Full Paper

Chinese Herbal Medicine, *Shengmai San*, Is Effective for Improving Circulatory Shock and Oxidative Damage in the Brain During Heatstroke

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Abstract. The aim of this study was to investigate the effect of *Shengmai San* (SMS), a traditional Chinese herbal medicine, on heatstroke-induced circulatory shock and oxidative damage in the brain in rats. Anesthetized rats were exposed to a high ambient temperature (43°C) to induce heatstroke. After the onset of heatstroke, the values of mean arterial pressure, cerebral perfusion pressure, cerebral blood flow, and brain partial pressure of O₂ were all significantly lower than those in normothermic controls. However, the values of intracranial pressure, brain and colonic temperatures, and brain levels of free radicals, lipid peroxidation, and cellular ischemia and damage markers were all greater in heatstroke rats compared with those of normothermic controls. Pretreatment or post-treatment with SMS significantly reduced the hypotension, intracranial hypertension, cerebral hypoperfusion and hypoxia and increased levels of ischemia and damage markers in the brain during heatstroke. The protective effects exerted by SMS pretreatment is superior to those of SMS post-treatment. The results demonstrate that SMS is effective for prevention and repair of circulatory shock and ischemic and oxidative damage in the brain during heatstroke.

Keywords: heatstroke, blood pressure, cerebral blood flow, *Shengmai San*, intracranial pressure

Introduction

There is considerable evidence for the involvement of free radicals and lipid peroxidation in the pathophysiology of brain ischemia-reperfusion (1, 2). For example, in a rat heat stroke model, we have shown that heat stroke-induced arterial hypotension and cerebral ischemia are associated with an increased production of free radicals (specifically, hydroxyl radicals, and superoxide), increased lipid peroxidation, and decreased enzymatic anti-oxidant defences in the brain (3). The heatstroke-induced circulatory shock and cerebral ischemia can be suppressed by pretreatment of rats with three free radical scavengers, namely, α -tocopherol, mannitol (4), and magnolol (5). These observations prompted us to think that free radical reactions play a

major role in initiation of circulatory shock and brain ischemic and oxidative injury during heatstroke.

Shengmai San (SMS), consisting of three herbal components, *Panax Ginseng* (PG), *Ophiopogon Japonicus* (OJ), and *Schisandra Chinensis* (SC), is routinely being used for treating coronary heart disease (6, 7). It was also found that SMS, but not PG, OJ, or SC, effectively suppressed the thiobarbituric acid reactive substance (TBARS) formation during reperfusion following ischemia, indicating that SMS improves the oxidative damage in the brain (8).

We studied here the effect of SMS on circulatory shock and brain oxidative and ischemic damage in heatstroke rats. The results obtained here show that SMS has an ability to prevent circulatory shock and brain oxidative and ischemic damage during the heat stroke and, moreover, that SMS administered right after the onset of heatstroke is still considerably effective for

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improving circulatory shock and oxidative damage in the brain.

Materials and Methods

Experimental animals

Adult Sprague-Dawley rats weighing between 238–262 g were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals ($n = 4/\text{group}$) were housed at a Ta (ambient temperature) of $22 \pm 1^\circ\text{C}$, with a 12-h light/dark cycle. Pelleted rat chow and tap water were available ad libitum. All protocols were approved by the Animal Ethics Committee of the Chi-Mei Medical Center (Tainan, Taiwan). Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approx. 8 h) by a single dose of urethane (1.4 g/kg body wt, i.p.). At the end of the experiments, control rats and any rats that had survived heatstroke were killed with an overdose of urethane.

Surgery and physiological parameter monitoring

The right femoral artery and vein of rats were cannulated with a polyethylene tubing (PE 50), under urethane anesthesia, for blood pressure monitoring and drug administration. The animals were positioned in a stereotaxic apparatus (Kopf model 1406; Grass Instrument Co., Quincy, MA, USA) to insert probes for measurement of ICP (intracranial pressure). ICP was monitored with a Statham P23AC transducer via a 20-gauge stainless-steel needle probe (diameter, 0.90 mm; length, 38 mm; Gould Instruments, Cleveland, OH, USA), which was introduced into the right lateral cerebral ventricle according to the stereotaxic coordinates of Paxinos and Watson (9) (7.7-mm interaural, 2.0 mm from the mid-line, and 3.5 mm from the top of the skull). All recordings were made on a four-channel Gould polygraph. Tco (colonic temperature) was monitored continuously by a thermocouple and both MAP (mean arterial pressure) and HR (heart rate) were monitored continuously with a pressure transducer. Tco was maintained at approx. 36°C using the electric thermal mat before the start of experiment.

