



Original Contribution

Moderate brain hypothermia started before resuscitation improves survival and neurobehavioral outcomes after CA/CPR in mice



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ABSTRACT

Aim of the study: No definitive experimental or clinical evidence exists whether brain hypothermia before, rather than during or after, resuscitation can reduce hypoxic-ischemic brain injury following cardiac arrest/cardiopulmonary resuscitation (CA/CPR) and improve outcomes. We examined the effects of moderate brain hypothermia before resuscitation on survival and histopathological and neurobehavioral outcomes in a mouse model.

Methods: Adult C57BL/6 male mice (age: 8–12 weeks) were subjected to 8-min CA followed by CPR. The animals were randomly divided into sham, normothermia (NT; brain temperature 37.5 °C), and extracranial hypothermia (HT; brain temperature 28–32 °C) groups. The hippocampal CA1 was assessed 7 day after resuscitation by histochemical staining. Neurobehavioral outcomes were evaluated by the Barnes maze (BMT), openfield (OFT), rotarod, and light/dark (LDT) tests. Cleaved caspase-3 and heat shock protein 60 (HSP70) levels were investigated by western blotting.

Results: The HT group exhibited higher survival and lower CA1 neuronal injury than did the NT group. HT mice showed improved spatial memory in the BMT compared with NT mice. NT mice travelled a shorter distance in the OFT and tended to spend more time in the light compartment in the LDT than did sham and HT mice. The levels of cleaved caspase-3 and HSP70 were non-significantly higher in the NT than in the sham and HT groups.

Conclusions: Moderate brain hypothermia before resuscitation improved survival and reduced histological neuronal injury, spatial memory impairment, and anxiety-like behaviours after CA/CPR in mice.

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1. Introduction

Despite many years of research on early defibrillation, cardiopulmonary resuscitation (CPR), and CPR medication, out-of-hospital cardiac arrest (OHCA) remains a significant cause of morbidity and mortality worldwide, with an estimated global annual incidence of sudden cardiac death of 4–5 million [1]. Therapeutic hypothermia (TH) is the only neuroprotective therapy shown to increase survival and decrease morbidity in adult OHCA patients [2]. Although the protective mechanisms of TH remain unclear, reports have suggested that ischemic apoptosis and reactive oxy-

gen species production during post-ischemic reperfusion are reduced by hypothermia [3].

Extensive research has focused on the implementation and effectiveness of TH as a post-resuscitation therapy. Recent data suggest that early TH application during CPR is superior to cooling initiated after resuscitation, resulting in both increased survival and improved neurologic outcomes [4]. Thus, experimental extracranial hypothermia during resuscitation after cardiac arrest (CA) reduced hippocampal injury [5], and TH during CPR significantly reduced myocardial infarction [6]. Ruttman et al. recently reported survival with favourable neurological outcomes in about a third of all accidental hypothermic non-avalanche OHCA patients [7], and several case series yielded similar survival rates [8]. Brown et al. estimated the survival rate of OHCA patients to be approximately 50% for primary hypothermic CA [9].

These findings suggest that inducing hypothermia in the brain parenchyma before, rather than during or after, resuscitation

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mitigates ischemic injury and improves outcomes after CA. Therefore, the authors hypothesized that brain cooling before resuscitation from CA/CPR achieves protective levels of brain hypothermia and results in improved neurological outcomes mimicking conventional TH after resuscitation. However, no definitive experimental or clinical evidence exists for this hypothesis. We used a well-established mouse model in which the brain and body temperatures can be independently controlled to analyse neurobehavioral and histopathological outcomes of CA/CPR started at different temperatures.

2. Materials and methods

2.1. Experimental animals

This study conformed to the National Institutes of Health guidelines for the care and use of animals in research. All experimental protocols were approved by the Chungbuk National University Institutional Animal Care and Use Committee (CBNUA-845-02) and reported in accordance with the ARRIVE guidelines. Male C57BL/6 adult mice (age: 8–12 weeks) were used. Mice were housed under a standard 12/12 h light/dark cycle and had free access to food and water.

2.2. Animal preparation

Anaesthesia was induced with 3% isoflurane and maintained with 1.5–2% isoflurane in oxygen-enriched air (fraction of inspired oxygen [FiO₂], 30%) via a face mask. Animals were endotracheally intubated using an intravenous 22G catheter connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany) set to a respiratory rate of 150 breaths/min. A PE-10 catheter was inserted into the right internal jugular vein for drug and fluid administration. Needle electrodes were placed subcutaneously on the chest for electrocardiogram (ECG) monitoring throughout the experimental procedures. Temperature probes were placed into the left ear canal and rectum. The auricular canal temperature has been shown to be similar to the brain parenchymal temperature during CA/CPR [5]. Rectal temperature was maintained near 37 °C during surgery with a heating lamp and pad.

2.3. CA and resuscitation

CA and CPR were performed as previously described with the addition of independent extracranial temperature control [5,10,11]. Briefly, mice were subjected to 8-min CA, which induced global cerebral ischemia and caused selective, delayed cell death of hippocampal CA1 neurons and neurobehavioral abnormalities in adult C57BL/6 mice [10,12], using active cooling or warming via a separate temperature control system to keep the brain hypothermic or normothermic before resuscitation. The animals were randomly divided into 3 groups. In the hypothermic brain group (HT), rapid cooling was started 15 min before CA using a polyethylene tubing coil connected to a temperature-controlled water bath (4.1 ± 2.3 °C) and a pump. The pericranial temperature was maintained at 31.8 ± 1.7 °C before resuscitation. In the normothermic brain group (NT), both the brain and body temperatures were maintained within the normal range throughout CA/CPR and early recovery (Fig. 1). CA was induced by injection of 50 µL of 0.5 M KCl via the jugular catheter, and confirmed by the appearance of asystole on the ECG and spontaneous breathing cessation. The endotracheal tube was disconnected from the ventilator, and anaesthesia was stopped. CPR was begun 8 min after CA induction by slow injection of 0.5–1.0 mL of epinephrine (16 µL of epinephrine per mL of 0.9% saline), chest compressions (approximately 300 per min), and ventilation with 100% oxygen (200 breaths/min). As soon as return to spontaneous circulation (ROSC) was achieved, defined as ECG activity with visible cardiac contractions, chest compressions

were stopped. Five minute post-resuscitation, the FiO₂ was decreased to 50%. When the spontaneous breathing rate reached 60 breaths/min, the endotracheal tube was removed. The animal was then placed into its home cage for complete recovery. Mice in the sham group (n = 8) underwent all procedures except for CA induction, cardiac compressions, and epinephrine injection.

