Original Article

Access this article online

Quick Response Code:

Website: http://www.braincirculation.org

DOI: 10.4103/bc.bc_119_23

Optimizing a mouse model of exertional heat stroke to simulate multiorgan and brain injuries

Yuliang Peng[1,2#], Zongping Fang[1,2#], Jing Li[1], Qi Jia[1], Hongwei Ma[1], Ling Li[1,3], You Wu[1], Xijing Zhang[1]

Abstract:

BACKGROUND: Exertional heat stroke (EHS) is a clinical entity characterized by abnormalities of the central nervous system (CNS) and is associated with multiple organ injury, some of which may be irreversible. It is valuable to establish an optimized model of EHS that is able to induce and assess damage to the CNS and multiple organs.

METHODS: We induced EHS by using an environmental chamber with adjustable temperature and humidity and a mice forced running wheel. The endpoint for the EHS was defined as either exhaustion or a core temperature of 42.5°C being reached. Injury to the liver, kidney, and CNS of mice in the EHS group was revealed through pathological studies using hematoxylin and eosin staining of harvested organs at different time points and detection of biomarkers. The depressive-like behavior of EHS mice was assessed through open field tests, forced swimming tests, and tail suspension tests.

RESULTS: The favorable environmental conditions for induction of EHS based on this presented model are 38°C, 70% RH. The EHS mice developed thermoregulatory dysfunction and experienced a significantly higher weight loss ratio compared to the SHE (sham heat exercise) group. The liver, kidney, and brain tissues of EHS mice were significantly damaged, and the pathological damage scores for each organ were significantly higher than those of the SHE group. In the open field test (OFT), compared to the SHE group, there was a significant reduction in the number and time of EHS mice entering the center of the open field. Additionally, there was a significant increase in immobile time during forced swimming test (FST) and tail suspension test (TST).

CONCLUSION: This study presents an improved animal model that has the potential to assess for neurological and multiple organ injury caused by EHS and simultaneously, while accurately reflecting the clinical characteristics observed in EHS patients.

Keywords:

Animal model, central nervous system, exertional heat stroke, multiple organ injury

Departments of [1]Critical Care Medicine, [3]Pediatric, Xijing Hospital, Fourth Military Medical University, Xi'an, [2]Department of Critical Care Medicine, Translational Research Institute of Brain and Brain-Like Intelligence, Fourth People's Hospital, School of Medicine, Tongji University, Shanghai, China

[#]Yuliang Peng and Zongping Fang contributed equally and shared the first authorship.

Address for correspondence:
Dr. Xijing Zhang, Department of Critical Care Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an, China.
E-mail: xjzhang0806@163.com
Dr. You Wu, Department of Critical Care Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an, China.
E-mail: yoyo19920210@163.com

Submission: 13-12-2023
Revised: 17-04-2024
Accepted: 19-04-2024
Published: 26-09-2024

Introduction

Heat stroke is a life-threatening clinical syndrome characterized by an increase in core temperature (>40°C) and the presence of central nervous system (CNS) abnormalities such as coma, ataxia, seizures, and impaired consciousness accompanied by multiorgan injury.[1] It is thought to be due to the imbalance between heat production and heat dissipation in the setting of exposure to heat or strenuous exercise. Depending on the cause and susceptible population, heat stroke is divided into classic heat stroke (CHS) and exertional heat stroke (EHS). The occurrence of CHS is attributed to passive exposure to elevated ambient temperatures (often accompanied by high humidity) and manifests in epidemic proportions during heat waves, particularly among older adults who frequently have

How to cite this article: Peng Y, Fang Z, Li J, Jia Q, Ma H, Li L, *et al*. Optimizing a mouse model of exertional heat stroke to simulate multiorgan and brain injuries. Brain Circ 2024;10:240-9.

preexisting medical conditions.[2] EHS occurs mainly in young and healthy individuals (typically marathoner, soldiers, construction workers, etc.) who, as per their occupation, are obligated to spend long periods of time in high temperatures.[3] The latest data indicate a rising trend in hospitalizations caused by EHS and heat-related exertional injuries among the civilian population of the United States.[4] In 2013, the US military experienced more than 1710 heat-related injuries, including 323 hospitalizations for EHS, most of which were related to exercise in hot temperatures.[5] Moreover, EHS is the third leading cause of death among athletes during sports activities.[6]

EHS patients often suffer severe acute kidney and liver injuries, which usually appear within hours of onset. In addition to their acute nature, these injuries tend to progress rapidly.[7-9] Some patients may be left with sequelae of CNS impairment, which mainly manifests as long-term memory loss, cognitive impairment, and ataxia.[10] Establishing a reliable preclinical model of EHS is crucial for developing clinical interventions to prevent multiorgan damage and adverse sequelae of the CNS.

There are well-established preclinical models for CHS in rodents.[8,11-13] However, this cannot be said about EHS. The differences in pathophysiology between EHS and CHS, as well as other factors related to mechanisms of heat production in muscles and the redistribution of blood during exercise, suggest that the more developed CHS model cannot be translated into the EHS model.[14,15] There currently exists a number of EHS models that have been previously described,[7,14,16,17] and although these contributed to many important findings about EHS, they still had many limitations. For instance, some findings from experiments done on such models were found to be inconsistent with current epidemiological data. Two examples of this would be that the applied electric shock can induce a superimposed stress response and that EHS has very high mortality rates.[18,19] In our research, we have developed a novel EHS model, in which adult male mice are trained to exercise at 38°C and 70% relative humidity (RH) on forced running machinery in attempting to induce survivable EHS conditions. This is in attempt to mimic the changes and characteristics typically seen in EHS patients. Second, we evaluated for CNS damage by looking for changes in the pathology level in the hippocampus and hypothalamus, in addition to changes in levels of CNS plasma markers that indicate injury. In short, this study aims to optimize existing EHS modeling systems by establishing a mouse model that mimics EHS-induced multiorgan system dysfunction and CNS injury inconsistent with the clinical characteristics seen in EHS patients.

