Salidroside pretreatment protects against myocardial injury induced by heat stroke in mice

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Abstract
Objective: To explore the protective effects and mechanisms of salidroside on myocardial injury induced by heat stroke (HS) in mice.

Methods: We pretreated mice with salidroside for 1 week and then established an HS model by exposure to 41.2°C for 1 hour. We then examined the effects of salidroside on survival. We also assessed the severity of cardiac injury by pathology, and analyzed changes in levels of myocardial injury markers, inflammatory cytokines, and oxidative stress.

Results: Salidroside pretreatment significantly reduced HS-induced mortality and improved thermoregulatory function. Salidroside also provided significant protection against HS-induced myocardial damage, and decreased the expression levels of cardiac troponin I, creatine kinase-MB, and lactate dehydrogenase. Moreover, salidroside attenuated HS-induced changes in the inflammation markers tumor necrosis factor-α, interleukin (IL)-6, and IL-10, and down-regulated the oxidative stress response indicated by thiobarbituric acid reactant substances, malondialdehyde, reduced glutathione, and superoxide dismutase.

Conclusions: Salidroside pretreatment protected against HS-induced myocardial damage, potentially via a mechanism involving anti-inflammatory and anti-oxidative effects.

Keywords
Salidroside, heat stroke, myocardial injury, systemic inflammatory response, oxidative stress, cytokine

Introduction
Global warming and the increasing intensity of global heatwaves have been associated...
with a corresponding increase in the number of deaths caused by heat stroke (HS). Previous studies suggested that HS is caused by the failure of thermal regulation during heat exposure, accompanied by severe acute phase reactions and changes in heat shock protein expression, with the interaction between cytotoxic, inflammatory, and clotting reactions caused by heat exposure ultimately leading to multiple organ damage. Although the cardiovascular system is considered to be the first system affected by HS, the mechanism is currently unclear.

Recent studies found that the pathophysiological changes in heat-induced disease were caused not only by direct damage due to heat exposure, but by the development of a systemic inflammatory response syndrome after thermal injury, which subsequently progresses to a sepsis-like response. Salidroside (C14H20O7) is a phenylethanol compound and the main active ingredient of Rhodiola. Salidroside has many biological functions including anti-fatigue, anti-tumor, and immune regulation activities, and has also demonstrated protective effects on the cardiovascular system and nerve cells. In this study, we aimed to explore the protective effects and mechanisms of salidroside on HS-induced myocardial injury in a murine model of HS.

Materials and methods

Animal preparation

All animal experiments were carried out in strict accordance with the animal experiment ethics guidelines set out by the Animal Ethics and Welfare Committee of Ningbo University (Approval No. AEWC-2017-33). Male mice (6–8 weeks old; Institute of Cancer Research) were maintained for 1 week at 22 ± 2°C and relative humidity of 50 ± 8%, with a 12-hour dark/light cycle, and provided with food and water ad libitum.

HS model

The mice were exposed to a temperature of 41.2°C and a relative humidity of 50% to 55% in an environmental chamber for 1 hour. The time when the mice were removed from the environmental chamber was set as 0 hours. The mice were then placed at a room temperature of 24°C and free access to food and drinking water was resumed immediately. Mice that survived to the seventh day after HS modeling were considered as survivors. We dynamically measured the rectal temperature of the mice during and for 72 hours after establishing the HS model using a thermocouple probe (RDTI13.5-A, Yuyao Jinruida Gas Appliances Co., Ltd., Ningbo, China) inserted directly into the rectum. We obtained detailed recordings including core body temperature, characteristics, consciousness, and body weight during establishment of the HS model. Mice were sacrificed by cervical dislocation 24 hours after successful establishment of the HS model and serum and heart tissue were obtained for subsequent experiments.

