major organs. The livers, spleens, and kidneys were weighed to calculate the organ coefficient. The results showed that AHSP administration did not significantly affect these organ coefficients (Table 4). Observation of the HE stained specimens under a microscope did not reveal any obvious pathological symptoms such as edema, degeneration, or necrosis in the liver and intestinal tissues.

Tables Table.1 Effect of AHSP on the activity of LDH, CK and the content of LD in the plasma of mice under heat stress ($\bar{x}$±SD n=6

Groups	Lactic dehydrogenase) U/ml	Lactic acid (mmol/L	Creatine kinase) U/ml
Control	5.553±0.618	2.947±0.406	0.478±0.052
AHSP	5.675±0.368	2.815±0.378	0.473±0.163
HS	6.615±0.948 [a]	3.924±0.985 [a]	0.692±0.193 [a]
AHSP+HS	6.290±1.056	3.256±0.565	0.540±0.181

a: Significantly different at P<0.05 (compared to the control group)

Table.2 Effect of AHSP on the activity of SOD, GSH-PX and the content MDA in the plasma of mice under heat stress ($\bar{x}$±SD n=6

Groups	Superoxide dismutase) U/ml	Glutathione peroxidase (U/ml	malonaldehyde) nmol/ml
Control	116.709±18.064	650.820±135.605	4.378±0.488
AHSP	111.621±23.520	658.197±107.947	4.503±0.476
HS	149.994±10.916 [A]	757.377±198.362	6.719±0.973 [A]
AHSP+HS	164.622±10.608 [b]	809.016±154.734	5.651±1.853

A: Highly significant different at P<0.01 (compared to the control group)
b: Significantly different at P<0.05 (compared to the control group)

Table.3 The weight change of mice in each group in AHSP acute toxicity test (g $\bar{x}$±SD n=14

Groups	days				
	1d	4d	7d	10d	13d
Control	29.507±0.763	30.779±0.969	31.143±0.936	32.279±0.795	34.221±1.079
AHSP	30.221±0.617	30.771±0.773	31.664±0.711	32.700±1.021	33.629±0.853

Table.4 The organ coefficient of mice in each group in AHSP acute toxicity test (g/g $\bar{x}$±SD n=14

Groups	Organ coefficient		
	Liver	Spleen	Kidney
Control	0.051±0.002	0.004±0.001	0.013±0.002
AHSP	0.053±0.003	0.004±0.001	0.016±0.001