Materials and Methods

Animals

All mice used in this model were males. This was done to eliminate the effect of estrogen which was shown in previous studies to improve the inflammatory response and cardiovascular dysfunction following heat stroke.[20] The Experimental Animal Center of the Air Force Military Medical University provided sexually mature adult pathogen-free C57BL/6 wild-type mice, aged approximately 8–9 weeks. The animals were accommodated in an animal room of specific-pathogen-free class, where the temperature was set at 24°C ± 0.5°C and the humidity maintained at 60% ± 2%, and maintaining the following circadian rhythm: daytime (08:00–20:00) and nighttime (20:00–08:00). Standard laboratory food and water were provided from time to time each day. With regard to handling animals in this research study, we followed the guidelines for the care and use of laboratory animals followed by institutions accredited by the Association for the Evaluation and Accreditation of Laboratory Animal Care. All experimental protocols involving animals were approved by the Air Force Military Medical University Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Overview of the exertional heat stroke protocol

The mice underwent a 6-day period of acclimatization training. The initial exercise involved a 15-min period of unrestricted activity, during which the mice were given the freedom to explore their surroundings on a running wheel. Following that was a very short recovery period (5 min). The training intensity was determined by adjusting the locomotor speed, and the training time was set to be 60 min/day. The starting speed was 9 r/min, and it was increased by 2 r/min/day to finally reach a maximum speed of 15 r/min by the 4th day. Finally, the mice rested for the remaining 2 days before the EHS trial was initiated. The running speed was maintained without the use of electric shock or any other form of manual stimulation.

The EHS model we constructed utilized a combination of an environmental chamber with a heating lamp (which allowed for accurate regulation of temperature and humidity) and a mice-forced running wheel [Figure 1a]. The experimental design described in the methods section is divided into three key stages [Figure 1b]. In the first stage, termed the preparation phase, an environmental chamber with adjustable temperature and humidity is used, and a heated lamp is installed inside. Next, a mice-forced running wheel is prepared and is placed inside the environmental chamber which

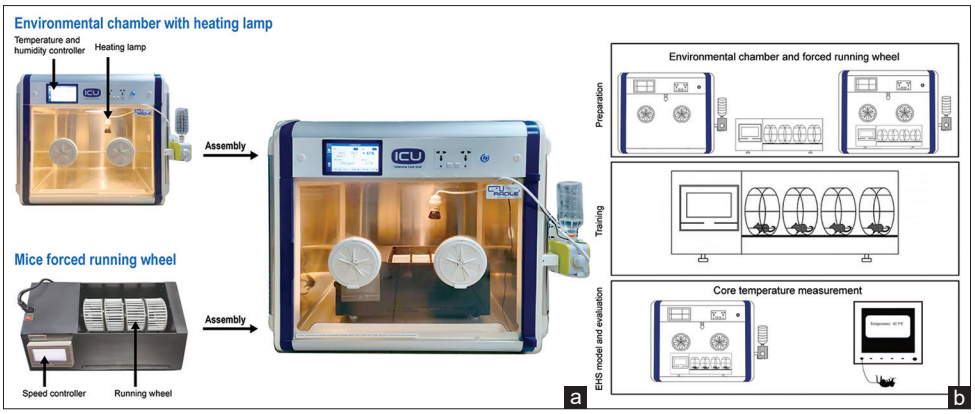


Figure 1: Overview of the protocol for the design and assessment of an exertional heat stroke (EHS) model. (a) The customized EHS module comprises an environmental chamber with a heating lamp and a mice-forced running wheel. (b) The protocol includes preparation, training and EHS model and evaluation. The environmental chamber was kept the environmental temperature at 38°C and 70% relative humidity. The forced running wheel was maintained at a constant speed of 15 r/min until each mouse has run to exhaustion under the customized EHS module. Finally, the core body temperature of the mice was measured

completes the construction of the model. In the second stage, the mice are trained to run continuously on the forced running wheel. Finally, the third stage is when EHS is induced. To induce EHS, the environmental chamber temperature (in degrees Celsius [°C]) and humidity (RH) were increased to achieve target values (37°C, 70% RH; 38°C, 70% RH; and 39°C, 70% RH). Once the environmental chamber temperature was calibrated to the target temperature (within approximately 2 h), the mouse was positioned on the running wheel. During the entire duration of the test, the running wheel maintains an unwavering velocity of 15 r/min. During this process, the body temperature of each mouse was monitored. The assessed endpoints for EHS in this test were signs of physical exhaustion (defined here as the apparent loss of consciousness, falling backward, or the inability to continue running or grab the wheel) and/or core body temperature (Tc) ≥42.5°C in mice. The running wheel was promptly halted, and the mice were removed from the forced run apparatus when it was observed that they rotated backward three times without exhibiting any signs of response. Such subjects were then weighed and placed back in the cage to allow for recovery at room temperature while continuing to monitor their Tc for the following 12 h.

Weight alteration

The mice were weighed before initiating the EHS protocol and when Tc T42.5°C or right after the onset of physical exhaustion. To calculate the change in weight, the following formula was used: weight before performing EHS – Weight at Tc T42.5°C or after onset of physical exhaustion/weight before performing EHS ×100%.

Biochemical analyses

All mice underwent EHS at set conditions of 38°C and 70% RH and were executed 3, 12, or 24 h following EHS onset. Moreover, an additional group (six mice) of sham heat exercise (SHE) mice were executed (at the same time) after matching exercise intensity and duration targets (maximum speed: 15 r/min and duration: 60 min) at set conditions of 25°C and 70% RH. Blood was collected in heparin-coated tubes which were spun at 2000 relative centrifugal force. A volume of 250 μl of plasma was taken from the sample and stored at −80°C. The plasma was sent to the Department of Clinical Laboratorial Examination of the Xijing Hospital, The Air Force Military Medical University for determination of blood urea nitrogen (BUN), creatinine (Cr), alanine transaminase (ALT), and aspartate aminotransferase (AST) levels. The concentrations of mouse Neuron-specific enolase (NSE) and S100 calcium-binding protein β (S100β) were determined by ELISA kits (Westang, Shanghai, China) according to the guided instructions.

Histopathological observations

All mice underwent EHS at set conditions of 38°C and 70% RH and were executed 3, 12, or 24 h following EHS onset. Moreover, an additional group (six mice) of sham SHE was executed (at the same time) after matching exercise intensity and duration targets (maximum speed: 15 r/min and duration: 60 min) at set conditions of 25°C and 70% RH. The fresh heart, liver, lung, kidney, and brain tissues were fixed in 4% paraformaldehyde for 12 h. After dehydration, they were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H and E). Subsequently, the sections were observed under a light microscope at magnifications of ×20 and ×40. Four nonoverlapping fields were randomly selected from each section to score the damage scores for each organ. The analysis of the sections was performed by two professional pathologists who were blinded to the experimental protocol. Each of them scored the degree

of organ injury on a scale of 0–4 (0: normal, 1: mild, 2: moderate, 3: severe, and 4: very severe).

Open field test

The mice were acclimated to their environment by being placed in the behavioral testing room 2 h before the commencement of the experiment. The mice were placed in a 40 cm × 40 cm × 40 cm polyvinyl chloride box in an open field test. A video tracking system recorded their total movement distance, number of entries into the central area, residence time in the central area, and immobility time within 5 min. The video-tracking system was utilized to measure the total distance covered by the mice within a 5 min timeframe, aiming to evaluate their motor function impairment and minimize potential interference with behavioral assessments. Then, the number of entries into the central area, residence time in the central area, and immobility time were recorded to assess the anxiety level of the mice, so as to indirectly reflect whether the brain function was disturbed. The mice's feces were cleaned, and the instrument was wiped with 75% ethanol to remove odors at the end of the test. The mice's movement and activity were recorded and analyzed using the ANY-maze system.