2.4. Measurements and outcomes

2.4.1. Health assessment score

Mice were weighed and their health assessed daily for 3 days after CA/CPR. The graded scoring systems ranged from 0 to 2, 0 to 3, or 0 to 5 depending on the behaviour assessed, with 0 indicating no deficit and the upper limit indicating the most impairment. The behaviours assessed included consciousness (0–3), interaction (0–2), ability to grab a wire top (0–2), motor function (0–5), and activity (0–2) [10]. The individual category scores were summated to generate an overall health assessment score.

2.4.2. Survival

Survival was monitored for 7 days after weaning of mechanical ventilation.

2.4.3. Neurobehavioral outcomes

Behavioural testing was carried out between the hours of 8 a.m. and 7 p.m. and performed by a blinded observer.

2.4.3.1. Barnes maze test. The Barnes maze test (BMT) was conducted as previously described with minor modifications [13]. The maze consisted of a white acrylic circular platform (91 cm in diameter) with 20 equally spaced holes and a black acrylic escape box (20 × 5 × 6 cm) along the perimeter. The maze was surrounded by 4 spatial cues at the height of the maze.

2.4.3.1.1. Acquisition trials. Each mouse was subjected to 4 acquisition trials per day for 3 days with an inter-trial interval of 10–15 min. Immediately prior to the first trial, the mouse was placed in the middle of the maze in a black starting cylinder (10 cm in diameter), and a buzzer (80–90 dB) was turned on. After 10 s, the chamber was lifted, and the mouse was pre-trained to enter the escape box by being guided to, and remaining in it for 2 min. Each acquisition trial began as the pre-training trial but the mouse was free to explore the maze. The trial ended when the mouse entered the goal tunnel or after 3 min had elapsed. Immediately after the mouse entering the tunnel, the buzzer was turned off and the mouse was allowed to stay in the tunnel for 1 min. After each trial, the maze was cleaned with 70% alcohol and rotated to eliminate intra-maze cues. The trials were recorded using a video tracking system (SMART; Panlab, Barcelona, Spain).

2.4.3.1.2. Probe trial. During a 90-s probe trial, conducted on POD 7, the escape tunnel leading to the target box was closed. The mice were allowed to explore the maze. Target and adjacent hole visits, path length, and latency to reach the target hole for the first time were recorded.

2.4.3.2. Open-field test. Locomotor activity was measured in a white open-top acrylic box (40 × 40 × 40 cm) with an illumination intensity of 20 lx at the box floor level for 30 min. The activity was automatically recorded using a video tracking system with the SMART 3.0 software. Distance travelled, time spent in the centre (25%) and area margins, and mean walking speed were measured.

2.4.3.3. Light/dark preference test. The apparatus consisted of black and white compartments (20 × 40 × 40 cm) separated by a connecting gate (5 × 8 cm). Each animal was individually placed in the centre of the bright compartment (facing away from the door), and the following parameters were measured for 5 min: latency of the initial movement from the light to dark area (transition), total number of transitions, and total time spent in the light area.

