

Effects of Heme Oxygenase 1 on Brain Edema and Neurologic Outcome after Cardiopulmonary Resuscitation in Rats

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Background: Heme oxygenase 1 (HO-1) has been shown to attenuate neuronal injury. Therefore, the authors examined whether HO-1 would reduce the brain damage caused by cardiac arrest.

Methods: Rats anesthetized with halothane were subjected to 8 min of cardiac arrest by asphyxia without any pretreatment (control group) or pretreated with an inducer of HO-1 (hemin group) or with an inhibitor of HO-1 (tin protoporphyrin group). Then, the animals were resuscitated with a standardized method. Brain water content (1 and 6 h after resuscitation), neurologic deficit score, viable neuronal counts, and caspase-3 immunostaining in the hippocampus (4 and 14 days) were evaluated.

Results: Water content in the hemin group was significantly reduced (mean \pm SD: $83.1 \pm 1.9\%$ for 1 h after resuscitation; $P = 0.03$) and significantly greater in the tin protoporphyrin group ($91.1 \pm 2.0\%$ for 1 h after resuscitation; $P = 0.035$) when compared with the control group ($88.2 \pm 2.4\%$). Water content of the cortex was nearly the same as that of the hippocampus. Neurologic deficit scores and neuronal survival were significantly better in the hemin group than in the control group on the 4th and 14th days. In rats that survived for 4 days, the amount of caspase 3–positive neurons was 27 ± 7 in the control group, whereas the value was 14 ± 6 in the hemin group ($P < 0.05$).

Conclusions: In rats resuscitated from cardiac arrest, induction of HO-1 by hemin reduced brain edema, improved neurologic outcome, and protected neurons against apoptosis.

CARDIAC arrest (CA) and resuscitation produce global ischemia and reperfusion damage to the brain, which lead to high mortality and delayed neuronal death.¹ Although attempts at restoration of spontaneous circulation (ROSC) have achieved great advancement, full recovery without neurologic dysfunction after CA is still a relatively rare event.²

Till now, the pathophysiology of global cerebral ischemia and reperfusion injury elicited by CA is still not clear. Brain edema has been documented in CA patients by computerized tomography or magnetic resonance imaging scanning and predicts a poor neurologic outcome.^{3,4} In addition, neuronal apoptosis and necrosis

occur over hours to days after ROSC from CA.⁵ Inhibition of these may reduce brain damage and improve function and survival.

Heme oxygenase 1 (HO-1) is an inducible form of heme oxygenase that degrades heme into carbon monoxide, iron, and bilirubin. Recent data have demonstrated potent neuroprotective effects of HO-1 in neuronal cell cultures,⁶ permanent cerebral ischemia,⁷ and organ transplantation.⁸ In addition, HO-1 has been shown to have antiinflammatory, antiapoptotic, and antiproliferative effects.⁹ However, the studies of HO-1 on neurologic outcome after CA and resuscitation are few. In this study, we investigated the effects of HO-1 on brain edema, neurologic outcome, and neuronal apoptosis after cerebral ischemia and reperfusion induced by CA and resuscitation.

Materials and Methods

Animal Groups

This study was approved by the Institutional Animal Care and Use Committee of Harbin Medical University in Harbin, Heilongjiang, China, and followed the national guidelines for the treatment of animals. Male Sprague-Dawley rats, weighing 300–350 g, were randomly assigned to four groups:

Sham group: Rats were anesthetized with halothane and oxygen (30%). The femoral artery and vein were cannulated with a polyethylene catheter. They were mechanically ventilated without CA procedures.

Control group: Under the same anesthetic conditions and preparations as the sham group, rats were subjected to asphyxial CA of 8 min, reversed by standard external cardiopulmonary resuscitation (CPR) followed by mechanical ventilation until 1 h after ROSC.

Hemin group: Rats received the same injury and the same resuscitation as described in the control group, but HO-1 was induced by intraperitoneal injection of 15 mg/kg hemin (Sigma, St. Louis, MO) 12 h before the CA.

SnPP group: Rats were treated as the control group, but HO-1 was inhibited by intraperitoneal injection of 30 μ mol/kg tin protoporphyrin IX (SnPP; Alexis, Lausen, Switzerland) 1 h before the CA.

The whole experiment was divided into two parts. In part 1, brains were harvested from 40 rats at 1 and 6 h after ROSC in sham, control, hemin, and SnPP groups

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(n = 5 for each time point). Brain water content measurements were performed in a temperature- and humidity-controlled room ($22^{\circ} \pm 3^{\circ}\text{C}$ and $65 \pm 5\%$). In part 2, 60 rats were divided randomly into four groups (sham group, n = 5; control group, n = 20; hemin group, n = 15; SnPP group, n = 20). On the 4th day after ROSC, we randomly chose and killed 5, 6, and 4 rats that survived in the control, hemin, and SnPP groups, respectively. Then, their brains were served for determination of the histopathologic changes. On the 14th day, the brains of all of the rats that survived were also reserved for the histopathologic examinations.