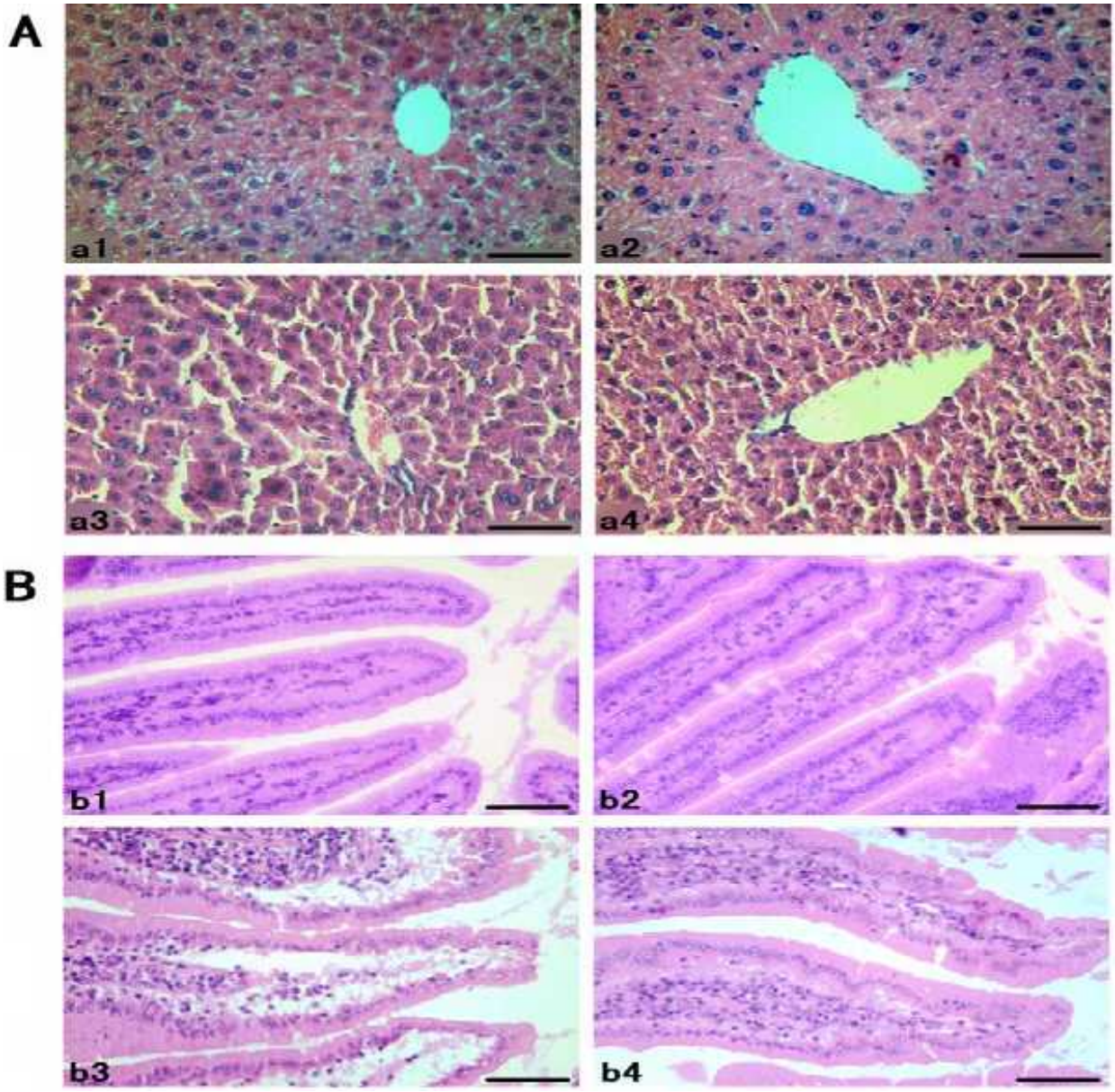

Fig. 1 A. The microscopic changes in the structure of mice livers. (a1) control group, neat arrangement of liver cells from the central vein towards the surrounding region, clear cell boundary. (a2) AHSP group, without any difference with the control group. (a3) HS group, HS caused a scattered arrangement of liver cells, increased intercellular cleft, blurred cell boundary and central venous congestion. (a4) AHSP+HS group, clear cell boundary relatively and reduced intercellular cleft and scattered arrangement of cells.

B. The microscopic changes in the structure of mice intestines. (b1) control group, very dense and orderly arrangement of columnar cells in the intestinal villi, clear goblet cells. (b2) AHSP group, without any difference with the control group. (b3) HS group, HS caused villi edema and fracture, loosened arrangement of columnar epithelial cells, less goblet cells. (b4) AHSP+HS group, dense arrangement of columnar cells relatively and reduced villi fracture.

A, B:(400), Hematoxylin & Eosin-Stain, Bar=50 ×m

DISCUSSION

Under normal circumstances, body temperature is closely regulated by matching heat production with heat loss via conduction, convection, radiation and evaporation. A high temperature makes it more difficult for animals to lose heat, and they accumulate heat, resulting in a series of physiological modifications and behavioral changes. In the present study, the mice exhibited rapid breathing, saliva secretion and listlessness at the prophase stage of heat stress, and they convulsed in the mid-stage. The saliva kept secreting and there was no urine output. Mice in the (AHSP+HS) group also exhibited the same symptoms, but the point at which they exhibited these was delayed. Furthermore, the anus temperature rose slower than in the HS group, and the time it took the anus temperature to reach 41°C was 3-8 minutes longer than in the HS group. This indicates that AHSP can enhance the anti-heat stress ability of mice, to some extent.