Animal groups

We randomly divided the mice into three groups (10 mice per group): normal temperature control (NC), with no treatment and kept at normal room temperature; vehicle saline + HS (V + HS), fed saline daily for 1 week before establishing the HS model; and salidroside + HS (S + HS), fed daily with different doses of salidroside (Zhejiang Reagent Factory, Hangzhou, China) (5, 25, and 50 mg/kg) for 1 week before establishing the HS model. The core body temperature of the mice was controlled at 37.0 to 37.5°C before the start of the experiment.
**Histopathology**

Heart tissues were removed and washed and placed in a 10% formalin solution for 48 to 72 hours, embedded in paraffin, and sections (2–3 μm) were cut using a rotary microtome (A130378, Ai Test Electronic Technology Co., Ltd., Shanghai, China). Tissues were dewaxed, rehydrated, and stained with hematoxylin–eosin, and pathological changes in heart tissue were observed under a light microscope. The severity of tissue damage was assessed according to previous studies as follows: normal (0 points); minor injury (0.5 points); mild injury (1 point); moderate injury (2 points), and severe injury (3 points).9

**Assessment of myocardial injury markers**

We analyzed serum myocardial injury-specific biomarkers in the different groups of mice. Mice were sacrificed by cervical dislocation 24 hours after establishment of the HS model. Blood samples were obtained by cardiac puncture, centrifuged at 2795 × g for 5 minutes at room temperature, and then stored at −80°C. The levels of cardiac troponin I (cTnI; eBioscience Co., San Diego, US), creatine kinase-MB (CK-MB; eBioscience Co.), and lactate dehydrogenase (LDH; Puzhen Biological Co., Shanghai, China) were determined by enzyme-linked immunosorbent assay (ELISA), according to the respective manufacturers’ instructions.

**Evaluation of inflammatory cytokines**

Heart tissue homogenate was centrifuged at 16,099 × g for 5 minutes at room temperature. The supernatant was removed and levels of tumor necrosis factor (TNF)-α (Abcam Co., Shanghai, China), interleukin (IL)-6 (Abcam Co.), and IL-10 (Abcam Co.) in the cardiac tissue cells were assessed using specific ELISA kits, according to the manufacturer’s instructions.

**Assessment of oxidative stress levels**

Heart tissue homogenate was centrifuged at 16,099 × g for 5 minutes at room temperature, and the supernatant was then stored at −80°C. Tissue levels of thiobarbituric acid reactant substances (TBARS) were detected by colorimetry (Haling Co., Shanghai, China). Malondialdehyde (MDA, Haling Co., Shanghai, China) was condensed with thiobarbituric acid to form a red product with a maximum absorption peak at 532 nm,10 and the colorimetric lipid content in the cardiac tissue was estimated by colorimetry. We also measured the absorbance at 600 nm, and the difference in absorbances at 532 nm and 600 nm was used to calculate the MDA content. We also measured the content of reduced glutathione (GSH) in heart tissue using dithiophenolic benzoic acid (Solarbio Co., Shanghai, China), and superoxide dismutase (SOD) using a SOD assay kit (Solarbio Co., Shanghai, China).11

**Statistical analysis**

All data were expressed as mean ± standard deviation (SD). Graphs were created using GraphPad Prism 5.20 (GraphPad Software Inc., La Jolla, CA, USA) and differences among the three groups were analyzed using two-way ANOVA with Dunnett’s post hoc test. P < 0.05 was considered to be significant.

**Results**

**Salidroside reduces HS-induced mortality**

HS can cause thermoregulatory dysfunction and mortality.12 We administered different doses of salidroside to mice for 1 week and then exposed them to HS at 41.2°C for 1 hour,7 to establish a mouse HS model. Mortality in the V + HS group was 95%, while daily salidroside pretreatment for 1 week resulted in a dose-dependent decrease in mortality, to 40% with
5 mg/kg, 25% with 25 mg/kg, and 10% with 50 mg/kg (P < 0.05, 0.001, and 0.001, respectively) (Figure 1a). We therefore performed subsequent experiments with 50 mg/kg salidroside as the study dose. We recorded the rectal temperature in the three groups of mice within 72 hours, and showed that the rectal temperature in the V + HS group declined progressively with time to a minimum of 31.2°C, and the mice eventually died. In contrast, the rectal temperature of mice in the S + HS group decreased rapidly to 37.7°C ± 0.4°C within 48 hours, and then stabilized at 37.3°C ± 0.1°C at 72 hours (Figure 1b).