Animal Preparation, CA, and Resuscitation

The rats had free access to food and water before the experiment. Anesthesia was induced with 3–5% halothane and oxygen (30%), which insufflated into a transparent chamber. The animal was immediately intubated orotracheally with a 14-gauge plastic catheter by direct laryngoscope and connected to a ventilator (Inspira ASV; Harvard Apparatus Inc., Holliston, MA). Tidal volume was adjusted to control arterial pH at 7.35–7.45, arterial carbon dioxide pressure at 35–45 mmHg, and arterial oxygen pressure greater than 90 mmHg. The rectal temperature was maintained at $37.5^{\circ} \pm 0.5^{\circ}\text{C}$ by a heating pad and a small warming lamp placed above the animal's head and body until 1 h after ROSC. A muscle relaxant (1.0 mg/kg vecuronium) was administered intravenously and as needed thereafter. After the surgical procedures, halothane was adjusted to as low as possible to prevent movement and maintain mean arterial pressure.

After baseline measurements and before the injury, halothane was washed out with 100% oxygen for 3 min followed by room air for 2 min with the tidal volume and frequency unchanged. CA was induced in all CA groups (control, hemin, and SnPP groups) by stopping mechanical ventilation and clamping the endotracheal tube at the end of exhalation for 8 min. CA was defined as a systolic blood pressure of 25 mmHg or less without arterial fluctuations. After 8 min of apnea and airway obstruction, CPR was initiated by unclamping the endotracheal tube and restarting mechanical ventilation with 100% oxygen and unchanged tidal volume at a frequency of 60 breaths/min. The resuscitation was continued by giving 0.01 mg/kg intravenous epinephrine, followed immediately by flushing the catheter with 1 mEq/kg intravenous sodium bicarbonate and applying sternal compressions at a rate of 200 per min until ROSC. Achievement of ROSC was defined when a spontaneous systolic blood pressure of 60 mmHg or greater was obtained. If CPR exceeded 2 min, the rat was excluded from the experiments. All of the physiology data were monitored and recorded using the BIOPAC MP150 physiometer (BIOPAC Systems Inc., Santa Barbara, CA).

After ROSC, rats were mechanically ventilated with 100% oxygen and invasively monitored for 1 h, maintain-

ing the rectal temperature as described two paragraphs previously. Blood samples were drawn for blood gases, glucose, and lactate measurements at baseline, 30 min after ROSC, and 60 min after ROSC, and appropriate ventilator adjustments were made. After a recovery period of 1 h, vascular catheters were removed and surgical wounds were sutured. All wounds were infiltrated with 0.25% bupivacaine (total dose 0.5 mg). Rats were then weaned from the ventilator, extubated, and placed in a chamber with 50% oxygen for another 1 h, after which they were returned to their cages with easily accessible food and water. Rats were given 6 ml isotonic saline subcutaneously 2 h after ROSC. Thereafter, rats were given $20 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ isotonic saline subcutaneously with 5% dextrose until they could eat and drink without assistance.

Brain Water Content Determined by Wet-Dry Method

At 1 and 6 h after ROSC, rats were decapitated, and the entire brain was removed in a standard fashion. The cortex, hippocampus, and brainstem of the brain were separated and weighed immediately after removal and then placed in a laboratory oven (105°C) for slow evaporation for 72 h. The dried samples were weighed, and the water content (%) was calculated as $(\text{wet weight} - \text{dry weight})/(\text{wet weight}) \times 100\%$.

Neurologic Deficit Evaluation and Survival Rate

All animals were evaluated before the experiment to ensure normal neurologic function. Neurologic deficit score (NDS) evaluations were performed by the same investigator, who was unaware of the group assignment. Consciousness and breathing, cranial nerves reflexes, motor function, sensory, and coordination were scored according to an NDS system (0–100% scale; 0 = normal, 100 = brain death), which was described for this model previously.¹⁰ Survival rates were also determined at 4 days after ROSC.

Neuronal Counts

For hippocampal neuronal survival analysis, the rats that survived for 4 and 14 days and those in the nonarrested sham group were deeply anesthetized with halothane, perfused through the heart with approximately 200 ml phosphate-buffered saline, 0.1 M, and perfusion fixed with 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde before paraffin embedding and sectioning. The 5- μm , hematoxylin and eosin-stained sections of the hippocampus (corresponding to bregma -3.3 cm according to the atlas of Paxinos and Watson¹¹) were examined. Viable pyramidal neurons were counted in the CA1 regions of the hippocampus with high-power field. Ischemic neurons were recognized by their pyknotic or karyorrhectic nuclei which lacked a clear nucleolus, whereas viable neurons were

defined as those cells showing distinct nucleus and nucleolus. One observer, who was blinded to the experimental protocol, counted the numbers of normal-appearing pyramidal neurons per high-power field ($\times 400$).

