

## Original Article

## Prolonged severe hemorrhagic shock at a mean arterial pressure of 40 mmHg does not lead to brain damage in rats

Ryosuke Mihara,<sup>1,2</sup> Akira Takasu,<sup>1</sup>  Kentaro Maemura,<sup>3</sup> and Toshiaki Minami<sup>2</sup><sup>1</sup>Department of Emergency Medicine, <sup>2</sup>Department of Anesthesiology, and <sup>3</sup>Department of Anatomy, Osaka Medical College, Osaka, Japan**Aim:** To examine whether prolonged hemorrhagic shock (HS) at a mean arterial pressure (MAP) of 40 mmHg leads to brain damage.**Methods:** Rats were anesthetized with sevoflurane. The HS model consisted of the following phases: I, pressure-controlled HS at a MAP of 40 mmHg; II, fluid resuscitation to normalize blood pressure; III, observations with outcome evaluations in terms of survival, overall performance categories, and neurological deficit scores, as well as evaluation of apoptosis in the hippocampus at 96 h. Each group of six rats was randomized into 60 min (group 1) or 75 min (group 2) each of phases I and II. Three sham rats were anesthetized for 150 min, and then awakened during phase III.**Results:** The three sham rats as well as five and two of the six rats in groups 1 and 2 ( $P < 0.05$ ), respectively, survived for up to 96 h. All survivors were functionally normal with overall performance category = 1 and neurological deficit score = 0 at 96 h. Apoptotic neurons were not found in the hippocampus.**Conclusions:** The higher mortality in group 2 suggested a more profound effect of HS compared with group 1. However, prolonged HS for 60 or 75 min did not cause functional damage or apoptosis in the hippocampus. These findings suggest that prolonged HS at a MAP of 40 mmHg, as a level at which cerebral blood flow seems preserved by autoregulatory mechanisms, does not lead to brain damage.**Key words:** Apoptosis, brain function, hemorrhagic shock, ischemia, resuscitation

## INTRODUCTION

IN CASES OF ongoing and life-threatening hemorrhage, attempts at restoring blood pressure to normal values might actually increase blood loss. Therefore, blood pressure is intentionally controlled at a lower level until fundamental treatments such as surgery or embolization are carried out on the bleeding sources.<sup>1,2</sup> Although the definitive blood pressure level has not been clarified, establishing a level from the viewpoint of brain protection is reasonable, because the brain is the organ that is most vulnerable to ischemia.<sup>3</sup> The mechanism for autoregulation of cerebral blood flow (CBF) may be preserved above a mean arterial pressure (MAP) of 50 mmHg.<sup>4</sup> Below that pressure, CBF greatly decreases; irreversible neurological damage occurs at

a MAP level of 30 mmHg.<sup>4–6</sup> Therefore, a MAP of 40 mmHg would be appropriate for the experimental setting because that level seems to be the minimum to prevent irreversible brain damage.

Apoptosis is a fundamental mode of cell death and differs from necrosis. Apoptosis occurs in neuronal cells in a variety of pathologic conditions, including hemorrhagic shock (HS) followed by resuscitation.<sup>7,8</sup> However, no study has assessed the effect of the hypotensive setting on brain function and the occurrence of apoptosis.

In this study, we aimed to clarify the effects of ischemia caused by prolonged HS at a MAP of 40 mmHg on brain function and the development of apoptosis in the hippocampus, which is the region in the brain that is most vulnerable to ischemia.

## METHODS

THIS STUDY WAS approved by our Institutional Animal Care and Use Committee and followed national guidelines for the treatment of animals. We carried out the experiments using 15 male albino Sprague–Dawley rats (mean body weight,  $380 \pm 28$  g; range, 340–430 g).

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## Experimental design

We used the three-phase HS model (Fig. 1). Hemorrhagic shock phase I included pressure-controlled HS at a MAP of 40 mmHg for 60 or 75 min. Resuscitation phase II included fluid resuscitation with a goal of rapidly achieving a MAP  $\geq 70$  mmHg with both shed blood and normal saline for 60 or 75 min. Observation phase III of up to 96 h included outcome evaluation in terms of survival, overall performance categories (OPC), neurologic deficit scoring (NDS), and morphology (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling [TUNEL] staining) of the brain. Both phases I and II were undertaken while rats were under sevoflurane anesthesia, but phase III was undertaken without anesthesia. Rats that survived up to 96 h were reanesthetized and killed with perfusion-fixation for TUNEL apoptosis evaluation.