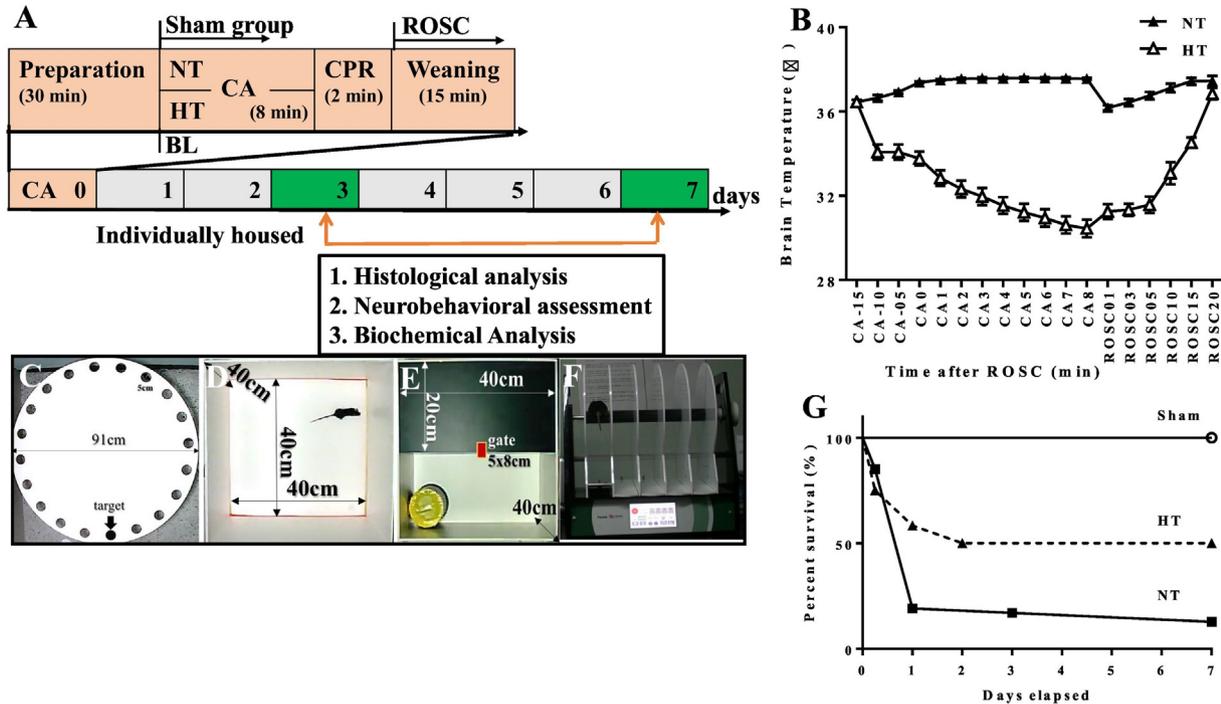


Fig. 1. Experimental design & Kaplan–Meier survival analysis. C57BL6 adult mice were subjected to extracranial hypothermia (HT) or normothermia (NT) before resuscitation. (A) Experimental timelines illustrate the CA/CPR process and the timing of histological, behavioural, and biochemical analyses. (B) Auricular canal temperature was measured as a proxy for brain parenchymal temperature throughout the CA/CPR sequence and plotted by time (e.g., ROSC10, 10 min after ROSC). (C–F) Neurobehavioral tests included the Barnes maze (C), open field (D), light/dark (E), and rotarod (F) tests. (G) Statistically significant improvement in survival was seen in the extracranial hypothermia (HT) group compared with that in the extracranial normothermia (NT) group. The log-rank statistic was 19.05 ($P < 0.0001$). BL, baseline; CA, cardiac arrest; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation.

2.4.3.4. Rotarod testing. Animals were tested on a rotarod treadmill (LE 8500, Panlab SL, Barcelona, Spain) with a diameter of 7 cm elevated 50 cm above the bottom of the apparatus and attached to a motor to control speed. Mice were placed on the rotarod at a starting speed of 4 rpm and acceleration of 0.5 rpm/s. The mice were subjected to 3 trials. In each trial, latency to fall and speed at fall were measured. Animals rested a minimum of 1 h between trials to avoid fatigue. The average latency to fall for the 3 trials was used as the measured parameter.

2.4.4. Histopathological outcomes

Seven days after CA/CPR, animals were anesthetized with 4% isoflurane and transcardially perfused with 0.9% cold saline followed by 10% formalin. The brains were removed, post-fixed with 10% formalin, and embedded in paraffin. Six-micrometer coronal sections were serially cut and stained with haematoxylin and eosin (H&E). The hippocampal CA1 region was analysed at 3 levels, 100 μm apart, beginning at -1.5 mm from bregma. Nonviable neurons were determined by hyper eosinophilic cytoplasm and dark pyknotic nuclei. The percentage of nonviable neurons was calculated for each brain region (average of 3 levels per section) as previously described [14]. All tissue specimens were assessed by a certified pathologist who was blinded to tissue information.

2.4.5. Western blotting

Bilateral hippocampus ($n = 4$ in each group) were dissected and sonicated for 40 s in ice-cold RIPA lysis buffer. The lysate was centrifuged at $12,000 \times g$ for 10 min at 4°C , and the supernatant was collected. Protein concentrations were determined using the Bradford method. Mouse anti-heat shock protein 70 (HSP70; ab5439, AbCam) and rabbit anti-caspase 3 (AB3623, Chemicon) were used as primary antibodies. The secondary antibody was a rabbit anti-alpha-tubulin HRP-linked antibody (AbC-2001, AbClon). The target

protein bands were quantified using a WesTM automated western blotting system (ProteinSimple, San Jose, CA, USA).

2.5. Statistical analysis

All data are presented as mean \pm SEM. Statistical evaluation of the data was performed using one-way ANOVA, parametric Student's t -test, or the nonparametric Wilcoxon-Mann Whitney two-sample rank test as appropriate. $P < 0.05$ was considered statistically significant. Data were analysed using the PASW/SPSSTM software, version 18 (IBM Inc., Chicago, USA) and GraphPad Prism 5.01 (GraphPad Prism Software, San Diego, CA, USA).

3. Results

3.1. Baseline characteristics and physiological parameters post-CPR

Mouse characteristics pre-arrest, intra-arrest, and post-resuscitation are shown in Table 1. There were no significant differences among the groups in baseline body weight, induction time, CPR duration, epinephrine dose, or time to ROSC. The NT group showed a significantly reduced body weight compared with the sham and HT groups on postoperative days (PODs) 1 and 2. The mean health assessment score in the NT group was also significantly different compared with that in the control and HT groups on PODs 1 and 2. There was no significant difference among the groups in the blood chemistry profile on POD 7.

3.2. Survival rates

Seven-day survival after CA was significantly decreased ($P < 0.001$) in the NT group (12.8%) compared to that in the sham (100%) and HT (50%) groups (Fig. 1G).

Table 1
Baseline characteristics and physiological parameters.

	Sham (n = 8)	NT (n = 10)	HT (n = 11)
Age (weeks)	9.1 ± 0.4	9.9 ± 1.4	9.8 ± 2.1
Body weight (baseline, g)	25.2 ± 2.7	23.4 ± 1.2	23.9 ± 1.7
Preparation time (min)	22.0 ± 1.7	31.0 ± 5.2	24.7 ± 4.0
CPR time (s)	–	137.8 ± 51.5	119.8 ± 21.0
Epinephrine dose (16 µg/mL)	–	0.7 ± 0.2	0.8 ± 0.2
ROSC rate (%)	–	100	90.9
Brain temperature (°C)	35.3 ± 1.3	37.4 ± 0.2	33.4 ± 0.8**
Body temperature (°C)	35.6 ± 1.3	36.6 ± 0.2	36.1 ± 1.3
Weight change (g)			
POD 1	23.9 ± 0.7	20.5 ± 0.2*	22.4 ± 1.1
POD 2	24.6 ± 0.8	20.8 ± 0.6*	22.7 ± 1.1
POD 3	25.0 ± 0.8	21.9 ± 0.8	23.8 ± 1.0
POD 7	24.1 ± 0.8	21.9 ± 0.5	23.5 ± 0.9
Health assessment score in surviving animals			
POD 1	0.4 ± 0.2	5.7 ± 1.5*	3.5 ± 0.6
POD 2	0.1 ± 0.1	3.0 ± 1.1*	2.2 ± 0.3
POD 3	0.0 ± 0.0	1.8 ± 0.9	0.5 ± 0.2
Blood chemistry data, POD 7			
Glucose (mg/dL)	266.0 ± 4.7	224.7 ± 16.2	230.0 ± 6.0
BUN (mg/dL)	20.5 ± 2.4	36.0 ± 15.5	14.5 ± 1.5
Creatinine (mg/dL)	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.1
Troponin I (ng/mL)	0.9 ± 0.2	1.3 ± 0.4	5.2 ± 2.7