Caspase-3 Immunohistochemistry

Additional brain sections were deparaffinized in xylene and rehydrated in graded alcohols. Slides were washed, and endogenous peroxidase was quenched by immersion in 3% hydrogen peroxide for 10 min. Heat-induced target retrieval was performed on all sections using a citrate-based target retrieval buffer and heated in an autoclave for 3 min. Subsequently, the slides were incubated with rabbit anticleaved caspase 3 (1:200; Cell Signaling Technology, Beverly, MA) overnight at 4°C, then washed in phosphate-buffered saline, and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Zhongshan Biotechnology Co., Beijing, China) for 1 h at room temperature. After the sections were washed with phosphate-buffered saline, the peroxidase reaction was developed by incubating the section in 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) solution. Finally, the sections were dehydrated through a gradient of ethanol solutions and then mounted and covered with a coverslip. Control

sections were processed identically and in parallel; however, they were incubated with phosphate-buffered saline instead of the primary antibodies. No labeling was detected in these controls. The same investigator who was blinded to the study intervention used microscopy to manually count the number of positively immunostained cells in the hippocampal CA1 region ($\times 400$).

Statistical Analysis

Rats were randomly assigned to groups by using a random number table. The experimental protocol was divided among researchers such that those responsible for the asphyxial CA model, NDS evaluation, data acquisition, and analysis were blind to each other's activities and results until data analyses were complete. Physiologic variables and viable neurons count were expressed as mean \pm SD and analyzed by one-way analysis of variance with Tukey correction for *post hoc* comparisons between multiple experimental groups. NDS were expressed as median (25th, 75th percentiles) and analyzed by the Kruskal-Wallis test followed by Mann-Whitney U analysis when the overall *P* value was significant. The Kaplan-Meier analysis and log-rank test were used to calculate the survival rates. Two-way analysis of variance and the Tukey *post hoc* test were used to compare

Table 1. Physiologic Variables in All Groups

	Sham (n = 15)	Control (n = 30)	Hemin (n = 25)	SnPP (n = 30)
MAP, mmHg				
Baseline	95 \pm 9	96 \pm 9	91 \pm 9	98 \pm 10
ROSC 30'	92 \pm 10	131 \pm 16*	142 \pm 13*	141 \pm 13*
ROSC 60'	92 \pm 11	89 \pm 14	89 \pm 7	92 \pm 12
HR				
Baseline	341 \pm 44	345 \pm 52	352 \pm 37	355 \pm 36
ROSC 30'	345 \pm 32	382 \pm 50	411 \pm 53	417 \pm 58
ROSC 60'	353 \pm 18	351 \pm 24	348 \pm 28	358 \pm 31
pH				
Baseline	7.42 \pm 0.07	7.44 \pm 0.05	7.45 \pm 0.06	7.43 \pm 0.05
ROSC 30'	7.41 \pm 0.08	7.40 \pm 0.08	7.43 \pm 0.06	7.40 \pm 0.07
ROSC 60'	7.40 \pm 0.06	7.42 \pm 0.05	7.41 \pm 0.05	7.41 \pm 0.04
Pao ₂ , mmHg				
Baseline	97.8 \pm 8.2	103.1 \pm 11.9	105.0 \pm 12.6	106.7 \pm 13.6
ROSC 30'	368.1 \pm 13.9	376.9 \pm 15.7	373.9 \pm 18.4	366.9 \pm 18.0
ROSC 60'	374.4 \pm 14.4	376.2 \pm 18.9	380.0 \pm 19.5	371.0 \pm 15.7
Paco ₂ , mmHg				
Baseline	39.7 \pm 2.7	39.2 \pm 3.5	39.6 \pm 4.0	41.6 \pm 4.6
ROSC 30'	39.0 \pm 3.6	37.2 \pm 4.1	39.7 \pm 4.2	39.1 \pm 4.7
ROSC 60'	39.7 \pm 2.9	40.1 \pm 4.4	39.7 \pm 3.4	39.8 \pm 4.0
Blood glucose, mg/dl				
Baseline	111.0 \pm 15.1	102.1 \pm 19.5	97.3 \pm 18.3	99.8 \pm 15.2
ROSC 30'	103.6 \pm 12.4	164.9 \pm 17.8*	172.1 \pm 19.9*	171.3 \pm 21.3*
ROSC 60'	99.1 \pm 12.0	151.2 \pm 19.8*	157.9 \pm 18.4*	153.7 \pm 15.7*
Lactate, mm				
Baseline	2.3 \pm 0.6	2.2 \pm 0.3	2.2 \pm 0.4	2.2 \pm 0.3
ROSC 30'	3.5 \pm 0.8	7.5 \pm 0.5*	7.5 \pm 0.5*	7.6 \pm 0.6*
ROSC 60'	3.3 \pm 0.7	7.4 \pm 0.5*	7.3 \pm 0.6*	7.5 \pm 0.6*

Values are presented as mean \pm SD.

* *P* < 0.05 vs. sham group.

HR = heart rate; MAP = mean arterial pressure; Paco₂ = arterial carbon dioxide pressure; Pao₂ = arterial oxygen pressure; ROSC = restoration of spontaneous circulation; SnPP = tin protoporphyrin.

Table 2. Variables in Asphyxial Cardiac Arrest Groups

	Control (n = 30)	Hemin (n = 25)	SnPP (n = 30)
Asphyxial time to CA, s	183 ± 24	188 ± 25	176 ± 20
CA time, s	297 ± 24	292 ± 25	304 ± 20
Maximal systolic BP after ROSC, mmHg	183 ± 25	185 ± 35	171 ± 28
Time of maximal systolic BP after ROSC, s	59 ± 11	62 ± 16	62 ± 14
Time requirement for ROSC, s	54 ± 12	50 ± 9	49 ± 10

Values are expressed as mean ± SD.