Materials

Constituents herbs of *Shengmai San* (SMS), *Panax ginseng* C.A. Mey (PG), *Ophiopogon japonicus* Ker-Gawl (OJ), and *Schisandra Chinensis* (Turcz.) Baill (SC), were the products of Jilin Sheng, Sichuan Sheng, and Jilin Sheng of P.R. China, respectively. The SMS preparation used in this study was kindly prepared by Sun Ten Pharmaceutical Co., Ltd. (Taipei, Taiwan).

Briefly, the three component herbs of SMS, OJ (48 g), and SC (24 g), were suspended in 1200 ml distilled water, soaked for 1 h, and then decocted for 1 h. The supernatant was filtered through gauze, and the filtrate was then freeze-dried. The dried filtrate was mixed with Neusilin FL2 as an additive to produce a granulated SMS preparation. The SMS granules were stored at -80°C until used. For each experiment, the SMS granules were weighed precisely and solubilized in distilled water to make the indicated final concentrations. The specimen number (091515) of SMS used in this study was recorded and stored for 10 years at Sun Ten Pharmaceutical Co., Ltd. The HPLC profile indicated that the chemical components contained at least Schizandrin and Ginsenoside. To evaluate the pharmacological efficacy of SMS, α -tocopherol (Sigma Chemical Co., St. Louis, MO, USA) was evaluated at the same time as a reference (positive control). Alpha-tocopherol was dissolved in corn oil (0.6 g in 1 ml corn oil).

Experimental groups

Animals were assigned randomly to one of the following four major groups:

One major group of rats were treated with an oral dose of distilled water (DW) (1 ml per rat) daily and consecutively for 7 days before initiation of heat stress or one single oral dose of DW (1 ml per rat) immediately after the onset of heatstroke. For heatstroke induction, animals were exposed to a Ta of 43°C (with relative humidity of 60% in a temperature-controlled chamber). At a certain point in the heatstroke group, when MAP and local cerebral blood flow (CBF) in the striatum of rat brains began to decrease from their peak levels, this moment was considered as the onset of heatstroke (3, 10). Immediately after the onset of heatstroke, heat stress was terminated and the animals were allowed to recover at room temperature (24°C). Our results showed that the latency for the onset of heatstroke (i.e., the interval between the start of heat exposure and the onset of heatstroke) were found to be about 70 min for the vehicle-treated heatstroke group. Then, both physiological parameters and survival time (interval between the initiation of heat exposure and animal death) were observed for up to 450 min (or at the end of experiments).

The second group of rats were treated with an oral dose of SMS (0.3–1.2 g/ml per rat, daily and consecutively for 7 days) or α -tocopherol (0.6 g/ml per rat, daily and consecutively for 7 days) 7 days before the start of heat exposure.

The third major group of rats were treated with one single oral dose of SMS (0.3–1.2 g/ml per rat) or α -

tocopherol (0.6 g/ml per rat) immediately after the start of heat exposure. Both the second and the third groups of rats were exposed to heat exposure (43°C for exactly 70 min) to induce heatstroke. Again, after 70-min heat exposure, the animals were allowed to recover at room temperature (24°C). These two groups of rats were used as drug-treated heatstroke groups.

The fourth major group of DW-treated rats were exposed to room temperature (24°C) and used as the normothermic controls.

Different group of animals were used for different sets of experiments: i) measurements of MAP, ICP, CPP (cerebral perfusion pressure = MAP – ICP), and striatal CBF, PO₂, and brain temperature (Tb); ii) determination of Tco, MAP, HR, and extracellular concentrations of glutamate, glycerol, lactate, pyruvate, and lactate/pyruvate ratio in the striatum; or iii) determination of the extent of lipid peroxidation and rate of $\cdot\text{O}_2^-$ generation in striatal mitochondria and extracellular levels of 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-DHBA in the striatum; or iv) determination of neuronal damage scores for different brain structures.