Body temperature represents the average rectal temperature during surgery. BUN, blood urea nitrogen; CA, cardiac arrest; CPR, cardiopulmonary resuscitation; HT, extracranial hypothermia before resuscitation; NT, normothermia; ROSC, return of spontaneous circulation; POD, postoperative day.

Asterisks indicate the level of significance of the difference between the NT and HT/ Sham groups.

* P < 0.05.

** P < 0.01.

3.3. HT reduces spatial memory deficits in the BMT

During the acquisition phase, there was a significant difference in latency to enter the target hole (total latency) and distance trav-

elled in the NT group compared with those in the sham or HT mice. The path length and total latency of the HT mice significantly decreased during training (Fig. 2A and B). In the probe trial on day 5 (short-term retention phase), there were statistically significant differences in mean primary latency and path lengths to the target and adjacent holes between the NT and HT group, indicating that brain hypothermia before resuscitation reduced CA/CPR-induced spatial memory deficits (Fig. 2C and D).

3.4. HT normalises global activity in the open-field test

NT mice showed a decrease in total distance travelled compared with that in the sham and HT mice. There were no significant differences in distance travelled in the centre among the 3 groups (Fig. 3A–C).

3.5. Anxiety-like behaviour in the light/dark preference test

NT mice tended to spend more time in the light compartment, showed increased initial transition latency, and made fewer transitions compared with sham or HT mice. However, the difference was not statistically significant (P = 0.32, Fig. 3D–F).

3.6. Rotarod performance

There was no significant difference among the sham, NT, and HT groups in rotarod test performance (Fig. 3G–H).

3.7. Histopathologic outcomes

Selective delayed cell death of hippocampal CA1 neurons was detected by H&E staining at 7 days following resuscitation. Neuronal damage was significantly reduced in the HT group (35.0 ± 3.4%) compared to that in the NT group (66.6 ± 2.8%; n = 5, P < 0.05; Fig. 4).

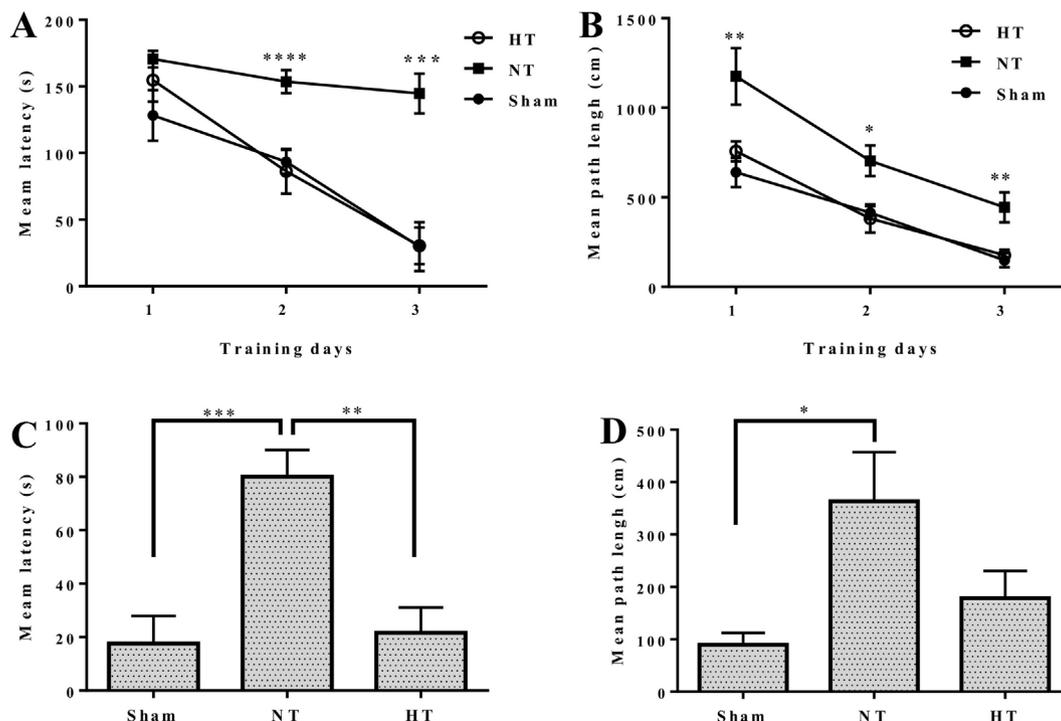


Fig. 2. Spatial learning and memory in animals following hypothermic (HT) or normothermic (NT) cardiac arrest and resuscitation. Latencies (A, C) and distances (B, D) to find the escape box in the Barnes maze test were measured. (A, B) acquisition trials; (C, D) day 5 retention trials. Asterisks indicate the level of significance of the difference between the HT and NT group: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (n = 8–10 per group; bars and whiskers show mean ± S.E.M.)