BP = blood pressure; CA = cardiac arrest; ROSC = restoration of spontaneous circulation; SnPP = tin protoporphyrin.

brain water content using group and time point (1 vs. 6 h) as independent factors. Significance was considered at the level of $P < 0.05$. Statistical analysis was performed using SPSS® software (SPSS Inc., Chicago, IL).

Results

A total of 128 rats were used in the whole experiment. Eight rats in the control group (resuscitation rate, 79% [30 of 38]), 10 rats in the hemin group (resuscitation rate, 74% [25 of 35]), and 10 rats in the SnPP group (resuscitation rate, 75% [30 of 40]) were excluded from the study because spontaneous circulation was not restored within 2 min. There were no statistical differences in resuscitation among these groups. Data from these animals were not included in the later analysis.

Physiologic Variables

As seen in table 1, there were no significant differences in any of the physiologic variables among the groups in the baseline period. The mean arterial pressure and heart rate were higher in all CA groups at 30 min after ROSC because epinephrine was given at the beginning of the CPR period. At 1 h after ROSC, the mean arterial pressure and heart rate returned to baseline levels. The levels of blood glucose and plasma lactate significantly increased in all CA groups at 30 and 60 min after ROSC.

Table 3. Brain Edema after Cardiac Arrest and Resuscitation

Group	Cortex		Hippocampus		Brainstem	
	1 h	6 h	1 h	6 h	1 h	6 h
Sham	81.4 ± 1.4	83.4 ± 1.6	82.6 ± 3.2	81.6 ± 3.2	74.9 ± 1.3	74.1 ± 0.7
Control	85.7 ± 2.0	83.1 ± 1.8	88.2 ± 2.4	85.9 ± 2.1	75.0 ± 0.5	74.9 ± 0.7
Hemin	82.4 ± 0.9	82.5 ± 1.1	83.1 ± 1.9	82.7 ± 2.7	74.5 ± 1.3	74.8 ± 1.1
SnPP	85.3 ± 1.7	83.9 ± 1.2	91.1 ± 2.0	89.5 ± 2.1	75.1 ± 1.3	75.4 ± 1.5

n = 5 for each time point in all groups. Values reflect % brain water content and are expressed as mean ± SD. Hemin group suppressed the increased water content in cortex ($P = 0.03$) and hippocampus ($P = 0.004$) when compared with control group at 1 h after restore of spontaneous circulation. There was no effect of time point after restoration of spontaneous circulation on brain water content in all regions ($P > 0.05$).

1 h = 1 h after restoration of spontaneous circulation; 6 h = 6 h after restoration of spontaneous circulation; SnPP = tin protoporphyrin.

Characteristics of CPR

As seen in table 2, time from the initiation of asphyxiation to CA was approximately 180 s in all groups. After CPR, the maximal systolic blood pressure and the time to achieve maximal systolic blood pressure and ROSC were not obviously different among the CA groups.

Brain Water Content after ROSC

As seen in table 3, water content of the cortex significantly increased in the control ($P = 0.03$) and SnPP groups ($P = 0.01$), with nearly the same extent between the two groups, compared with 81.4%, which is mean of the corresponding value in the sham group. However, water content of the cortex did not significantly increase in the hemin group (control vs. hemin, $P = 0.03$). Water content of the hippocampus increased in the control group when compared with the sham group ($P = 0.001$), and the SnPP group showed a higher value than the control group ($P = 0.035$), whereas the hemin group suppressed the increased water content in this region (control vs. hemin, $P = 0.004$). Water content of the brainstem was not significantly different among the groups ($P = 0.78$). There was no effect of time point after ROSC on brain water content in all regions ($P > 0.05$), and there was no interaction between factors ($P > 0.05$).

Overall Survival Rate and NDS after ROSC

All rats in the sham group (100% [5 of 5]) survived well throughout the 4-day observation period. In all CA groups, 14 rats died on day 1; 5 of them died of intrac-table pulmonary injury (edema and pneumothorax) after CPR (2 in the control group, 1 in the hemin group, and 2 in the SnPP group). From day 2 to day 4 after ROSC, 11 other rats, 5 in the control group, 1 in the hemin group, and 5 in the SnPP group, died because of stupor or severe cachexia. Ten rats (50% [10 of 20]) in the control group, 12 rats (80% [12 of 15]) in the hemin group, and 8 rats (40% [8 of 20]) in the SnPP group survived until day 4. The difference of 4-day survival rates in the hemin and SnPP groups were not significant when compared with that of the control group (hemin vs. control, $P =$