Forced swimming test

Two hours before the forced swimming test (FST), mice were placed in the behavioral testing room for acclimation. Subsequently, warm water (23°C–24°C) was injected into a transparent cylindrical vessel measuring 20 cm in diameter and 45 cm in height, raising the water column to a height of 30 cm. The mice were placed in a circular container, and their activity was recorded with a video camera for 6 min, and the ANY-maze system analyzed their activity within a timeframe of 2–6 min. The immobility time analysis of mice was employed to evaluate the level of depression in mice, which indirectly indicated the dysfunction of brain activity.

Tail suspension test

The mice were acclimated to their environment by being placed in the behavioral testing chamber 2 h before the commencement of the tail suspension test (TST). Then, they were placed in a white plastic box measuring 30 cm × 30 cm × 50 cm for the test. The tape was affixed 1–2 cm from the distal end of the mice tail, and subsequently, the mice were suspended from the top of the plastic box. The mice's activity was recorded by a video camera for a duration of 6 min, and the ANY-maze system analyzed their activity within a timeframe of 2–6 min. The laboratory should be kept quiet, and the operator should avoid wearing perfume or any strong odors. The immobility time analysis of mice was employed to evaluate the level of depression in mice, which indirectly indicated the dysfunction of brain activity.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 9.0 software. The experimental data are presented as the mean ± standard error of the mean. Survival was expressed as a percentage. Comparisons between the two groups were made using the Student's-*t*-test, and multiple comparisons were made using one-way analysis of variance (ANOVA). *Post hoc* comparisons with Bonferroni correction were conducted when necessary. To confirm the results, at least three independent experiments were performed. Statistical significance was defined as a two-tailed $P \leq 0.05$.

Results

Exertional heat stroke causes thermoregulatory dysfunction and mortality

Mice suffering from EHS lose the ability to maintain thermoregulatory balance.[15] This is evident by the changes seen in Tc after 12 h after the onset of EHS in each group. The typical thermal profiles expected at each temperature are shown in Figure 2a-d. Upon exposure to increasing environmental temperatures (Tenv; 37°C and 38°C) at a set humidity (70% RH), the Tc of mice was noted to increase immediately following the start of exercise, rising to over 41°C in almost all subjects. The temperature is subsequently maintained at this level for a period of time, followed by a rapid increase to 42°C [Figure 2b and c]. In contrast, in mice exposed to 39°C, the body temperature rose rapidly to 42°C [Figure 2d].

Once the endpoints of the EHS protocol were reached, the mice were taken out and restored at room temperature (25°C, 50% RH), and we monitored their Tc for the following 12 h. A rapid drop in Tc to the hypothermic range was noted just after 30 min of recovery. Conversely, Tc could repeatedly be increased by 1°C–2°C during exercise in SHE animals, reaching an average maximum of 38°C [Figure 2a].

The survival rates in mice following EHS induction were measured under each of the three conditions (increasing temperatures and high humidity) used in this EHS model within 24 h of onset. The survival rate of mice at 38°C, 70% RH was 70%, which is similar to the survival rate of EHS in human patients [Figure 2e]. However, at 39°C and 70% RH, the survival rate of mice was only 20%, significantly lower than that of patients with EHS clinically. At 37°C, 70% RH, the survival rate of mice was 100%, which was identical that of mice in the SHE groups. Moreover, all animals lost weight during the test period, but the EHS mice had a high weight loss ratio of 6.5% compared to the SHE group mice [Figure 2f, one-way ANOVA, $^{***}P < 0.001$; $^{**}P < 0.01$]. Consequently, the favorable environmental

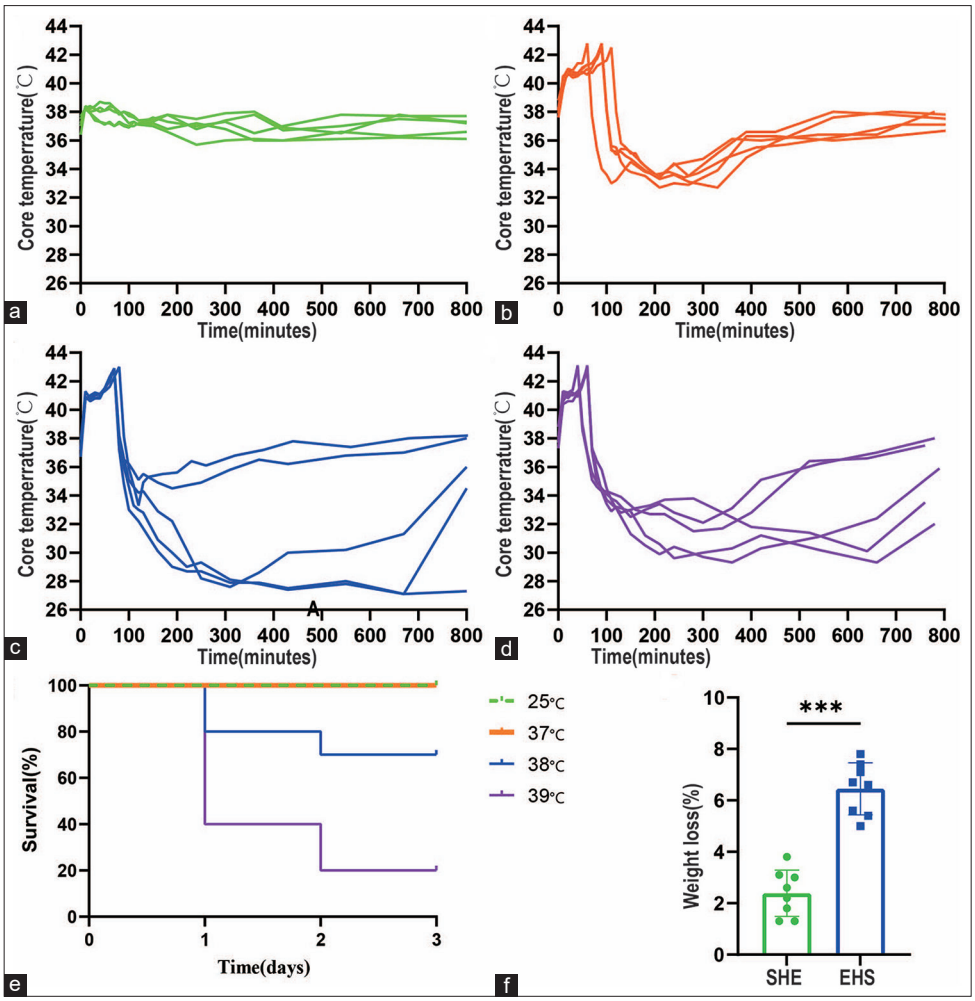


Figure 2: Temperature curve of mice during and 12 h after exertional heat stroke (EHS), survival rate at 3d after EHS, and weight loss during EHS. (a-d) The core temperature of mice exposed to 25°C, 37°C, 38°C, or 39°C at a set 70% relative humidity (RH) was monitored at 10 min intervals; (e) survival rate of mice with EHS within 3 d under four different conditions of environmental chamber temperature and RH; (f) percent body weight loss from different groups after the onset of EHS. Mean ± standard error of the mean; ***$P < 0.001$, $n = 8$, Statistics by one-way analysis of variance