Heat stress can disturb the metabolism of the body and subsequently cause changes in the enzyme activity and concentration in the plasma. LDH is an important enzyme involved in the energy metabolism of the body, and thus can be used as an indicator to evaluate the myocardial anaerobic metabolism of skeletal muscle, liver, and kidney (Tian *et al*. 2000). LD, the end product of anaerobic glycolysis, is generated through the oxidation of pyruvate by LDH. Hence, LD levels can also reflect the oxygen supply, metabolic status, and perfusion of the tissues. CK is an organ-specific enzyme and shows the highest concentration in skeletal muscles. Hence, elevated CK activity is an important sign for skeletal muscle malnutrition and dysfunction (Sandercock *et al*. 2006). In the present study, we found that under heat stress conditions, the LDH activity in mouse plasma was significantly increased, indicating that heat stress can enhance anaerobic metabolism and increase LD. However, AHSP can reduce the LDH activity and LD concentration to some extent, indicating that AHSP can alleviate the enhancement of anaerobic respiration by LDH and the accumulation of the metabolic end product, and thus can reduce the damage of anaerobic glycolysis to the body. The increased CK activity by heat stress indicated that heat stress caused tissue damage, and thus enzymes can enter the blood through the cell membrane. Our results indicated that AHSP can reduce the enzyme activity in heat stress treated mice, which further confirmed that AHSP can mitigate the reduction of glycogen content and the insufficiency of muscle energy supply under heat stress conditions.

The release of cortisol and catechol under heat stress conditions can cause the formation of radicals such as O_2^- and OH^- (Freeman and Crapo. 1982), which in turn can destroy the balance of the oxidation and antioxidant systems in the body (Lin *et al*. 2010). SOD, an important antioxidant enzyme in the body, can eliminate free superoxide anion radicals to protect cells (Wang *et al*. 2008). GSH-Px, another important antioxidant enzyme, can specifically catalyze the reduction reaction of H_2O_2 by glutathione (GSH) to eliminate active oxygen and lipid hydroperoxide (Xi *et al*. 2007). MDA is the end product of the lipid peroxidation reaction caused by the attacking of free radicals on the unsaturated fatty acids in the plasma membrane. Its concentration can reflect the degree of lipid peroxidation and thus can indirectly reflect the degree of cell damage in the body (Wang *et al*. 2008). In the present study, heat stress treated mice exhibited increased SOD and GSH-Px activities, indicating an increased free radical scavenging ability of the body. However, the increased MDA concentration suggested that the damage caused by lipid peroxidation could not be eliminated on time. Our results further indicated that AHSP administration led to a further increase of SOD and GSH-Px activities but a decrease in the MDA concentration, indicating that AHSP can further enhance the enzymatic antioxidant system of the body and reduce the lipid oxidation during heat stress to protect the permeability and integrity of the cell membrane.

Heat stress can cause pathological changes in a variety of animal tissues and organs, including the redistribution of the blood, generation of free radicals, changes in the endocrine system, and damage to the immune system by aldosterone, cortisol, and corticosterone. The damage to the digestion system is mainly on the mucosa, including epithelial cell loss, edema of lamina propria, intestinal villi fracture (Hu *et al*. 2009), reduced intestinal mucosal immunity, and destroyed ecological balance of the intestinal microenvironment. The damage to the liver includes acute vesicular degeneration, disturbed nutrient metabolism, reduced detoxification ability, and bile synthesis and excretion disorders (Ning *et al*. 2003). Consistently, our study indicated that heat stress caused a series of changes in the liver cells such as swelling, increased intercellular cleft, villi edema and fracture, and central lacteal dispersion, whereas AHSP mitigated the injury caused by heat stress in the livers and intestines. Further research would be required to elucidate the relevant mechanism.

With the application of discovery toxicology in the toxicological research of new drugs, drug toxicology studies have gradually shifted from the traditional two-stage pattern (pre-clinical evaluation and clinical evaluation) to the four-stage full evaluation model, which consists of early discovery toxicology, preclinical evaluation, clinical evaluation, and post-marketing supervision. The safety and efficacy of new drugs is crucial for the successful development of the drugs. According to the FDA in the United States, safety factors

have resulted in failure in approximately 30% of the new drug developments. If the drug toxicity can be evaluated earlier, the development of highly toxic drugs can be avoided (Johnson and Wolfgang. 2001). Hence, we performed acute toxicity test on AHSP. Our results indicated that the drug treated mice failed to show any obvious pathological change. The body weight and the organ coefficients of the livers, spleens, and kidneys were not affected by AHSP. Observation of HE stained specimens under microscope did not reveal any obvious pathological symptoms in the liver and intestinal tissues. These preliminary safety test results indicated that AHSP is probably safe for use. A full toxicological evaluation of AHSP should be conducted in the future.

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