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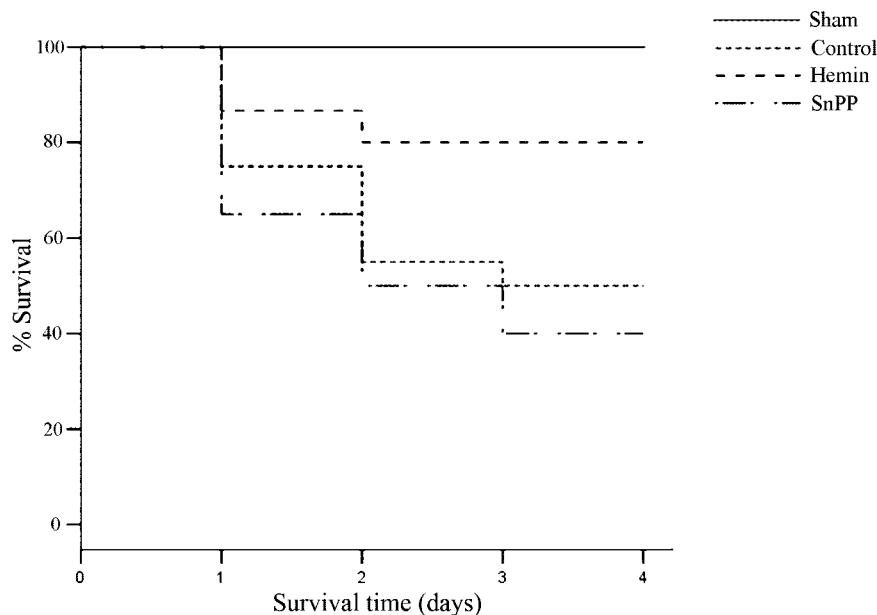


Fig. 1. Kaplan-Meier plot of cumulative survival 4 days after cardiac arrest and resuscitation in sham ($n = 5$), control ($n = 30$), hemin ($n = 25$), and tin protoporphyrin (SnPP; $n = 30$) groups.

0.085; SnPP vs. control, $P = 0.518$; fig. 1). Despite the fact that necropsies were performed on all dead rats, the causes of death remained unclear in every rat.

As seen in figure 2, neurologic deficits were noted in all CA groups on the 4th and 14th days after ROSC. Rats in the hemin group showed lower NDS on the 4th day (P

$= 0.004$) when compared with control group, and this protection effect continued to the 14th day ($P = 0.02$).

Neuronal Counts

As seen in figure 3, the hippocampal CA1 neuron counts were determined in the sham, control, hemin, and SnPP group. At 4 days of recovery, rare neuronal death occurred in the CA1 region of sham-operated rats. After 8 min of CA and resuscitation followed by 4 days of recovery, neuronal density in the control and SnPP groups was much lower than that in the sham group; nuclear pyknosis, karyorrhexis, and vacuolization were also seen in the control and SnPP groups. However, the neuronal density and cell morphology were well preserved in the hemin group. For comparison, the numbers of viable neurons per high-power field ($\times 400$) in the CA1 region of all CA groups were significantly lower than that of the sham group. The hippocampal CA1 counts were 29 ± 6 in the hemin group, which was significantly more than the counts in the control group (8 ± 4 ; $P < 0.05$) but still less than that in the sham group (49 ± 5 ; $P < 0.05$). The CA1 cell counts in the SnPP group (5 ± 3) were less than those in the control group, but the difference was not significant. For the rats that were allowed to recover for 14 days, CA1 neuron counts in the control group (5 ± 2) were similar to those in the SnPP group (4 ± 3). The CA1 neuron counts in the hemin group (28 ± 4) were significantly higher as compared with those in the control and SnPP groups ($P < 0.05$). There were not significantly fewer CA1 neurons in the 14-day-recovery rats as compared with the 4-day-recovery rats in any group.

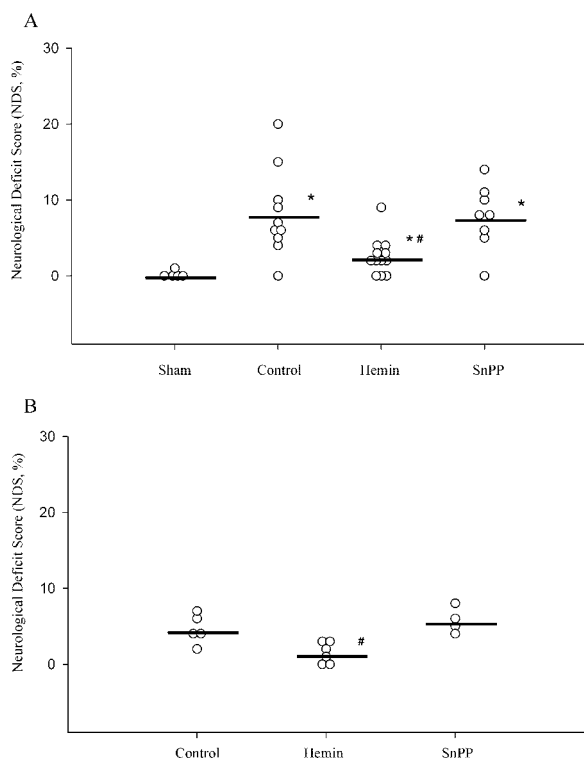


Fig. 2. Neurologic deficit score in all groups on the 4th (A) and 14th (B) days after asphyxial cardiac arrest. Open circles indicate values for individual animals. Horizontal bars indicate group median values for neurologic deficit score. * $P < 0.05$ versus sham group. # $P < 0.05$ versus control group. SnPP = tin protoporphyrin.