**Salidroside protects against HS-induced heart damage**

The heart tissue in the NC group had a regular structure with clear myofibrils, intact cardiomyocytes, and normal activity (Figure 2a). However, the structure in the V + HS group was disordered, with broken myofibrils, edematous myocardial cells, and interstitial infiltration of a large number of inflammatory cells (Figure 2b). Myocardial cell edema was reduced in the S + HS group, cell viability was increased, myofibrils were relatively clear, and the number of interstitial inflammatory cells was decreased (Figure 2c).

**Salidroside reduces HS-induced increases in myocardial enzymes**

Changes in serum levels of cTnI, CK-MB, and LDH in the three groups of mice are shown in Figure 3. Serum levels of all three enzymes were within the normal ranges in the NC group, but were all significantly increased in the V + HS group compared with the NC group, and significantly decreased in the S + HS compared with the V + HS group (both P < 0.001).

**Salidroside attenuates HS-induced inflammation**

We investigated the effects of salidroside on HS-induced inflammation by measuring serum levels of the inflammatory factors TNF-α, IL-6, and IL-10. Serum levels of TNF-α and IL-6 were significantly increased while IL-10 levels were significantly decreased in the V + HS group.
6 hours after establishing the model (all $P < 0.01$), with peak or trough values reached at 24 hours (Figure 4). However, TNF-$
abla$ and IL-6 levels were not significantly elevated in the S $+$ HS group and the increase in IL-10 levels was relatively mild compared with the NC mice, and levels of TNF-$
abla$ and IL-6 were significantly decreased and IL-10 levels were significantly elevated ($P < 0.05$) compared with the V $+$ HS group at 24 hours after HS model establishment.

**Salidroside protects against HS-induced cardiomyocyte damage by down-regulating oxidative stress**

TBARS and MDA activities were significantly increased and the activities of GSH and SOD were significantly decreased in cardiomyocytes in the V $+$ HS group (all $P < 0.05$) (Figure 5), suggesting that HS increased the oxidative stress response in cardiomyocytes. However, the TBARS and MDA activities were significantly decreased and the GSH and SOD activities were significantly increased in the S $+$ HS compared with the V $+$ HS group (all $P < 0.05$), and all tended to normalize. These results suggested that salidroside might protect against HS-induced cardiomyocyte injury by down-regulating oxidative stress.

**Discussion**

In the present study, we investigated the effects of salidroside on HS-induced mortality in mice. Mice were pretreated with different doses of salidroside for 1 week and then exposed to 41.2°C for 1 hour. The results showed that pretreatment with salidroside significantly decreased HS-induced mortality in a dose-dependent
manner, with the lowest mortality (10%) at a daily oral dose of 50 mg/kg. In addition, salidroside rapidly reduced the rectal temperature to $37.7 \pm 0.4^\circ C$ within 48 hours, and stabilized it at $37.3 \pm 0.1^\circ C$ at 72 hours, indicating that salidroside could significantly improve HS-induced thermo-regulatory dysfunction in mice.

Cardiac dysfunction is a leading cause of death in patients with heat-related diseases. HS-induced cardiac dysfunction often manifests as significant arrhythmias, heart failure, and even cardiac arrest. Quinn et al. showed that HS severely damaged myocardial cells, resulting in vacuolar changes and partial necrosis. Salidroside has many functions, including anti-oxidation, anti-fatigue, anti-tumor, immune regulation, and free radical scavenging. The results of the current study suggested that salidroside pretreatment could also protect against HS-induced myocardial damage in mice.