Measurement of extracellular glutamate, glycerol, lactate/pyruvate, and hydroxyl radicals in the striatum

Animals were anesthetized with urethane intraperitoneally. The animal's head was mounted in a stereotaxic apparatus (Davis Kopf Instruments, Tujunga, CA, USA) with the nose bar positioned 3.3-mm below the horizontal line. Following a midline incision, the skull was exposed and a burr hole was made in the skull for the insertion of a dialysis probe (4 mm in length; CMA/12; Carnegie Medicine, Stockholm, Sweden). The microdialysis probe was stereotactically implanted into the striatum, according to the atlas and coordinates of Paxinos and Watson (9). The coordinates for the right striatum were: 9.7-mm interaural, 2.0 mm from the midline, and 4.5 mm from the top of the skull. As described previously (10), an equilibrium period of 60 min without sampling was allowed after probe implantation. The microdialysis probes were perfused at 2 $\mu\text{l}/\text{min}$ with a sterile isotonic solution containing 147 mmol/l Na⁺, 4.0 mmol/l K⁺, 2.3 mmol/l Ca²⁺, and 156 mmol/l Cl⁻; and the dialysates were sampled in microvials. The dialysates were collected every 10 min in a CMA/140 fraction collector. Aliquots of dialysates (5 μl) were injected into a CMA 600 Microdialysis analyzer for measurement of lactate, glycerol, pyruvate, and glutamate. All reagents required for analysis were obtained from CMA Microdialysis.

The concentrations of hydroxyl radicals were measured by a modified procedure based on the hydroxy-

lation of sodium salicylate by hydroxyl radicals, leading to production of 2,3-DHBA and 2,5-DHBA (11–13). These two compounds were then measured in dialysates by HPLC with electrochemical detection. A Ringer's solution (0.860 g of NaCl, 0.030 g of KCl and 0.033 g of CaCl₂ per 100 ml) containing 2 mmol/l sodium salicylate was perfused through the microdialysis probe at a constant flow rate (1.2 $\mu\text{l}/\text{min}$). A reverse-phase C₁₈ column [phase II; particle size, 3 μm ; 100 $\times$ 3.2 mm (length $\times$ internal diameter); BioAnalytic Systems, West Lafayette, IN, USA] was used, and the mobile phase consisted of a mixture of 75 mmol/l monochloroacetic acid, 0.7 mmol/l disodium EDTA, 1.5 mmol/l sodium 1-octanesulfonate, and 45 ml/l acetonitrile (pH 3.0). The retention times of 2,3-DHBA and 2,5-DHBA were 9.07 and 5.44 min, respectively.

Measurement of lipid peroxidation

In a separate set of animals, 15 min after the onset of heatstroke, each group of animals was decapitated. The striatum was removed from the brain. A fluorescence assay procedure was used to measure lipid peroxidation in this brain area (14). The brain area was homogenized with water for 2 s in an ultrasonic tissue disrupter. The homogenates were mixed with 400 μl of ice-cold chloroform and 200 μl of methanol and then vortex-mixed for 1 min and left on ice for 15 min. After centrifugation at 8000 $\times g$ for 5 min, 400 μl of the chloroform layer was transferred to another tube containing 100 μl of methanol and then scanned with a spectrofluorometer. The lipid peroxidation was determined by measuring the levels of malondialdehyde and its dihydropyridine polymers at 356 nm excitation and 426 emission.

Isolation of mitochondria and preparation

All steps in the isolation of mitochondria and the preparation of submitochondrial particles were performed at ice-cold temperature. Mitochondria from the brain were isolated as described by Ozawa et al. (15). Briefly, the striatum was dissected out, rinsed and homogenized in 10 vol (w/v) of ice-cold isolation buffer [0.3 mol/l mannitol and 0.1 mmol/l EDTA (pH 7.4)]. The homogenate was centrifuged at 600 $\times g$ for 10 min, and the supernatant was recentrifuged at 10000 $\times g$ for 10 min. The resulting pellet was resuspended in 40 ml of isolation buffer and centrifuged at 5000 $\times g$ for 10 min. The pellet was resuspended in 30 mmol/l potassium phosphate buffer (pH 7.0).

Submitochondrial particles were made by resuspending the mitochondrial preparation in 4 vol of 30 mmol/l potassium phosphate buffer (pH 7.0). This mixture was sonicated three times for 30 s each at 1-min intervals.

The sonicated mitochondria were centrifuged at $8250 \times g$ for 10 min to sediment unfragmented mitochondria, and the supernatant was recentrifuged at $80000 \times g$ for 40 min to pellet the mitochondrial particles. The pellet was resuspended in 30 mmol/l potassium phosphate buffer and used for further assay or storage at -80°C (16, 17).