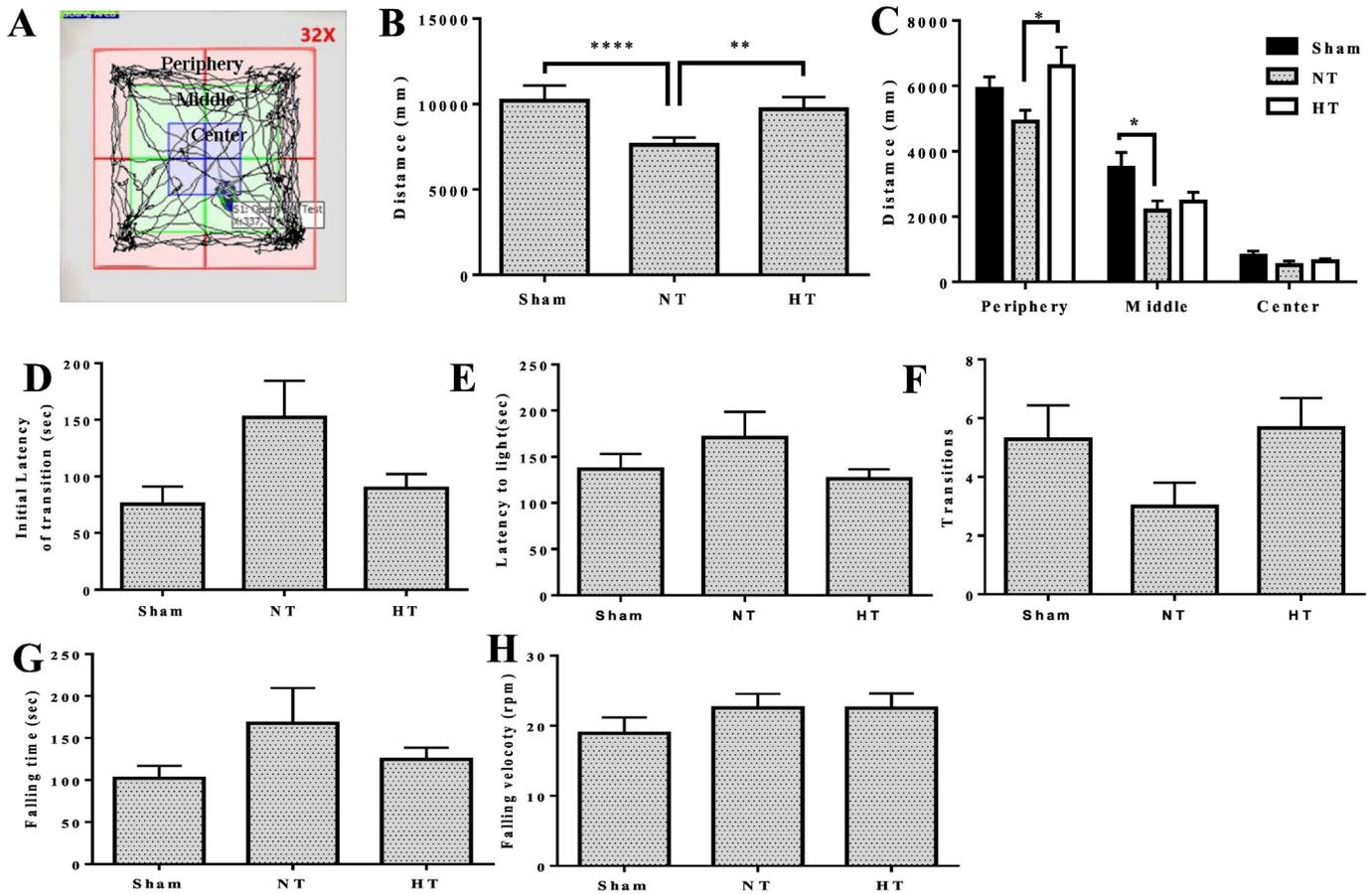


Fig. 3. Locomotor activity, anxiety-like behaviour, and motor performance of animals following hypothermic (HT) or normothermic (NT) cardiac arrest and resuscitation. *Locomotor activity:* (A) representative trace of the open field experiment. (B) Total distance travelled. (C) Distances travelled in the centre, middle, and periphery of the arena. *Anxiety-like behaviour* (D–F) there were no significant differences in initial transition latency, time spent in the lighted compartment, or number of transitions in the light-dark test. *Rota-rod test:* (G) time to fall. (H) Speed at fall. Data are expressed as means \pm S.E.M. ($n = 6-8$ per group) and analysed by one-way analysis of variance followed by Tukey's post hoc test. Asterisks indicate the level of significance of the difference between the indicated groups: *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ ($n = 6-8$ per group; bars and whiskers show mean \pm S.E.M.).

3.8. Cleaved caspase-3 and HSP70 levels

To investigate apoptotic and oxidative stress injury in hippocampal tissues, the protein levels of cleaved caspase-3 and HSP70 were investigated by western blotting at 7 days after CPR. The NT group showed increased mean levels of cleaved caspase-3 and HSP70 compared to those in the control and HT groups; however, the differences were not significant (caspase-3, $P = 0.236$; HSP70, $P = 0.159$; Fig. 5).

4. Discussion

The main finding of this study is that moderate brain hypothermia started before resuscitation improved survival after CA/CPR in mice. In addition, HT reduced CA/CPR-induced cognitive deficits. These results indicate that the brain parenchymal temperature before resuscitation may be a crucial determinant of CA/CPR outcomes.

Numerous studies have shown that TH protects from ischemia-reperfusion injury and that post-CPR TH improves CA patient outcomes [3,15,16]. The protective effects of TH against brain injury may be attributable to a reduction in brain metabolism, inhibition of excitatory amino acid release and oxidative stress, attenuation of the immune/inflammatory response, and modification of cell death signalling pathways [5,17,18]. In traditional TH, the brain temperature is lowered through body cooling after resuscitation.