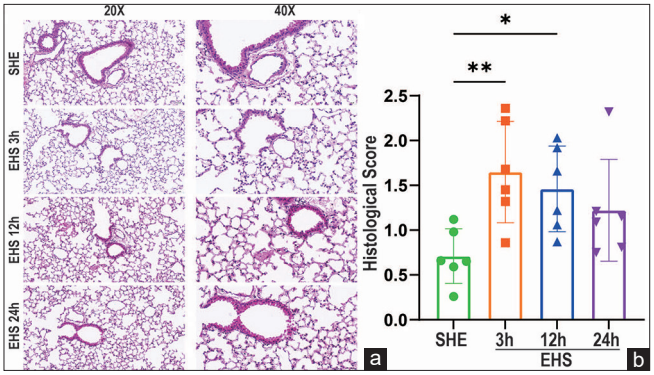


Figure 3: Representative histopathology of lung injury in exertional heat stroke (EHS). (a) Representative images of hematoxylin and eosin staining of the lung from sham heat exercise mice or 3 h, 12 h, and 24 h after EHS. Scale bars = 50 μm and 20 μm. (b) Pathological scores among groups. Mean ± standard error of the mean; *$P < 0.05$, **$P < 0.01$; $n = 6$, Statistics by one-way analysis of variance

conditions for induction of EHS based on this presented model are 38°C, 70% RH.

Exertional heat stroke causes severe multiple organ dysfunction

To assess organ damage in this model, we harvested the heart, liver, kidney, and lungs at different time points (3 h, 12 h, and 24 h) following execution of mice exposed to 38°C and 70% RH. No significant pathological changes were noted in the heart tissues of the mice in the EHS group compared to the SHE groups at all time points [Supplementary Figure 1a]. Moreover, the pathology assessment [Supplementary Figure 1b] showed that the pathology scores at all time points in mice in the EHS group were not significantly different from those in the SHE groups. Findings of neutrophil infiltration were found in the lung tissues of the EHS group at 3 h, but no pathological changes were seen at 12 h and 24 h [Figure 3a]. Pathology scores at 3 h and 12 h were significantly higher in the EHS group than in the SHE groups [Figure 3b, one-way ANOVA, **$P < 0.01$; *$P < 0.05$). The liver and kidneys are known to

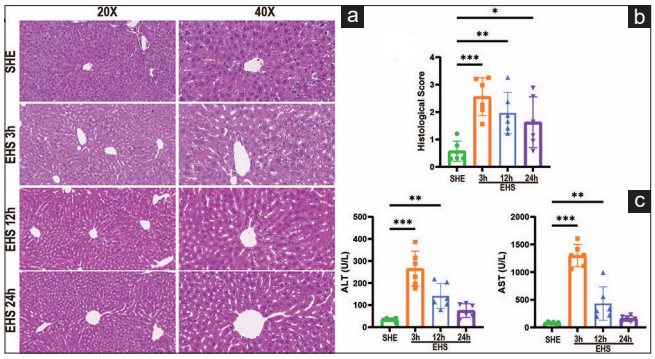


Figure 4: Representative histopathology and biochemical analyses of liver injury in exertional heat stroke (EHS). (a) Representative images of hematoxylin and eosin staining of liver from sham heat exercise mice or 3 h, 12 h, and 24 h after EHS. Scale bars = 50 μm, and 20 μm. (b) Pathological scores among groups. Mean ± standard error of the mean (SEM); ***P* < 0.01; ****P* < 0.001, *n* = 6, Statistics by one-way analysis of variance (ANOVA). (c) Analysis of serum alanine aminotransferase and aspartate aminotransferase. Mean ± SEM; **P* < 0.05; ***P* < 0.01; ****P* < 0.001, *n* = 6, Statistics by one-way ANOVA

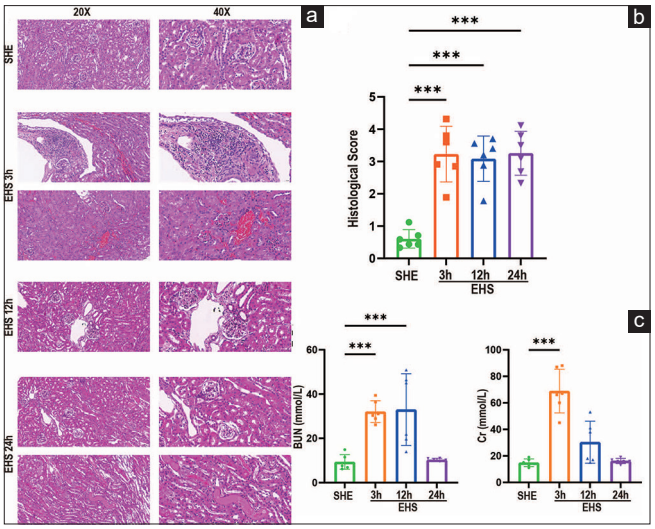


Figure 5: Representative histopathology and biochemical analyses of kidney injury in exertional heat stroke (EHS). (a) Representative images of hematoxylin and eosin staining of kidney from sham heat exercise mice or 3 h, 12 h, and 24 h after EHS. Scale bars = 50 μm, and 20 μm. (b) Pathological scores among groups. Mean ± standard error of the mean (SEM); ****P* < 0.001, *n* = 6, statistics by one-way analysis of variance (ANOVA). (c) Analysis of blood urea nitrogen and creatinine. Mean ± SEM; ****P* < 0.001, *n* = 6, Statistics by one-way ANOVA

be very sensitive to thermal damage,[21] and our results are in accordance with this. For instance, vacuolar degeneration and swelling of hepatocytes were seen at each time point in the EHS group compared to the SHE groups [Figure 4a]. The pathology scores in the EHS group were significantly higher compared to those in the SHE groups (one-way ANOVA, ****P* < 0.001; ***P* < 0.01; **P* < 0.05), with the most severe scores observed at 3 h [Figure 4b]. Moreover, renal tissues assessed at 3 h in the EHS group showed a small amount of tubular mild hydropathy, interstitial vascular stasis, and lymphocytic infiltration around the renal pelvis. This is in comparison to findings at 12 h and 24 h, where dilated glomerular capillaries, swollen tubular epithelial cells, and occasional tubular protein were seen in renal tissues of the EHS group [Figure 5a]. Subsequently, pathological assessment showed significant renal pathology scores at all time points in the EHS groups [Figure 5b, one-way ANOVA, ****P* < 0.001).