$\cdot\text{O}_2^-$ generation monitoring

The rate of $\cdot\text{O}_2^-$ generation by submitochondrial particles was measured as described by Boveris (18). Both the test and reference cuvettes contained 20 to $40 \mu\text{l}$ of submitochondrial homogenates, 0.1 mol/l potassium phosphate buffer (pH 7.4), $7.2 \mu\text{mol/l}$ cytochrome c, $0.6 \mu\text{mol/l}$ antimycin A, and 7 mmol/l succinate. SOD (superoxide dismutase; 200 units/ml; Sigma, St. Louis, MO, USA) was added to the reference cuvette. The reduction of cytochrome c was monitored spectrophotometrically at 550 nm. As both the test and reference cuvettes contained identical ingredients, except that the latter included SOD, the measured rate of cytochrome c reduction was specific because of its interaction with $\cdot\text{O}_2^-$.

Measurements of CBF, brain PO_2 , and T_b

A 100- μm -diameter thermocouple and two 230- μm fibers were attached to the oxygen probe. This combined probe measures oxygen, temperature, and microvascular blood flow. The measurement requires OxyLite™ and OxyFlo™ instruments. OxyLite™ 2000 (Oxford Optro-nix, Ltd., Oxford, UK) is a two-channel device (measuring PO_2 and temperature at two sites simultaneously), whereas OxyFlo™ 2000 is a two-channel laser Doppler perfusion monitoring instrument. The OxyLite™ has been designed to operate in conjunction with the OxyFlo™. The combination of these two instruments provides simultaneous tissue blood flow, oxygenation, and temperature data. Under urethane anesthesia, the animal was placed in a stereotaxic apparatus, and the combined probe was implanted into the striatum using the atlas and coordinates of Paxinos and Watson (9). The probe calibration parameters were transferred from the probe packaging to the OxyLite™ Instrument using the bar code wand. For each PO_2 input on the OxyLite™, there is a corresponding temperature input. A thermocouple may be attached to these temperature inputs using the thermocouple adapters provided. The temperature measurement serves two purposes: i) to automatically compensate the PO_2 measurement and ii) to continuously monitor tissue temperature. OxyLite™ is a laser Doppler flow meter whose primary purpose is to measure real-time microvascular red blood cell perfusion. Laser Doppler signals were recorded in BPU (blood

perfusion units), which are a relative unit scale defined using a carefully controlled motility standard. The OxyFlo™ is calibrated before leaving the factory using a motility standard solution of carefully selected latex spheres undergoing Brownian motion. The OxyFlo™ is a stable instrument and should not under normal circumstances require recalibration.

Neuronal damage score

At the end of the experiments, animals were killed by an overdose of sodium pentobarbital, and the brains were fixed in situ and left in the skull in 10% neutral buffered formalin for at least 24 h prior to removal from the skull. The brain was removed and embedded in paraffin blocks. Serial ($10 \mu\text{m}$) sections through the striatum were stained with haematoxylin/eosin for microscopic evaluation. The extent of cerebral neuronal damage was scored on a scale of 0–3, modified from the grading system of Pulsinelli et al. (19), in which 0 is normal, 1 indicates approx. 30% of the neurons are damaged, 2 indicates that approx. 60% of the neurons are damaged, and 3 indicates that 100% of the neurons are damaged. Each hemisphere was evaluated independently without the examiner knowing the experimental conditions.

Statistical analyses

All data except neuron damage score are expressed as means $\pm$ S.E.M. and were analyzed by two-way analysis of variance (ANOVA). Duncan's multiple-range test was used for post hoc multiple comparison among means. Neuron damage scores are expressed as the median followed by the first and third quartile. For the neuronal damage data, the Wilcoxon signed rank test was used when only two groups were compared. The Wilcoxon tests convert the scores or values of a variable to ranks, require calculation of a sum of the ranks, and provide critical values for the sum necessary to test the null hypothesis at a given significant level. A *P* value less than 0.05 was considered as statistical significance.

Results

Effects of heat exposure on both latency for onset of heatstroke and survival time

Table 1 summarizes the effects of heat exposure ($T_a = 43^\circ\text{C}$ for 70 min) on survival time in DW- and drug-treated rats. The survival time values obtained from eight rats pretreated with oral administration of DW solution daily and consecutively for 7 days before the initiation of heat stress were found to be 95 ± 5 min. The values for the survival in rats pretreated with 7-day consecutive oral administration of DW before heat stress

Table 1. Effects of heat exposure (HE, 43°C for 70 min) on survival time in distilled water (DW)-treated, *Shengmai San* (SMS)-treated, and α -tocopherol (AT)-treated rats