Recent studies have shown that early application of TH during resuscitation can also be beneficial [4,5]. In contrast, our study investigated the effects of selective cerebral cooling started before resuscitation. Brain hypothermia before resuscitation may mimic accidental hypothermic CA and exert protective effects against global hypoxic brain injury.

Accidental hypothermia is defined as a trunk or core temperature of $<35^\circ\text{C}$ [19]. Brain oxygen consumption decreases by $\sim 6\%$ per 1°C fall in core temperature [20], resulting in improved tolerance for reduced blood flow. Neurologically intact (cerebral performance scale 1–2) CA survivors with the longest no-flow time [21], manual [22] and mechanical [23] CPR, total resuscitation [24], and intermittent CPR [25], as well as the lowest survived core temperature [26] and persistent ventricular fibrillation [27], all suffered accidental hypothermic CA. Survival without neurologic impairment in these cases may have been possible because of decreased requirement for cerebral oxygen at reduced temperatures. We used a CA mouse model to show that moderate brain hypothermia during CA/CPR improved survival and overall health recovery.

Cognitive deficits are a common problem in CA survivors. The hippocampus is involved in learning and memory and is susceptible to hypoxic-ischemic injury after CA [28,29]. We used the BMT to assess learning and memory. Spatial memory is generally believed to be dependent on an intact hippocampus, and cued learning is partially dependent on the hippocampus and on other structures such as the superior colliculus and dorsal striatum [29]. NT mice displayed behavioural deficits in hippocampus-dependent learning

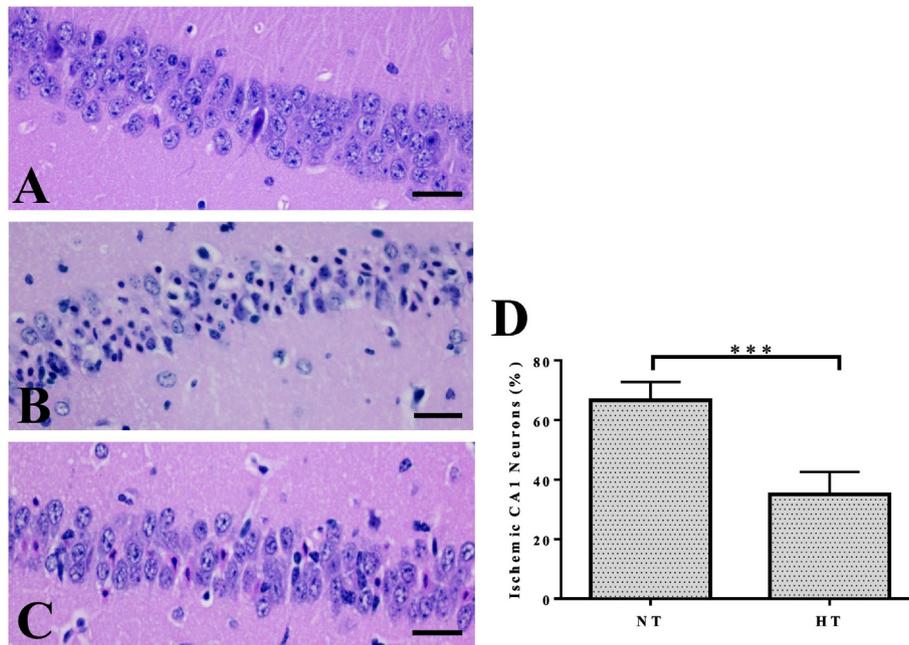


Fig. 4. Neuronal death in the hippocampal CA1 following 8-min cardiac arrest (CA). Representative photomicrographs of hippocampal CA1 neurons in adult mice following sham (A), normothermic (B), or extracranial hypothermic (C) CA and cardiopulmonary resuscitation (CPR). Hippocampal sections were stained with haematoxylin-eosin 7 days after the CA procedure. (D) Quantification of ischemic neurons in the CA1 region of the hippocampus 7 days after CA/CPR. Significantly fewer injured neurons were present in HT mice than in NT mice; ***, $P < 0.001$. Magnification; $400\times$, scale bars = $20\ \mu\text{m}$.

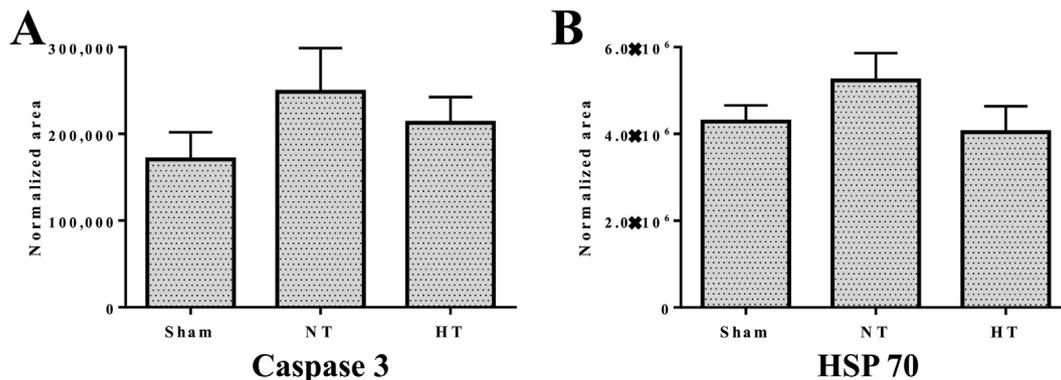


Fig. 5. Automated capillary western blotting. (A) Protein levels of cleaved caspase-3, heat shock protein 70 (HSP70), and α -tubulin in hippocampal brain tissues of adult mice 7 days after sham, normothermic (NT), or extracranial hypothermic (HT) cardiac arrest and resuscitation. (B, C) Graphs show densitometric quantification of the western blotting bands. The cleaved caspase-3 and HSP70 levels are normalized by that of α -tubulin in each sample ($n = 4$ per group).

paradigms, suggesting that hippocampal function was compromised after CA. This result is in agreement with reports of post-CA spatial memory deficits in both rats and mice [17,29]. However, we showed for the first time that HT begun before resuscitation ameliorated hippocampus-dependent spatial memory dysfunction in a CA model. Reduced loss of hippocampal neurons may underlie the HT-associated spatial memory improvement.