ALT and AST, which are markers of liver injury, were found to be significantly elevated following EHS reaching maximum levels at 3 h compared to SHE controls, before gradually decreasing at 12 h and 24 h [Figure 4c, one-way ANOVA, ****P* < 0.001; ***P* < 0.01]. In addition, the levels of BUN and Cr were measured to assess kidney function. BUN levels can be reflective of the glomerular filtration rate and the degree of renal injury. In our study, EHS mice had high levels of BUN and Cr at 3 h and 12 h [Figure 5c, one-way ANOVA, ****P* < 0.001].

Exertional heat stroke causes severe central nervous system damage

The brain tissue is very sensitive to high temperatures. Hence, it is inevitable to sustain CNS injury following EHS. CNS injury in EHS mice was evaluated through pathological studies of H- and E-stained brain tissue. Two main brain regions, namely, the hippocampus and the hypothalamus, were selected for analysis. These areas were assessed at 3 h, 12 h, and 24 h following EHS onset. H and E staining was done on the hypothalamus first, and subsequent analysis of the slides revealed at 3 h, the presence of cell body contraction, nucleus fixation, and massive vacuolar degeneration in the EHS group compared to no such findings seen in SHE groups. The same findings were seen in the EHS group slides at 12 h and 24 h but with a lesser degree of damage [Figure 6a]. The pathology scores in the EHS group were significantly higher compared to those in the SHE groups (one-way ANOVA, ****P* < 0.001; ***P* < 0.01), with the most severe scores observed at 3 h [Figure 6b]. Shifting the sight toward the hippocampus, pathology slides of hippocampal neurons, and vertebral cells in the EHS group at 3 h revealed the presence of edema, vacuolation, and sparse cellular arrangement compared to no such findings in the SHE groups. The same findings were seen at 12 h and 24 h with a similar degree of damage [Figure 6c]. Hence, hippocampal pathology scores were significantly higher in the EHS group than in the SHE groups at all time points [Figure 6d, one-way ANOVA, ****P* < 0.001]. The levels of serum NSE and S-100β can be employed for the assessment of brain injury severity. The results of serum ELISA showed significant increases in NSE and S100β at 3 h, 12 h, and 24 h after the onset of EHS [Figure 6e and f, one-way ANOVA, ****P* < 0.001; ***P* < 0.01; **P* < 0.05].

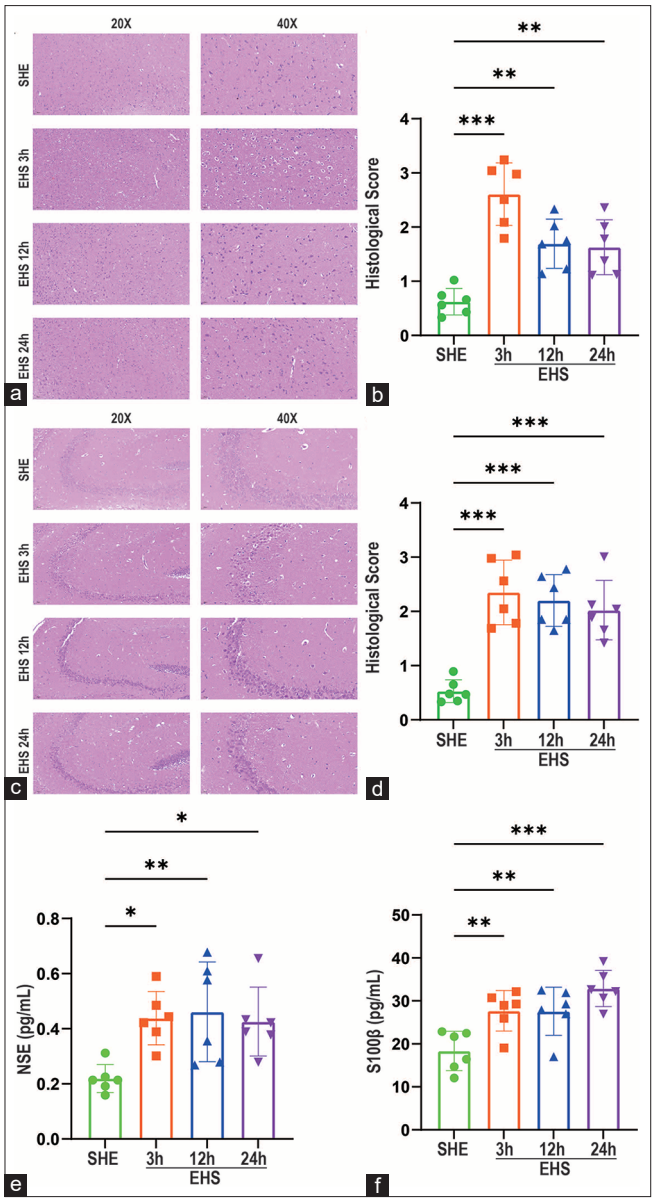


Figure 6: Representative histopathology and biochemical analyses of brain injury in exertional heat stroke (EHS). (a and c) Representative images of hematoxylin and eosin staining of the brain from sham heat exercise mice or 3 h, 12 h, and 24 h after EHS. Scale bars = 50 μm, and 20 μm. (b and d) Pathological scores among groups. Mean ± standard error of the mean (SEM); $^{**}P < 0.01$; $^{***}P < 0.001$, $n = 6$, Statistics by one-way analysis of variance (ANOVA). (e and f) Analysis of neuron-specific enolase and calcium binding protein β (S100β). Mean ± SEM; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, $n = 6$, Statistics by one-way ANOVA

Exertional heat stroke causes depressive-like behavior

We conducted a behavioral test 48 h after the induction of EHS to assess the mice's brain function by evaluating their depression-like behavior. The OFT [Figure 7a and b], FST [Figure 7g], and TST [Figure 7i] were conducted. In the OFT, there were no differences in total movement distance [Figure 7c], but a decrease in both the number of entries into the central zone [Figure 7d, Student's *t*-test, $^{**}P < 0.01$] and the time spent in the central zone [Figure 7e, Student's *t*-test, $^{**}P < 0.01$]. In addition, there was an increase in immobility time [Figure 7f, Student's *t*-test, $^{**}P < 0.01$] observed in the EHS group compared to the SHE group. The immobility time [Figure 7h and j, Student's *t*-test, $^{***}P < 0.001$; $^{*}P < 0.05$) was similarly increased in EHS mice during both the FST and TST.