Caspase-3 Staining

Caspase 3-positive cells revealed apoptotic neurons of the hippocampal CA1 region in all animals (fig. 4). On

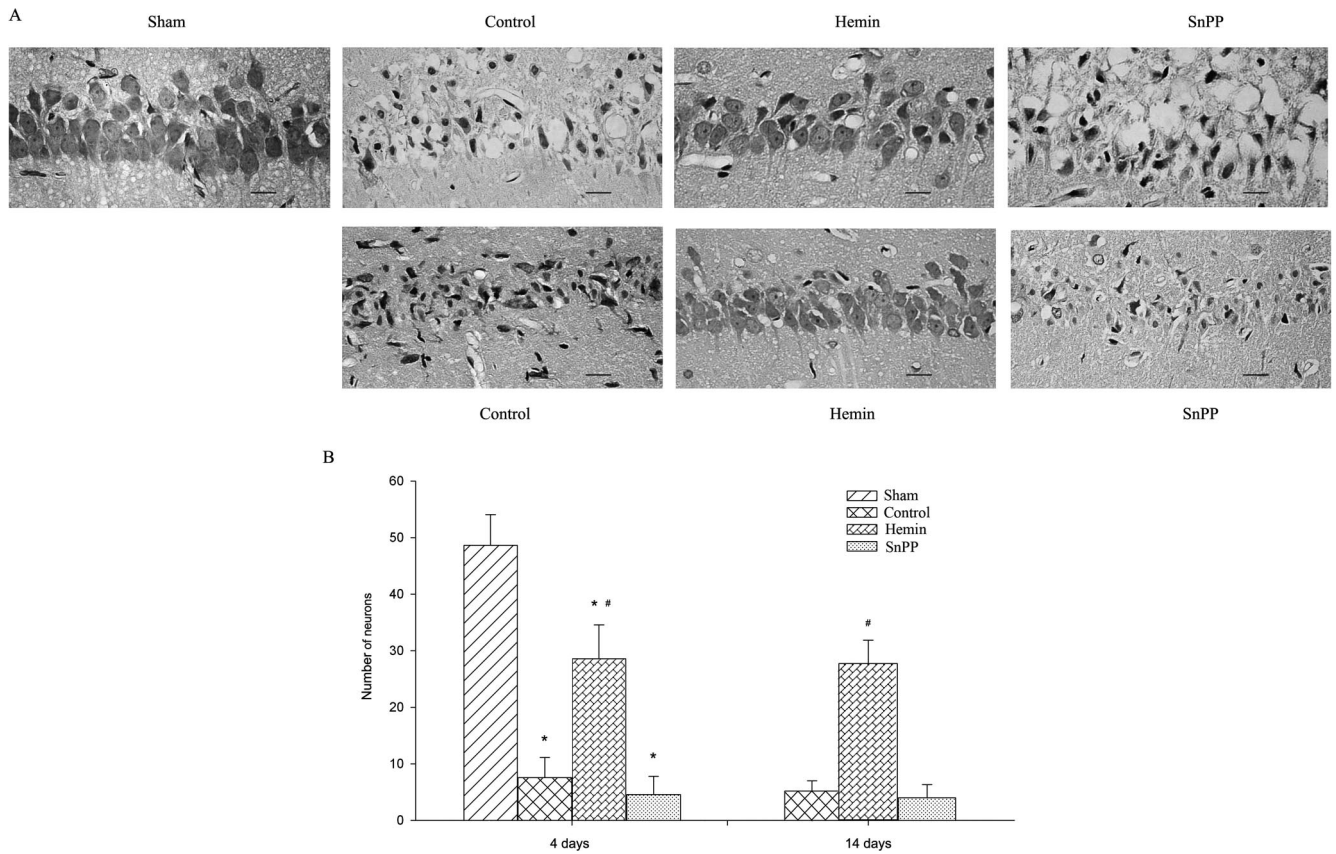


Fig. 3. (A) Representative histologic images of the hippocampal CA1 region from rats belonging to sham (n = 5), control (n = 5), hemin (n = 6), and tin protoporphyrin (SnPP; n = 4) groups on the 4th day (upper panel) and the 14th day (lower panel) (n = 5, 6, and 4 in control, hemin, and SnPP groups, respectively). All images were captured at 400× magnification. Scale bar indicates 100 μm. (B) Number of survival neurons in the CA1 region of the rat hippocampus belonging to different groups. *P < 0.05 versus sham group. # P < 0.05 versus control group.

the 4th day after ROSC in the hippocampal CA1 region, 27 ± 7, 14 ± 6, and 37 ± 5 caspase 3–positive neurons were observed in the control, hemin, and SnPP groups, respectively; these values were much greater than that of the sham group (3 ± 3; P < 0.05). Caspase 3–positive cells in the hemin group were fewer when compared with the control group on the 4th day (P < 0.05). However, this difference was not significant on the 14th day.

Discussion

In the current study, rats pretreated with hemin showed decreased water content in the cortex and hippocampus at 1 h after ROSC. In the long-term study, the hemin group had an improved NDS and displayed more neuron counts and less neuronal apoptosis in the hippocampal CA1 region from 4 to 14 days after ROSC. On the other hand, pretreatment with SnPP exacerbated these changes.