CA is associated with high rates of depression, anxiety, and post-traumatic stress disorder [29]. We showed increased anxiety-like behaviour in mice following normothermic CA, as revealed by reductions in total distance travelled in the open-field test and in time spent in the light compartment in the light/dark preference test. HT during CA/CPR reduced the anxiety-like behaviours. There is greater variability in neurobehavioral changes in the NT group, such as in BMT, LDT, and Rota rod. Notably, 8-min CA in NT situations may contribute to greater individual variability in global cerebral ischemia-related injury. HT may mitigate global cerebral ischemia-related injury with reduced individual variability.

Normothermic CA induced a loss of hippocampal neurons, which was reduced by HT begun before resuscitation. In addition,

normothermic CA increased, albeit non-significantly, the hippocampal levels of cleaved caspase-3 and HSP70 compared to those in the sham and HT groups at 7 days after CA/CPR. Multiple factors influence CA-induced neuronal death, including excitatory synaptic input, oxidative stress, inflammation, mitochondrial dysfunction, and disrupted intracellular Ca^{2+} homeostasis. These factors activate JNK, cytochrome c, and caspases-3, -8, and -9 in hippocampal CA1 pyramidal neurons [30]. Increased caspase-3 immunoreactivity after normothermic CA and its reduction by HT may indicate that HT ameliorates caspase-3-induced apoptosis after CA/CPR [31–34]. Whereas the previous studies investigated caspase-3 expression within 72 h after CA/CPR, we did so at 7 day post-CA, which may explain why our results were not statistically significant.

HSP70 is a ubiquitous protein induced by exposure to stress conditions such as infection, inflammation, toxins, and hypoxia. HSP70 plays multiple roles in cellular homeostasis as an intracellular chaperone [35]. A recent clinical study in comatose post-CA patients showed that HSP70 levels were decreased significantly in survivors, but not in non-survivors, and predicted 30-day mor-

tality regardless of age, sex, complications, and the acute physiology and chronic health evaluation score [36]. Consistent with this observation, we found low HSP70 expression in the HT group as compared with the NT group. This reduction was likely associated with decreased necrotic cell death caused by severe hypoxia-ischemia followed by reperfusion.

This study has several limitations. First, no post-resuscitation HT group was included. Because the brain temperature in our test group remained reduced throughout the CA-CPR-weaning sequence, we cannot rule out a post-resuscitation HT effect. Further study will be needed to clarify the difference in neuroprotective effects between pre- and post-resuscitation HT on CA outcomes. Second, only moderate brain hypothermia was applied. Mild or severe hypothermia before resuscitation remains to be studied. Third, small animals were used, and CA was induced by KCl. Thus, our findings cannot be extrapolated to female mice, larger animals or humans, or to CA caused by other aetiologies. Fourth, we analysed protein expression and histological outcomes at 7 days after CA/CPR. Earlier post-CA/PCR time points need to be examined.

5. Conclusions

Moderate brain hypothermia before resuscitation improved survival and reduced CA1 neuronal injury, spatial memory impairment, and anxiety-like behaviours after CA/CPR in mice.

Conflicts of interest

The authors have no conflict of interest to report.