Discussion

EHS is a serious fatal disease thought to be due to the effects of increased core body temperature, secondary to intense physical exertion, on various body systems.[2] It usually manifests as more severe symptoms, more rapid development, and higher morbidity and mortality than normal heat exhaustion.[22-24] Despite the use of rapid cooling and aggressive supportive therapy, patients with heat stroke often develop irreversible multiorgan dysfunction syndrome including permanent CNS damage.[25,26] Previously studied models rarely focused on the detrimental sequelae of EHS, and instead put unnecessary emphasis on constructing the model design, including the use of electrical stimulation, anesthesia, or predetermined Tc thresholds.[17,27] The previous EHS models utilized plant growth incubators to replicate the high temperature and humidity environment, but these models suffered from issues such as inconsistent temperature control and limited ability to observe mouse conditions.[15] Therefore, in the current study, we set out to develop an optimized animal model of EHS. The model established in this study effectively maintains the high temperature and humidity conditions in the environmental chamber, allowing real-time observation of mice during EHS. In addition, multiple mice can be accommodated simultaneously. The model was designed to focus on the signs, symptoms, and multiorgan dysfunction observed in patients with EHS.[14] In addition, the model allowed the study of potential mechanisms involved in the onset of EHS and within the recovery period. It can also investigate the impact of various intervention methods on thermoregulation, as well as assess organ dysfunction and rehabilitation indexes through functional tests conducted in high-temperature conditions.

EHS is common in young people, such as athletes, soldiers, and in any occupation that requires working in hot environments. Therefore, the selection of sexually mature mice aged 8–9 weeks for this study was based on the appropriateness of this age range in representing patients with EHS. After 1 week of acclimatization, the mice were formally tested at 9–10 weeks. At this age, mice had better grasp and crawling abilities, which may be consistent with the prevalence of EHS in young and middle-aged adults. Some studies, however, such as the one done by King *et al*., used relatively older mice aged 4 months.[7] However, we believe that the issue

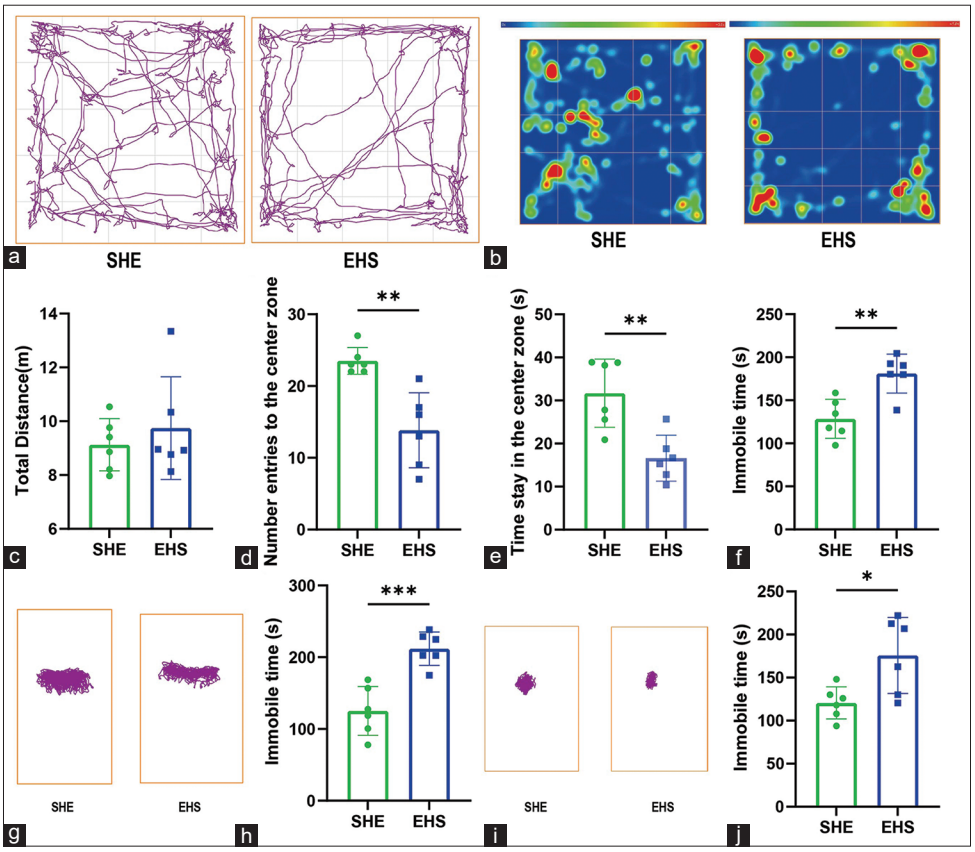


Figure 7: The impact of exertional heat stroke (EHS) on depressive-like behavior. (a) Typical tracking chart of the OPF between the sham heat exercise (SHE) mice and the EHS mice. (b) Typical heat map of the OPF between the SHE mice and the EHS mice. (c) The difference between SHE and EHS in the total distance of motion in OFT. (d) The difference between SHE and EHS in the number of entries to the central area in OFT. (e) The difference between SHE and EHS in the time stay in the central area in OFT. (f) The difference between SHE and EHS in the immobility time in the OFT. Mean ± standard error of the mean (SEM); $^{**}P < 0.01$, $n = 6$, Statistics by Student's-*t*-test. (g) Typical tracking chart of the forced swimming test (FST) between the SHE mice and the EHS mice. (h) The difference between SHE and EHS in the immobility time in the FST. Mean ± SEM; $^{***}P < 0.00$, $n = 6$, Statistics by Student's-*t*-test. (i) Typical tracking chart of the tail suspension test (TST) between the SHE mice and the EHS mice. (j) The difference between SHE and EHS in the immobility time in the TST. Mean ± SEM; $^{*}P < 0.05$, $n = 6$, Statistics by Student's-*t*-test

with using older mice is that they may have impaired thermoregulation and immune regulation.

In our study, we designed three EHS conditions with relatively high temperature and humidity (37°C, 70% RH; 38°C, 70% RH; and 39°C, 70% RH). Under high temperature and humidity conditions, the body temperature of mice quickly rose to over 41°C. This was reflected in an initial net gradient (from Tc to Tenv) of 0.5°C–3°C throughout the exercise period, allowing for some radiant heat loss. Based on the Tc curve, it appears that mice exposed to Tenv of 37°C or 38°C exhibit adequate compensatory mechanisms to maintain Tc at about 41°C. However, in the face of increasing metabolic heat production, it reaches a particular point where it is too high to compensate and this is when Tc begins to rise rapidly, leading to EHS.