Twelve rats were randomized into experimental group 1 (phase I and II for 60 min each for a total of 120 min) or group 2 (phase I and II for 75 min each for a total of 150 min) to compare the survival rate between groups. To create a sham group, three rats were anesthetized, underwent catheter insertion, and were observed for 150 min.

## Preparation and baseline measurements

Rats were anesthetized in a jar with nitrous oxide/oxygen ( $N_2O:O_2$ , 50%:50%) plus 5% sevoflurane. After weighing, animals were placed in the supine position and maintained with

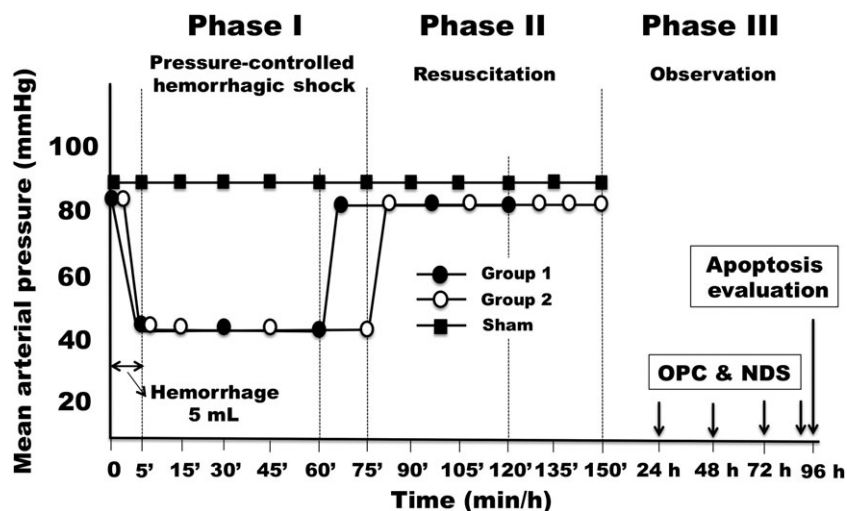
$N_2O/O_2$  (50%/50%) and sevoflurane 2% anesthesia with spontaneous breathing through a cone mask. The anesthesia level was fixed throughout the HS and resuscitation phases. The rectal temperature was maintained at  $37.5 \pm 0.5^\circ\text{C}$ . Through a sterile incision in the left groin, a polyethylene catheter (PE50) was inserted approximately 2 cm into the femoral artery for pressure monitoring and blood sampling. A similar catheter (PE60) was inserted into the left femoral vein and advanced into the inferior vena cava (by 5 cm) for blood withdrawal. Each catheter was connected to pressure transducers. Electrocardiogram, heart rate, arterial blood pressure, and rectal temperature were continuously recorded using a polygraph (PowerLab 4/26; ADInstruments, Bella Vista, Australia). These preparative procedures were completed within 45 min.

After the above preparation, a period of 5 min was allowed for the animals to reach a steady state, and then baseline data were recorded.

Arterial blood samples (0.3 mL each) were drawn to monitor pH,  $PaO_2$ ,  $PaCO_2$ , base excess, and hemoglobin (measured using an ABL 80 blood gas analyzer; Radiometer, Copenhagen, Denmark) at baseline and at the end of phases I and II (75 and 150 min after baseline in the sham group). Each sampled blood volume was replaced with an equivalent volume of normal saline.

## HS phase I

Immediately after performing baseline measurements, HS was induced with 5 mL of blood withdrawal at a steady rate



**Fig. 1.** Diagram of a three-phase hemorrhagic shock (HS) rat model: phase I, HS; phase II, resuscitation; phase III, observation. Group 1 (closed circles), phase I (60 min) and phase II (60 min) for a total of 120 min. Group 2 (open circles), phase I (75 min) and phase II (75 min) for a total of 150 min. Sham (closed squares), observation for 150 min. OPC, overall performance categories; NDS, neurologic deficit scoring.

for 5 min through the vena cava catheter, resulting in a transient decrease in MAP to approximately 30–40 mmHg. The start of the volume-controlled hemorrhage was designated as hemorrhage shock time zero (HST<sub>0</sub>). The initial withdrawn blood was preserved in heparinized syringes (blood : heparin = 1 mL:1 U) at 25°C for reinfusion during the resuscitation phase. Mean arterial pressure was then controlled at 40 mmHg with further blood withdrawal or reinfusion through the venous catheter. The additional withdrawn blood was also preserved in heparinized syringes for use during the following resuscitation regimen.