A characteristic feature of brain damage caused by CA is brain edema, which increases the capillary–tissue diffusion distance and impairs oxygen and waste exchange, ultimately leading to loss of brain function.⁴ The patho-

physiologic mechanisms of brain edema caused by CA or cerebral ischemia have been discussed by various authors.^{12–18} Among them, N-methyl-D-aspartate (NMDA) receptor-mediated aquaporin 4 (AQP4)-dependent brain edema plays an essential role.¹⁹ The cellular acidification after CA, which is induced by energy failure and anaerobic glycolysis, induces neuronal membrane depolarization. This triggers calcium influx through voltage-sensitive Ca²⁺ channels, which further depolarizes the neuronal membrane and stimulates the release of massive amounts of glutamate. The elevation of the extracellular glutamate causes a prolonged and excessive activation of postsynaptic NMDA receptors, which regulates the activation of AQP4. AQP4, the most abundant aquaporin protein throughout the central nervous system, participates in water movement and cerebral edema formation after CA and resuscitation.^{15,16}

By using HO-1 knockout mice, Ahmad *et al.*²⁰ found that unilateral intrastriatal injection of NMDA produced a significantly larger lesion volume *in vivo* and lower cell viability in cultured neurons *in vitro*, leading the authors to propose that HO-1 is neuroprotective against brain damage mediated by NMDA through HO-1 itself or its metabolites (carbon monoxide, Fe²⁺, and bilirubin). In

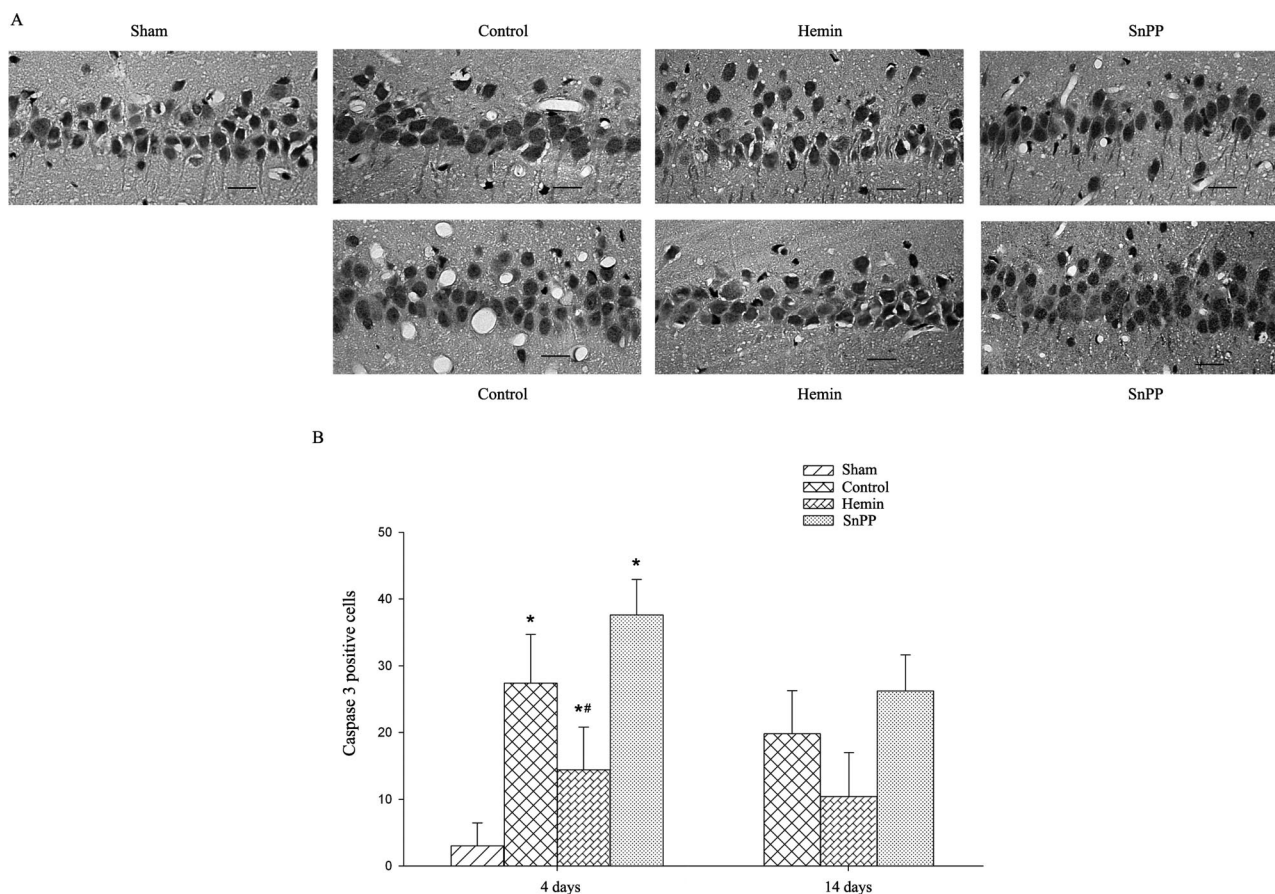


Fig. 4. (A) Representative images of caspase 3-positive neurons in the hippocampal CA1 region from rats belonging to sham ($n = 5$), control ($n = 5$), hemin ($n = 6$), and tin protoporphyrin (SnPP; $n = 4$) groups on the 4th day (upper panel) and the 14th day (lower panel) ($n = 5, 6,$ and 4 in control, hemin, and SnPP groups, respectively). All images were captured at $400\times$ magnification. Scale bar indicates $100\ \mu\text{m}$. (B) Number of caspase 3-positive neurons in the CA1 region of the rat hippocampus belonging to different groups. * $P < 0.05$ versus sham group. # $P < 0.05$ versus control group.