Treatments	Survival time (min)
1. Daily oral dose for consecutive 7 days before initiation of HE	
1) DW (1 ml)-treated rats kept at 24°C	450 $\pm$ 2
2) DW (1 ml)-treated rats kept at 43°C	95 $\pm$ 5*
3) SMS (0.3 g/ml)-treated rats kept at 43°C	142 $\pm$ 10*,†,‡
4) SMS (0.6 g/ml)-treated rats kept at 43°C	276 $\pm$ 25*,†,‡
5) SMS (1.2 g/ml)-treated rats kept at 43°C	334 $\pm$ 41*,†,‡
6) AT (0.6 g/ml)-treated rats kept at 43°C	157 $\pm$ 9*,†,§,‡
2. Single one oral dose immediately after the onset of heatstroke	
1) DW (1 ml)-treated rats kept at 24°C	450 $\pm$ 3
2) DW (1 ml)-treated rats kept at 43°C	92 $\pm$ 4*
3) SMS (0.3 g/ml)-treated rats kept at 43°C	133 $\pm$ 9*,†
4) SMS (0.6 g/ml)-treated rats kept at 43°C	185 $\pm$ 28*,†
5) SMS (1.2 g/ml)-treated rats kept at 43°C	258 $\pm$ 39*,†
6) AT (0.6 g/ml)-treated rats kept at 43°C	132 $\pm$ 8*,†,§

Data represent means $\pm$ S.E.M. of eight rats per group. All normothermic controls were killed with an overdose of urethane about 450 min after the start of experiment (or at the end of the experiment). All groups exposed to 43°C had HE withdrawn at 70 min. * P <0.05, compared with group 1); † P <0.05, compared with group 2); § P <0.05, compared with group 4); and ‡ P <0.05, compared with respective control groups of single one oral injection immediately after the onset of heatstroke (ANOVA followed by Duncan's test).

were indisquishable from those of the rats treated with one single, oral administration of DW immediately after the onset of heatstroke. Either 7-day consecutive, oral administration before heat stress or one single oral administration immediately after the onset of heatstroke of SMS (0.3–1.2 g/ml) or α -tocopherol (0.6 g/ml) significantly increased the survival time during heatstroke. The protective effects exerted by SMS or α -tocopherol pretreatment is superior to those of SMS posttreatment. In addition, the protective effects exerted by SMS seem superior to those exerted by α -tocopherol in terms of survival during heatstroke.

Effects of heat exposure on physiological and biochemical parameters

Both Figs. 1 and 2 show the effects of heat stress (43°C) on MAP, ICP, CPP, CBF, brain PO₂, and brain temperature in rats pretreated with consecutive, daily, oral administration of DW solution or SMS (0.6 g/ml) for 7 days before the initiation of heat stress (Fig. 1) or one single oral dose of DW or SMS (0.6 g/ml) right after the onset of heatstroke (Fig. 2). As shown in these 2 figures, 10–30 min after the onset of heatstroke, all the MAP, CPP, CBF, and brain PO₂ values were significantly lower as compared with those of the pre-heat exposure controls (P <0.05). On the other hand, the values of both ICP and brain temperature were significantly greater as compared with those of the pre-heat

exposure controls (P <0.05). The heatstroke-induced arterial hypotension, intracranial hypertension, decreased cerebral perfusion, cerebral ischemia, and decreased oxygenation in brain were significantly attenuated by treatment with daily consecutive oral administration of SMS for 7 days (Fig. 1) before heat stress or one single oral dose (0.6 g/ml) of SMS right after the onset of heatstroke (Fig. 2). However, the increased brain temperature attendant with heatstroke was not affected by treatment with SMS (Figs. 1 and 2).

Both Figs. 3 and 4 show the effects of heat stress (43°C) on MAP, Tco, HR, and values of glutamate, glycerol, lactate, pyruvate, and lactate/pyruvate in the extracellular fluids of striatum. As compared with those of pre-heat controls, the values of Tco and striatal concentrations of glutamate, glycerol, lactate, and lactate/pyruvate ratio were significantly higher in rats 10–30 min after the onset of heatstroke. On the other hand, the values of MAP, HR, and striatal concentration of pyruvate were significantly lower in rats after the onset of heatstroke. Again, it was found that the heatstroke-induced arterial hypotension, bradycardia, as well as the increased striatal concentrations of glutamate, glycerol, and lactate/pyruvate ratio were significantly attenuated by treatment with SMS solution either oral administration of SMS daily and consecutively for 7 days before heat stress (Fig. 3) or one single oral dose (0.6 g/ml) of SMS right after the onset of heatstroke

Fig. 1. Effects of heat stress on MAP, ICP, CPP, CBF, brain PO₂, and brain temperature (Tb) in rats pretreated with an oral dose of distilled water (1 ml) (open circle, n=8) or SMS (0.6 g in 1 ml) (closed circle, n=8) daily and consecutively for 7 days. *Significantly different from the pre-heat control values; †significantly different from rats pretreated with distilled water (P<0.05, ANOVA followed by Duncan's test). The arrow indicates the onset of heatstroke.