Tc in mice exposed to 39°C increased rapidly, reaching 42°C, suggesting that the subjects were simply unable to disperse the excess heat generated by the high metabolic load over the small gradient between Tc and Tenv. After being allowed to recover at room temperature, the Tc of EHS mice exhibited a rapid decline toward the lower temperature range. This drop was similar to that seen in the CHS mice model.[11] We monitored the survival rate over 24 h and compared the survival rates of mice under the various conditions they were exposed to. We found that the survival rate of mice at 37°C was 100%, which was not consistent with the survival rate seen in EHS patients. The survival rate of mice under the condition of 39°C was 50%, which is not reflective of the survival rate seen in human EHS patients and can present difficulties for the subsequent study on the recovery period of EHS. Finally, we chose the condition of 38°C to be the most favorable for EHS to occur because the survival rate of mice under this condition was 70%, which is consistent with the survival rate of EHS patients and alleviates the difficulties for the EHS recovery study.

Several different mechanisms may contribute to neurological dysfunction or confusion that occur in EHS, including reduced cerebral blood flow due to the combined demands of exercising muscles and heat

exchange, dehydration, persistent hypocapnia,[4,28] loss of blood–brain barrier integrity,[29] and the direct neurological effects of hyperthermia.[30] In this study, we evaluated dehydration as a potential culprit in the development of neurological dysfunction. We noticed that following EHS onset in mice, they had a significantly higher weight loss ratio of 6.5% compared to the SHE group. Moreover, we demonstrated the presence of multiorgan damage in the liver and kidney as detected by histology within a short window of recovery and also relied on serum biomarkers to assess the time course of ongoing damage. The increase in these measurements can indicate the progression of multiorgan dysfunction rather than solely reflecting damage to individual organs. The results of serum biomarker proteins are contingent upon their production and clearance rates. Consequently, in cases of kidney or liver injury where excessive production occurs or clearance is impeded, there may be an accumulation that does not correspond to the extent or duration of the injury. This may partly explain the somewhat unexpected time course of the responses observed in mice, which exhibit a rather dramatic increase or decrease in magnitude over time. Previous models also identified elevated liver and kidney function indicators in EHS mice,[27] but there was a lack of definitive histopathological examination. In this study, HE staining revealed that the liver and kidney tissues of EHS mice exhibited the most severe damage at 3 h, while levels of ALT and AST reached their peak at 3 h and gradually declined at 12 and 24 h. The elevated ALT and AST levels were consistent with severe liver injury. Brain injury as a characteristic manifestation of heat attack disease can lead to CNS dysfunction.[31] In the CHS mouse model, heat stroke leads to dysfunction of iron metabolism and iron regulatory protein in the hippocampus, as well as spatial memory impairment in mice.[32] However, this has not been reported in EHS models. Our study found that EHS mice exhibited depressive-like behavior, indicating a dysfunction in brain function and further confirming damage to the CNS.

Limitations of the study

In this study, multiple organs of EHS mice were assessed to elucidate the damage caused by EHS on various bodily systems, and the establishment of an EHS model was also validated. The detection of certain organ injuries remains limited due to experimental technology constraints. The study did not investigate any potential damage to the gastrointestinal system or alterations in the gut microbiota. The role of gastrointestinal barrier integrity in the development of EHS remains unclear. Moreover, there is a lack of detection for both coagulation and platelet functions in the blood system. Therefore, in future studies, we will improve the detection of various systems in EHS mice more effectively.

There remain many unanswered questions about EHS that need to be addressed. For instance, it is unclear why certain individuals are more susceptible to EHS under the same environmental conditions. In addition, it is important to consider the effects of certain underlying diseases that are currently not recognized, such as the role of the immune system in organ dysfunction, coordination of movement, and the impact of acute exercise on the development of organ damage.[21] This will help us gain a more comprehensive understanding of this disease and its contributing factors. Before human studies, these questions were mainly addressed in animal models. The model could potentially be utilized in the future to elucidate the underlying mechanisms of EHS and facilitate the development of interventions aimed at delaying the onset of EHS or preventing subsequent multiorgan and CNS injury. In conclusion, the protocol we present in this study establishes guidelines for implementing a reliable preclinical EHS model in mice and promises to identify potential pitfalls to avoid when reproducing the approach in other settings and in future studies.

Conclusion

The present study involved the construction of EHS models under three distinct conditions characterized by high temperature and humidity levels, followed by the subsequent measurement of mice survival rates within 24 hours post-disease onset. The survival rate of mice at 38°C and 70% RH was found to be 70%, which is comparable to the survival rate observed in human patients with exertional heat stroke. Additionally, we demonstrated the presence of histologically detected damage of varying severity in the central nervous system, liver, and kidney shortly after recovery, while also utilizing serum biomarkers to assess the temporal progression of sustained damage. In conclusion, the protocol presented in this study provides comprehensive guidelines for the implementation of a robust preclinical EHS model in mice.

Author contributions

Xijing Zhang: Concepts, Design,Guarantor; Yuliang Peng: Data acquisition, Data analysis; You Wu: Experimental studies; Zongping Fang: Definition of intellectual content; Jing Li: Manuscript preparation; Qi Jia: Manuscript editing, Manuscript review; Hongwei Ma: Statistical analysis; Ling Li: Literature search.

Ethical statement

The study was approved by the Ethics Committee of the Fourth Military Medical university (approval number:

IACUC-20241414, dated on 2024.02.01). Patients were consented by an informed consent process that was reviewed by the Ethics Committee of the Fourth Military Medical university and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Financial support and sponsorship

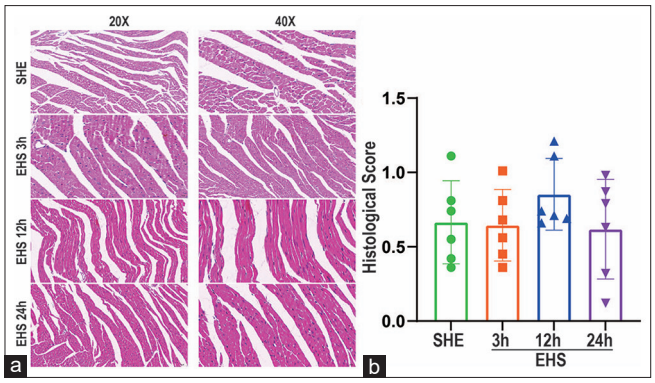
This work was supported by grants from the National Natural Science Foundation of China to (81871603) and the Discipline Boost Program of the First Affiliated Hospital of the Fourth Military Medical University (XJZT21J08) to Xijing Zhang, and the National Natural Science Foundation of China (82171322) and the Natural Science Foundation of Shaanxi Province of China (2022KJXX-102) to Zongping Fang.