this study, we demonstrated that induction of HO-1 with hemin decreased brain water content in the cortex at 1 h and in the hippocampus at both 1 and 6 h after CA resuscitation, whereas the administration of SnPP tended to exacerbate brain water content in the hippocampus and cortex. Therefore, it is likely that the decreased brain water content in hemin group was through the induction of HO-1 and inhibition of NMDA receptor-mediated AQP4-dependent brain edema.

Caspase 3 is the major effector caspase involved in apoptotic pathways. In previous studies, it has been documented that caspase 3 triggers the execution phase of apoptosis.²¹⁻²³ The activation of caspase 3 is considered to be the most specific mark of the execution of apoptosis. The current study showed that the number of variable neurons decreased and the caspase 3-positive neurons increased significantly in the hippocampal CA1 region in all CA groups. The induction of HO-1 preserved the viable neurons well and inhibited neuronal apoptosis significantly. The NDS also had an obvious improvement in the hemin group.

Heme oxygenase 1 has been shown to be neuroprotective *in vivo*²⁴ and *in vitro*.²⁵ The mechanism by

which high expression of HO-1 protects the brain may include several factors: (1) a neuroprotective effect against acute excitotoxicity induced by NMDA through HO-1 itself or its metabolites,²⁰ (2) inhibition of apoptosis by regulating cellular prooxidant iron and carbon monoxide,²⁶ (3) prevention of inflammation reactions by targeting chemokines and proinflammatory cytokines after very early ischemia and reperfusion injury,²⁷ and (4) a decrease of the production of oxygen free radicals.⁶

Numerous studies have established direct evidence that massive outpouring of superoxide radicals during cerebral ischemia and reperfusion injury provokes damage to lipids, DNA, and proteins, leading to neuronal death.^{28,29} Moreover, although tissue perfusion is restored, neuronal damage continues. Idris *et al.*³⁰ reported that free radicals increased rapidly after onset of CA and peaked early during reperfusion in a swine model of CPR. Another study also suggests that oxidative damage plays a prominent role in CA and sudden death which can affect survival and outcome.³¹ In addition, it has been reported that superoxide potently triggers mitochondrial swelling and the release of proteins involved in activation of apoptotic pathways.^{32,33}

The products from the metabolism of heme by HO-1 contribute to cellular defenses against oxidative stress injury. In neural cell culture experiments, a minute amount of bilirubin is capable of protecting cells from 10,000-fold higher concentrations of the oxidant hydrogen peroxide.³⁴ The iron released by HO-1 also increases the synthesis of ferritin, which stores free iron and has well-known cytoprotective properties.³⁵ Carbon monoxide, a newly discovered gas mediator from heme metabolism, has the ability to inhibit some of the enzymes involved in arachidonic acid metabolism,³⁶ bind heme moieties that can inactivate nitric oxide synthase activity,³⁷ which can produce nitric oxide and damage neurons through activation of poly (adenosine diphosphate ribose) synthetase,³⁸ relax smooth muscle and block platelet aggregation,³⁹ and elevate cyclic guanosine monophosphate levels in neurons, which eventually leads to the suppression of apoptosis.⁴⁰ Therefore, the improvement of neurologic function in the hemin group might be due to the induction of HO-1, resulting in less oxide damage and neuronal apoptosis, and these effects may be through HO-1 itself directly or its metabolites indirectly.

This study has several limitations. First, the study was designed to determine the maximal effects of HO-1 on CA-elicited brain damage; therefore, hemin, the inductor of HO-1, was given before CA. This is not clinically practical or possible. However, administering a therapeutic agent before the onset of the injuries has often been used as an effective method to study the mechanisms of the injuries in laboratory settings. Further studies are needed to reveal neuroprotective effects of HO-1, given during or after resuscitation. Second, the evaluation of brain water content in this study was terminated 6 h after ROSC, when maximal brain edema was observed at 1 h within 6 h of ROSC.⁴¹ The effect of HO-1 on CA-elicited brain edema beyond 6 h of ROSC is unknown. Therefore, further studies are needed to investigate the time course of brain edema beyond 6 h of ROSC and the effect of HO-1 on CA-elicited brain edema in the later phases.

In conclusion, the current results suggest that induction of HO-1 by administration of hemin may contribute to reduce CA-elicited brain edema in the early phases, improve neurologic function, and protect neurons against apoptosis in the hippocampal CA1 region. Our finding, therefore, may suggest a new therapeutic strategy to improve the survival and prognosis after CA and resuscitation.

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