(Fig. 4).

Effects of heat exposure on lipid peroxidation, [•]O₂⁻ generation rate, and DHBA production

Table 2 Summarizes the extent of lipid peroxidation and rate of [•]O₂⁻ generation in the striatum as well as the extracellular concentration of DHBA in the striatum. In DW-treated rats, both the degree of lipid peroxidation

and rate of [•]O₂⁻ generation and the extracellular level of DHBA in the striatum were, in all cases, greater than in normothermic controls 20 min after the onset of heatstroke. Again, the heat stress-induced enhancement of the extent of lipid peroxidation, the rate of [•]O₂⁻ generation, and DHBA formation in the striatum were attenuated by treatment with an oral dose of SMS (0.6 g/ml) either daily and consecutively for 7 days

Fig. 2. Effects of heat stress on MAP, ICP, CPP, CBF, brain PO₂, and brain temperature (Tb) in rats treated with single one oral dose of distilled water (1 ml) (open circle, n = 8) or SMS (0.6 g in 1 ml) (closed circle, n = 8) right after the onset of heatstroke. *Significantly different from the pre-heat control; †significantly different from the distilled water-treated rats ($P < 0.05$, ANOVA followed by Duncan's test). The arrow indicates the time of heatstroke onset and drug injection.

before the initiation of heat stress or only one oral dose immediately after the onset of heatstroke.

Effects of heat exposure on neuronal damage score values

The effects of heat exposure on neuronal damage in

different brain structures of rats pretreated or posttreated with DW or SMS was determined. Again, it was found that the scores for cerebral neuronal damage in heatstroke rats receiving daily and consecutively an oral dose of SMS for 7 days before heat stress or one single oral dose of SMS (0.6 g/ml) immediately after the onset

Fig. 3. Effects of heat stress on Tco, MAP, HR, and the extracellular concentrations of glutamate, glycerol, lactate, pyruvate, and lactate/pyruvate ratio in the striatum of rats treated with an oral dose of distilled water (open circle, n = 8) or SMS (0.6 g in 1 ml) (closed circle, n = 8) daily and consecutively for 7 days. *Significantly different from the pre-heat control; †significantly different from the distilled water-treated rats ($P < 0.05$, ANOVA followed by Duncan's test). The arrow indicates the time of heatstroke onset.

Fig. 4. Effects of heat stress on Tco, MAP, HR, and the extracellular concentrations of glutamate, glycerol, lactate, pyruvate, and lactate/pyruvate ratio in the striatum of rats treated with single one oral dose of distilled water (open circle, $n = 8$) or SMS (0.6 g in 1 ml) (closed circle, $n = 8$) right after heatstroke onset. *Significantly different from the pre-heat control; †significantly different from the distilled water-treated rats ($P < 0.05$, ANOVA followed by Duncan's test). The arrow indicates the time of heatstroke onset and drug injection.

of heatstroke were significantly lower ($P < 0.05$) than those of the respective DW controls. A typical example for neuronal damage during heatstroke is depicted in Fig. 5. Under microscopic examination, neuron damages

developed in different degrees. Damaged neurons appeared shrunken with structureless and/or eosinophilic cytoplasm and shrunken nucleus (Fig. 5). Neuronal damage scores in the SMS-treated groups were

Table 2. Effects of heat exposure (43°C for 70 min) on the extent of lipid peroxidation and rate of $\cdot\text{O}_2^-$ generation in tissue homogenates and the extracellular concentration of DHBA in the striatum of rats treated with distilled water (DW) or *Shengmai San* (SMS)