Acknowledgments

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References

- [1] Chugh SS, Reinier K, Teodorescu C, et al. Epidemiology of sudden cardiac death: clinical and research implications. *Prog Cardiovasc Dis* 2008;51:213–28.
- [2] Mangus DB, Huang L, Applegate PM, Gatling JW, Zhang J, Applegate II RL. A systematic review of neuroprotective strategies after cardiac arrest: from bench to bedside (part I - protection via specific pathways). *Med Gas Res* 2014;4:9.
- [3] Karnatovskaia LV, Wartenberg KE, Freeman WD. Therapeutic hypothermia for neuroprotection: history, mechanisms, risks, and clinical applications. *Neurohospitalist* 2014;4:153–63.
- [4] Scolletta S, Taccone FS, Nordberg P, Donadello K, Vincent JL, Castren M. Intra-arrest hypothermia during cardiac arrest: a systematic review. *Crit Care* 2012;16:R41.
- [5] Hutchens MP, Fujiyoshi T, Koerner IP, Herson PS. Extracranial hypothermia during cardiac arrest and cardiopulmonary resuscitation is neuroprotective in vivo. *Ther Hypothermia Temp Manag* 2014;4:79–87.
- [6] Yannopoulos D, Zviman M, Castro V, et al. Intra-cardiopulmonary resuscitation hypothermia with and without volume loading in an ischemic model of cardiac arrest. *Circulation* 2009;120:1426–35.
- [7] Ruttman E, Dietl M, Kastenberger T, et al. Characteristics and outcome of patients with hypothermic out-of-hospital cardiac arrest: experience from a European trauma center. *Resuscitation* 2017;120:57–62.
- [8] Khorsandi M, Dougherty S, Young N, et al. Extracorporeal life support for refractory cardiac arrest from accidental hypothermia: a 10-year experience in Edinburgh. *J Emerg Med* 2017;52:160–8.
- [9] Brown DJ, Brugger H, Boyd J, Paal P. Accidental hypothermia. *N Engl J Med* 2012;367:1930–8.
- [10] Deng G, Yonchek JC, Quillinan N, et al. A novel mouse model of pediatric cardiac arrest and cardiopulmonary resuscitation reveals age-dependent neuronal sensitivities to ischemic injury. *J Neurosci Methods* 2014;222:34–41.
- [11] Kida K, Shirozu K, Yu B, Mandeville JB, Bloch KD, Ichinose F. Beneficial effects of nitric oxide on outcomes after cardiac arrest and cardiopulmonary resuscitation in hypothermia-treated mice. *Anesthesiology* 2014;120:880–9.
- [12] Kofler J, Hattori K, Sawada M, et al. Histopathological and behavioral characterization of a novel model of cardiac arrest and cardiopulmonary resuscitation in mice. *J Neurosci Methods* 2004;136:33–44.
- [13] Patil SS, Sunyer B, Hoger H, Lubec G. Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze. *Behav Brain Res* 2009;198:58–68.
- [14] Allen D, Nakayama S, Kuroiwa M, et al. SK2 channels are neuroprotective for ischemia-induced neuronal cell death. *J Cereb Blood Flow Metab* 2011;31:2302–12.
- [15] Benson-Cooper KA. Therapeutic hypothermia is independently associated with favourable outcome after resuscitation from out-of-hospital cardiac arrest: a retrospective, observational cohort study. *N Z Med J* 2015;128:33–7.
- [16] Wang XP, Lin QM, Zhao S, Lin SR, Chen F. Therapeutic benefits of mild hypothermia in patients successfully resuscitated from cardiac arrest: a meta-analysis. *World J Emerg Med* 2013;4:260–5.
- [17] Kurinami H, Shimamura M, Ma T, et al. Prohibitin viral gene transfer protects hippocampal CA1 neurons from ischemia and ameliorates postischemic hippocampal dysfunction. *Stroke* 2014;45:1131–8.
- [18] Cronberg T, Lilja G, Rundgren M, Friberg H, Widner H. Long-term neurological outcome after cardiac arrest and therapeutic hypothermia. *Resuscitation* 2009;80:1119–23.
- [19] Deslarzes T, Rousson V, Yersin B, Durrer B, Pasquier M. An evaluation of the Swiss staging model for hypothermia using case reports from the literature. *Scand J Trauma Resusc Emerg Med* 2016;24:16.
- [20] Truhlar A, Deakin CD, Soar J, et al. European Resuscitation Council Guidelines for Resuscitation 2015: Section 4. Cardiac arrest in special circumstances. *Resuscitation* 2015;95:148–201.
- [21] Althaus U, Aeberhard P, Schupbach P, Nachbar BH, Muhlemann W. Management of profound accidental hypothermia with cardiorespiratory arrest. *Ann Surg* 1982;195:492–5.
- [22] Lexow K. Severe accidental hypothermia: survival after 6 hours 30 minutes of cardiopulmonary resuscitation. *Arctic Med Res* 1991;50(Suppl. 6):112–4.
- [23] Piacentini A, Volonte M, Rigamonti M, Guastella E, Landriscina M. Successful prolonged mechanical CPR in a severely poisoned hypothermic patient: a case report. *Case Rep Emerg Med* 2012;2012:381798.
- [24] Meyer M, Pelurson N, Khabiri E, Siegenthaler N, Walpoth BH. Sequela-free long-term survival of a 65-year-old woman after 8 hours and 40 minutes of cardiac arrest from deep accidental hypothermia. *J Thorac Cardiovasc Surg* 2014;147:e1–2.
- [25] Boue Y, Payen JF, Torres JP, Blancher M, Bouzat P. Full neurologic recovery after prolonged avalanche burial and cardiac arrest. *High Alt Med Biol* 2014;15:522–3.
- [26] Gilbert M, Busund R, Skagseth A, Nilsen PA, Solbo JP. Resuscitation from accidental hypothermia of 13.7 degrees C with circulatory arrest. *Lancet* 2000;355:375–6.
- [27] Nordberg P, Ivert T, Dalen M, Forsberg S, Hedman A. Surviving two hours of ventricular fibrillation in accidental hypothermia. *Prehosp Emerg Care* 2014;18:446–9.
- [28] Fugate JE, Moore SA, Knopman DS, et al. Cognitive outcomes of patients undergoing therapeutic hypothermia after cardiac arrest. *Neurology* 2013;81:40–5.
- [29] Wilder Schaaf KP, Artman LK, Peberdy MA, et al. Anxiety, depression, and PTSD following cardiac arrest: a systematic review of the literature. *Resuscitation* 2013;84:873–7.
- [30] Cui D, Shang H, Zhang X, Jiang W, Jia X. Cardiac arrest triggers hippocampal neuronal death through autophagic and apoptotic pathways. *Sci Rep* 2016;6:27642.
- [31] Suh GJ, Kwon WY, Kim KS, et al. Prolonged therapeutic hypothermia is more effective in attenuating brain apoptosis in a Swine cardiac arrest model. *Crit Care Med* 2014;42:e132–42.
- [32] Lee JH, Kim K, Jo YH, et al. Effect of valproic acid on survival and neurologic outcomes in an asphyxial cardiac arrest model of rats. *Resuscitation* 2013;84:1443–9.
- [33] Qin J, Wang P, Li Y, et al. Activation of Sigma-1 receptor by cutamesine attenuates neuronal apoptosis by inhibiting endoplasmic reticulum stress and mitochondrial dysfunction in a rat model of asphyxia cardiac arrest. *Shock* 2019;51:105–13.
- [34] Zhou T, Lin H, Jiang L, et al. Mild hypothermia protects hippocampal neurons from oxygen-glucose deprivation injury through inhibiting caspase-3 activation. *Cryobiology* 2018;80:55–61.
- [35] Karlin S, Brocchieri L. Heat shock protein 70 family: multiple sequence comparisons, function, and evolution. *J Mol Evol* 1998;47:565–77.
- [36] Jenei ZM, Szeplaki G, Merkely B, Karadi I, Zima E, Prohaszka Z. Persistently elevated extracellular HSP70 (HSPA1A) level as an independent prognostic marker in post-cardiac-arrest patients. *Cell Stress Chaperones* 2013;18:447–54.