Conflicts of interest

There are no conflicts of interest.

References

1. Epstein Y, Yanovich R. Heatstroke. N Engl J Med 2019;380:2449-59.
2. Bouchama A, Abuyassin B, Lehe C, Laitano O, Jay O, O'Connor FG, *et al.* Classic and exertional heatstroke. Nat Rev Dis Primers 2022;8:8.
3. Adams T, Stacey E, Stacey S, Martin D. Exertional heat stroke. Br J Hosp Med (Lond) 2012;73:72-8.
4. Nelson NG, Collins CL, Comstock RD, McKenzie LB. Exertional heat-related injuries treated in emergency departments in the U.S., 1997-2006. Am J Prev Med 2011;40:54-60.
5. Armed Forces Health Surveillance Center (AFHSC). Update: Heat injuries, active component, U.S. Armed Forces, 2013. MSMR 2014;21:10-3.
6. Howe AS, Boden BP. Heat-related illness in athletes. Am J Sports Med 2007;35:1384-95.
7. King MA, Leon LR, Mustico DL, Haines JM, Clanton TL. Biomarkers of multiorgan injury in a preclinical model of exertional heat stroke. J Appl Physiol (1985) 2015;118:1207-20.
8. Leon LR, Blaha MD, DuBose DA. Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery. J Appl Physiol (1985) 2006;100:1400-9.
9. Roberts GT, Ghebeh H, Chishti MA, Al Mohanna F, El Sayed R, Al Mohanna F, *et al.* Microvascular injury, thrombosis, inflammation, and apoptosis in the pathogenesis of heatstroke: A study in baboon model. Arterioscler Thromb Vasc Biol 2008;28:1130-6.
10. Dematte JE, O'Mara K, Buescher J, Whitney CG, Forsythe S, McNamee T, *et al.* Near-fatal heat stroke during the 1995 heat wave in Chicago. Ann Intern Med 1998;129:173-81.
11. Leon LR, DuBose DA, Mason CW. Heat stress induces a biphasic thermoregulatory response in mice. Am J Physiol Regul Integr Comp Physiol 2005;288:R197-204.
12. Leon LR, Gordon CJ, Helwig BG, Rufolo DM, Blaha MD. Thermoregulatory, behavioral, and metabolic responses to heatstroke in a conscious mouse model. Am J Physiol Regul Integr Comp Physiol 2010;299:R241-8.
13. Quinn CM, Duran RM, Audet GN, Charkoudian N, Leon LR. Cardiovascular and thermoregulatory biomarkers of heat stroke severity in a conscious rat model. J Appl Physiol (1985) 2014;117:971-8.
14. King MA, Leon LR, Morse DA, Clanton TL. Unique cytokine and chemokine responses to exertional heat stroke in mice. J Appl Physiol (1985) 2017;122:296-306.
15. He SX, Li R, Yang HH, Wang ZQ, Peng YM, Huang JH, *et al.* Optimization of a rhabdomyolysis model in mice with exertional heat stroke mouse model of EHS-rhabdomyolysis. Front Physiol 2020;11:642.
16. Costa KA, Soares AD, Wanner SP, Santos Rd, Fernandes SO, Martins Fdos S, *et al.* L-arginine supplementation prevents increases in intestinal permeability and bacterial translocation in male Swiss mice subjected to physical exercise under environmental heat stress. J Nutr 2014;144:218-23.
17. Hubbard RW. Effects of exercise in the heat on predisposition to heatstroke. Med Sci Sports 1979;11:66-71.
18. Hubbard RW, Bowers WD, Matthew WT, Curtis FC, Criss RE, Sheldon GM, *et al.* Rat model of acute heatstroke mortality. J Appl Physiol Respir Environ Exerc Physiol 1977;42:809-16.
19. Hubbard RW, Matthew WT, Criss RE, Kelly C, Sils I, Mager M, *et al.* Role of physical effort in the etiology of rat heatstroke injury and mortality. J Appl Physiol Respir Environ Exerc Physiol 1978;45:463-8.
20. Chen SH, Chang FM, Niu KC, Lin MY, Lin MT. Resuscitation from experimental heatstroke by estrogen therapy. Crit Care Med 2006;34:1113-8.
21. Sawka MN, Leon LR, Montain SJ, Sonna LA. Integrated physiological mechanisms of exercise performance, adaptation, and maladaptation to heat stress. Compr Physiol 2011;1:1883-928.
22. Casa DJ, Armstrong LE, Kenny GP, O'Connor FG, Huggins RA. Exertional heat stroke: New concepts regarding cause and care. Curr Sports Med Rep 2012;11:115-23.
23. Zeller L, Novack V, Barski L, Jotkowitz A, Almog Y. Exertional heatstroke: Clinical characteristics, diagnostic and therapeutic considerations. Eur J Intern Med 2011;22:296-9.
24. Leon LR, Bouchama A. Heat stroke. Compr Physiol 2015;5:611-47.
25. Leon LR. Hypothermia in systemic inflammation: Role of cytokines. Front Biosci 2004;9:1877-88.
26. Yeo TP. Heat stroke: A comprehensive review. AACN Clin Issues 2004;15:280-93.
27. Lin PH, Huang KH, Tian YF, Lin CH, Chao CM, Tang LY, *et al.* Exertional heat stroke on fertility, erectile function, and testicular morphology in male rats. Sci Rep 2021;11:3539.
28. González Alonso J, Crandall CG, Johnson JM. The cardiovascular challenge of exercising in the heat. J Physiol 2008;586:45-53.
29. Kiyatkin EA, Sharma HS. Permeability of the blood-brain barrier depends on brain temperature. Neuroscience 2009;161:926-39.
30. White MG, Luca LE, Nonner D, Saleh O, Hu B, Barrett EF, *et al.* Cellular mechanisms of neuronal damage from hyperthermia. Prog Brain Res 2007;162:347-71.
31. Bouchama A, Knochel JP. Heat stroke. N Engl J Med 2002;346:1978-88.
32. Liu J, Wan M, Zhang Y, Zhang S, Zhang H, Wu S. Dysfunction of Iron metabolism and iron-regulatory proteins in the rat hippocampus after heat stroke. Shock 2019;51:780-6.



Supplementary Figure 1: Representative histopathology of heart injury in exertional heat stroke (EHS). (a) Representative images of hematoxylin and eosin staining of heart from sham heat exercise mice or 3 h, 12 h, and 24 h after EHS. Scale bars = 50 μm and 20 μm. (b) Pathological scores among groups. Mean ± standard error of the mean; not significant (*P* (gn. 05), *n* = 6, Statistics by one-way analysis of variance