Treatment	Lipid peroxidation (RFU)	Rate of $\cdot\text{O}_2^-$ generation (nmol/min per mg protein)	DHBA (% baseline)
1. Daily oral administration for consecutive 7 days before initiation of HE			
1) DW (1 ml)-treated rats kept at 24°C	1.58 $\pm$ 0.11	6.91 $\pm$ 0.68	100 $\pm$ 4
2) DW (1 ml)-treated rats kept at 43°C	2.26 $\pm$ 0.13*	9.15 $\pm$ 0.82*	726 $\pm$ 27*
3) SMS (0.6 g in 1 ml)-treated rats kept at 43°C	1.64 $\pm$ 0.09 [†]	7.07 $\pm$ 0.88 [†]	150 $\pm$ 5* [†]
2. Single one oral dose immediately after the onset of heatstroke			
1) DW (1 ml)-treated rats kept at 24°C	1.54 $\pm$ 0.09	6.88 $\pm$ 0.66	100 $\pm$ 5
2) DW (1 ml)-treated rats kept at 43°C	2.31 $\pm$ 0.14*	9.18 $\pm$ 0.72*	731 $\pm$ 29*
3) SMS (0.6 g on 1 ml)-treated rats kept at 43°C	1.77 $\pm$ 0.10 [†]	7.15 $\pm$ 0.56 [†]	142 $\pm$ 4* [†]

Data are means $\pm$ S.E.M. of eight rats per group. RFC, relative fluorescence units. For the determination of lipid peroxidation and rate of $\cdot\text{O}_2^-$ generation in the homogenates and extracellular concentration of DHBA of striatum, samples were obtained 85 min after the initiation of heat exposure or 15 min after the onset of heatstroke or at the equivalent time after injection of DW for the rats at 24°C. * P <0.05, compared with DW-treated rats at 24°C; [†] P <0.05, compared with DW-treated rats at 43°C (ANOVA followed by Duncan's test).

significantly lower than those in the DW-treated groups (Table 3).

Discussion

Excessive accumulation of glutamate and lactate/pyruvate ratio is a well-known marker of cellular ischemia, whereas glycerol is a marker of how severely cells are affected by ongoing pathology (20–25). Indeed, both present and previous (26, 27) results have shown that both cerebral ischemia and injury that occurred during heatstroke are associated with an increased production of glycerol, lactate/pyruvate ratio, and glutamate in the brain. Our results further demonstrated that PO_2 in the rat brain was greatly reduced after the onset of heatstroke. Thus, it appears that the excessive accumulation of glycerol, glutamate, and lactate/pyruvate ratio in the brain may be secondary to cerebral ischemia and injury in rats. Probably, the most striking findings of the present study is that oral administration of SMS for a consecutive 7-day procedure before the initiation of heat exposure or one single, oral dose of SMS immediately after the onset of heatstroke significantly attenuated the increased cerebral levels of glycerol, glutamate, and lactate/pyruvate ratio and prevented the cerebral ischemia and injury and arterial hypotension during heatstroke. The protective effects exerted by the former were superior to the later in terms of survival time during heatstroke. In addition, it was found that the protective effects exerted by SMS seemed superior to those by α -tocopherol. In the present study, SMS is soluble in water, whereas α -tocopherol is dissolved in corn oil. As shown in our previous (4)

and present results, control injection of water or corn oil had an insignificant effect on heatstroke responses. These indicate that the difference in their preparation did not influence the results in Table 1 and the comparison of the potency between SMS and α -tocopherol.

When rodents are exposed to external heat stress, they have both decreased stroke volume and decreased peripheral vascular resistance produced arterial hypotension (28). Both arterial hypotension and intracranial hypertension eventually led to cerebral hypoperfusion and hypoxia after the onset of heatstroke, as demonstrated in the present results. Cerebral hypoperfusion to below the autoregulatory level caused cerebral ischemia, which led to neurological damage and the onset of central nervous system syndromes associated with heatstroke (29). As demonstrated in the present and previous (4) results, the prolongation of survival in heatstroke rats with SMS or α -tocopherol treatment was found to be related to enhancement of mean arterial pressure and local cerebral blood flow, as well as reduction in both intracranial hypertension and cerebral neuronal damage during heatstroke. The augmentation of CBF in rats treated with SMS or α -tocopherol may be brought about by higher cerebral perfusion pressure resulting from lower intracranial pressure (due to reduction in cerebral edema and cerebrovascular congestion) and higher mean arterial pressure during heatstroke (29).

There is evidence that both circulatory shock and cerebral ischemia are associated with increased production of free radicals (specifically, hydroxyl radicals, and superoxide), increased lipid peroxidation, and decreased enzymatic anti-oxidant defences in the brain of heatstroke-affected rats (3). Pretreatment with α -tocopherol

Fig. 5. Histopathological examination of neuronal damage. Top panel, brain in a normoathermic rat. Middle panel, brain in a heatstroke rat with distilled water resuscitation; neurons appeared shrunken. Bottom panel, brain in heatstroke rat treated with SMS (0.6 g in 1 ml) immediately after the onset of heatstroke. Animals were killed 85 min after the start of heat exposure or 15 min after the onset of heatstroke for hematoxylin and eosin stain (original magnification $\times 545$). Scale bar, 50 μm .