HS affects human cell membranes and enzymes and causes direct damage to cardiomyocytes, resulting in partial myocardial cell lysis, hemorrhage, and necrosis.
Furthermore, hyperpyrexia dehydration causes tissue hypoperfusion and increased oxygen needs, and myocardial cells become hypoxic and necrotic, while skin vasodilation due to water loss causes heat redistribution, insufficient blood volume, increased cardiac output, electrolyte imbalance, and disruption of the sodium-potassium pump, causing myocardial ischemia, necrosis, arrhythmia, and heart failure. Myocardial markers are released into the blood by HS-damaged cardiomyocytes, and their elevated levels can thus predict myocardial damage and the severity of HS. Although LDH is not a cardiac-specific injury indicator, it can help to determine the severity of myocardial cell membrane damage. In our study, serum cTnI, CK-MB, and LDH levels were all significantly increased by HS, and these increases were all attenuated by salidroside pretreatment. These results suggest that myocardial markers were elevated early in HS, and that salidroside pretreatment could significantly reduce these myocardial markers and protect against HS-induced heart injury.

Systemic inflammatory response syndrome secondary to HS activation is considered to be the main cause of multiple organ dysfunction, and inhibition of the inflammatory

Figure 4. Salidroside (50 mg/kg) pretreatment decreased the expression levels of TNF-α and IL-6 and increased the level of IL-10 in heat stroke-induced myocardial injury mice. Values expressed as mean±SD; *P < 0.01 compared with NC group; #P < 0.05 compared with V + HS group. NC, normal control (n = 10); V + HS, vehicle saline + heat stroke (n = 10); S + HS, salidroside + heat stroke (n = 10).
response in HS mice was shown to reduce the degree of organ damage and improve survival. The current results showed that levels of the pro-inflammatory factor TNF-α and the anti-inflammatory factor IL-10 were significantly elevated by HS, while IL-10 levels were significantly decreased. However, salidroside pretreatment could significantly reverse these effects, suggesting that it has potentially important anti-inflammatory ability.

TBARS and MDA are commonly used as indicators to evaluate lipid peroxide formation. GSH acts as an antioxidant to decompose H₂O₂, and SOD takes two superoxide molecules and makes them into one H₂O₂ and one H₂O. In the present study, salidroside pretreatment significantly decreased the HS-induced increases in TBARS and MDA and increased the levels of GSH and SOD, suggesting that salidroside protected against HS-induced cardiomyocyte damage by down-regulating the oxidative stress response.

Salidroside is a phenylethanol compound with multiple biological functions, including anti-fatigue, anti-tumor, and immune regulation activities, and with

Figure 5. Salidroside decreased expression of TBARS and MDA and increased expression of GSH and SOD in heart tissues. Values expressed as mean ± SD; *P < 0.05 compared with NC group; **P < 0.05 compared with V + HS group. TBARS, tissue thiobarbituric acid reactant substances; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; NC, normal control (n = 10); V + HS, vehicle saline + heat stroke (n = 10); S + HS, salidroside + heat stroke (n = 10).
protective effects on the cardiovascular system and nerve cells. However, the molecular mechanisms by which salidroside protects against HS-induced myocardial injury remain unclear, and further studies using cultured human cardiomyocytes are needed to clarify the protective mechanism of salidroside against HS-induced myocardial injury.

The current study had some limitations. It was an animal experimental study, and further studies are needed to confirm the results in humans. More studies are also needed to elucidate the underlying pathophysiology in terms of its cytology and molecular biology.