or mannitol 30 min before the onset of heat exposure significantly attenuated heat stroke-induced arterial hypotension, cerebral ischemia and neuronal damage, and the increased free radical formation and lipid peroxidation in the brain (4). Results obtained here clearly demonstrated that SMS had a strong activity to prevent

the cerebral oxidative stress and neuronal damage produced by cerebral ischemia during heatstroke. Pretreatment with SMS successfully prevented both the increased production of free radicals (specifically, hydroxyl radicals and superoxide) and increased lipid peroxidation in the brain of heat stroke-affected rats. Moreover, it was found that SMS administered immediately after the onset of heatstroke was still effective both in preventing circulatory shock and free radicals accumulation and increased lipid peroxidation in the brain already damaged to a considerable extent during heatstroke. The similar results have been demonstrated in a cerebral ischemia-reperfusion injury model (8). When SMS was injected directly into rat duodenum 2 h before cerebral ischemia by bilateral carotid artery occlusion, TBARS formation during reperfusion following ischemia was almost completely suppressed in the brain. The loss of glutathione peroxidase activity after the ischemia reperfusion was also effectively prevented by the SMS pre-administration. It was found that SMS also effectively suppressed the TBARS formation even when it was administered after 45 min reperfusion following ischemia, indicating that SMS improves the oxidative damage already established in the brain. Likewise, the decrease of glutathione peroxidase activity was minimized in the damaged brain by the post-treatment with SMS.

Both our present and previous results (3, 4) have shown that free radical reactions play a major role in initiation of heatstroke-induced circulatory shock and cerebral ischemic injury. However, many other biochemical processes are also involved in establishing oxidative damage in the brain (1). For example, an endotoxin given systemically can elicit an increase of inducible nitric oxide synthase (iNOS)-dependent NO production in the nucleus tractus solitarius (NTS) and induce arterial hypotension (30). Heatstroke rats displayed arterial hypotension, endotoxemia, cerebral ischemia (3, 31), and reduced baroreceptor reflex response (32). Our recent results (10) further demonstrated that the heatstroke-induced ischemia, iNOS overexpression, and NO overproduction in rat brain can be suppressed by pretreatment with aminoguanidine (an iNOS inhibitor). It is likely that SMS may alleviate arterial hypotension as well as cerebral ischemia exhibited during the onset of heatstroke by reducing the iNOS-dependent NO overproduction. Of course, this contention needs future verification.

It has been implicated that many biochemical and physiological processes are involved in establishing oxidative damage in the brain (1). As suggested before by Wang et al. (8), antioxidant therapy using a single active compound will have inherent limitations. SMS

Table 3. Effects of heat exposure (HE, 43°C for 70 min) on neuronal damage in different brain structures of rats treated with distilled water (DW) or *Shengmai San* (SMS)

Treatment	Neuronal damage score (0–3)		
	Striatum	Hypothalamus	Hippocampus
1. Daily oral dose for consecutive 7 days before the initiation of HE			
1) DW (1 ml)-treated rats at 24°C	0 (0, 0)	0 (0, 0)	0 (0, 0)
2) DW (1 ml)-treated rats at 43°C	2 (2, 2)*	2 (2, 2)*	2 (2, 2)*
3) SMS (0.6 g in 1 ml)-treated rats at 43°C	1 (0, 1) [†]	1 (0, 1) [†]	1 (0, 1) [†]
2. One single oral dose immediately after the onset of heatstroke			
1) DW (1 ml)-treated rats at 24°C	0 (0, 0.75)	0 (0, 1)	0 (0, 1)
2) DW (1 ml)-treated rats at 43°C	2 (2, 2)*	2 (2, 2)*	2 (2, 2)*
3) SMS (0.6 g in 1 ml)-treated rats at 43°C	1 (0, 1) [†]	1 (0, 1) [†]	1 (0, 1) [†]

For the determination of neuronal damage score, animals were killed 85 min after the initiation of heat exposure (or 15 min after onset of heatstroke). Data are presented as the median, followed by the first (Q1) and third (Q3) quartile. * $P < 0.05$, compared with DW-treated rats at 24°C; [†] $P < 0.05$, compared with DW-treated rat sat 43°C (Wilcoxon test).

with antioxidant activity are an attractive formula in overcoming this limitation because they consist of three herbal constituents having different physiological functions. The data in the present study indicate that SMS could be a potential herbal medicine for treating circulatory shock and oxidative ischemic damage in the brain during heatstroke.

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