In summary, our results suggest that salidroside pretreatment could significantly reduce HS-induced mortality and improve thermoregulatory function, and could also protect against HS-induced myocardial damage. Salidroside may protect against myocardial damage via anti-inflammatory and anti-oxidative mechanisms.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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References
1. Patz JA, Campbell-Lendrum D, Holloway T, et al. Impact of regional climate change on human health. Nature 2005; 438: 310–317.
2. Piver WT, Ando M, Ye F, et al. Temperature and air pollution as risk factors for heat stroke in Tokyo, July and August 1980–1995. Environ Health Perspect 1999; 107: 911–916.
3. Marfisi A, Genty A, Ferry T, et al. Fever as a prognostic factor for heat stroke. Presse Med 2008; 37: 406–411.
4. Krau SD. Heat-related illness: a hot topic in critical care. Crit Care Nurs Clin North Am 2013; 25: 251–262.
5. Xu MC, Shi HM, Gao XF, et al. Salidroside attenuates myocardial ischemia-reperfusion injury via PI3K/Akt signaling pathway. J Asian Nat Prod Res 2013; 15: 244–252.
6. Zhong H, Xin H, Wu LX, et al. Salidroside attenuates apoptosis in ischemic cardiomyocytes: a mechanism through a mitochondria-dependent pathway. J Pharmacol Sci 2010; 114: 399–408.
7. Chatterjee S, Premachandran S, Sharma D, et al. Therapeutic treatment with L-arginine rescues mice from heat stroke-induced death: physiological and molecular mechanisms. Shock 2005; 24: 341–347.
8. Zhang W, Peng M, Yang Y, et al. Protective effects of salidroside on mitochondrial functions against exertional heat stroke-induced organ damage in the rat. Evid Based Complement Alternat Med 2015; 2015: 504567.
9. Deng XY, Chen JJ, Li HY, et al. Cardioprotective effects of timosaponin B II from Anemarrhenae asphodeloides Bge on isoproterenol-induced myocardial infarction in rats. Chem Biol Interact 2015; 240: 22–28.
10. Ohkawa H, Ohishi N and Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351–358.
11. Kumar M, Kasala ER, Boddukuru LN, et al. Baicalein protects isoproterenol induced myocardial ischemic injury in male Wistar rats by mitigating oxidative stress and inflammation. Inflamm Res 2016; 65: 613–622.
12. Rousseau JM, Villevieille T, Schiano P, et al. Reversible myocardial dysfunction after exertional heat stroke. Intensive Care Med 2001; 27: 328–329.
13. Wang X, Yuan B, Dong W, et al. Humid heat exposure induced oxidative stress and apoptosis in cardiomyocytes through the angiotensin II signaling pathway. *Heart Vessels* 2015; 30: 396–405.

14. Quinn CM, Duran RM, Audet GN, et al. Cardiovascular and thermoregulatory biomarkers of heat stroke severity in a conscious rat model. *J Appl Physiol* 2014; 117: 971–978.

15. Fan H, Zhao Y, Zhu JH, et al. Thrombocytopenia as a predictor of severe acute kidney injury in patients with heat stroke. *Ren Fail* 2015; 37: 877–881.

16. Hausfater P, Doumenc B, Chopin S, et al. Elevation of cardiac troponin I during non-exertional heat-related illnesses in the context of a heatwave. *Crit Care* 2010; 14: R99.

17. Quinn CM, Audet GN, Charkoudian N, et al. Cardiovascular and thermoregulatory dysregulation over 24 h following acute heat stress in rats. *Am J Physiol Heart Circ Physiol* 2015; 309: H557–H564.

18. Lim CL and Mackinnon LT. The roles of exercise-induced immune system disturbances in the pathology of heat stroke: the dual pathway model of heat stroke. *Sports Med* 2006; 36: 39–64.

19. Inci S, Ozcan OE and Kilinc K. Time-level relationship for lipid peroxidation and the protective effect of alpha-tocopherol in experimental mild and severe brain injury. *Neurosurgery* 1998; 43: 330–335.

20. Imam SZ and Ali SF. Selenium, an antioxidant, attenuates methamphetamine-induced dopaminergic toxicity and peroxynitrite generation. *Brain Res* 2000; 855: 186–191.