## Resuscitation phase II

At HST<sub>60</sub> or HST<sub>75</sub>, resuscitation was begun by reinfusion of the withdrawn heparinized blood at 1.0 mL/100 g body weight for 1 min, followed by infusion of normal saline at 2.0 mL/100 g body weight for 4 min to reach the goal of raising the MAP to  $\geq 70$  mmHg. If the MAP did not reach 70 mmHg, the same resuscitation regimen was repeated one more time. At HST<sub>120</sub> or HST<sub>150</sub>, veins were knotted, catheters were removed, and the groin incision was closed. The rat then received s.c. injections of 10 mL dextrose 5% (because the rat would be unable to ingest food or water for some time after anesthesia) and N<sub>2</sub>O and sevoflurane were discontinued and replaced with oxygen.

## Observation phase III

After awakening, the rat was moved to an observation chamber for 4 days. Food and water were available, and functional outcome evaluations were carried out every 24 h from day 1 to day 4. Overall, neurologic function was assessed in terms of OPC (1, normal; 2, moderate disability; 3, severe disability; 4, coma; 5, death) and NDS (0–10%, normal; 100%, brain death), by methods modified for rats from cerebral resuscitation studies in dogs.<sup>9,10</sup> The NDS consists of five components: reduced level of consciousness, abnormal cranial nerve function, abnormal motor and sensory function, and reduced coordination.

## Apoptosis evaluations

After functional evaluation on day 4, the surviving rats were anesthetized with 5% sevoflurane, a median sternotomy was carried out, and then rats were killed with perfusion-fixation with a solution of buffered 4% paraformaldehyde infused into the left cardiac ventricle at 90 cmH<sub>2</sub>O pressure. The right cardiac atrium was opened for venous drainage, and the solution was infused until the drainage fluid was clear. The cranium was then

opened. The brain was removed and placed into paraformaldehyde solution.

The TUNEL staining to evaluate DNA fragmentation was carried out according to the protocol in the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Briefly, 5-mm thick dewaxed sagittal sections of the right brain hemisphere, including the hippocampus, were incubated with 0.3% hydrogen peroxide in methanol for 30 min at 24°C to block endogenous peroxidase activity. Sections were treated with proteinase K (20  $\mu$ g/mL) for 15 min at 24°C followed by incubation with TUNEL reaction mixture for 1 h at 37°C. For the negative control for staining, the specimen was incubated with buffer without terminal transferase instead of the TUNEL reaction mixture. For the positive control for DNA fragmentation, the section was treated with 0.05 U/ $\mu$ L DNase I (Takara, Shiga, Japan) for 15 min at 37°C prior to the TUNEL reaction. Subsequently, sections were incubated with a converter-POD (Roche, Mannheim, Germany) for 30 min at 37°C and then reacted with diaminobenzidine substrate solution for 10 min at 24°C. Sections were cut to 5  $\mu$ m thick and inspected with a microscope (Nikon ECLIPSE E600; Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (VB7000; Keyence, Osaka, Japan).

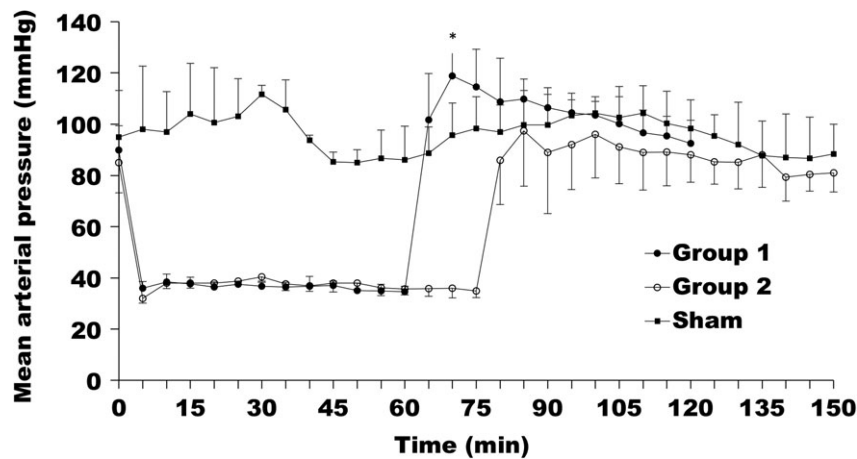
## Statistical analysis

All data are expressed as the mean  $\pm$  standard deviation. Hemodynamic and physiologic variables in experimental groups 1 and 2 were compared using repeated-measures ANOVA and the unpaired *t*-test. The paired *t*-test was used for comparisons of physiologic variables between the baseline and subsequent time points in each group. Survival time was determined using the Kaplan–Meier procedure. The survival rate was compared using Fisher's exact test. In all tests, *P*-values of  $< 0.05$  were considered statistically significant. A group size of six rats allowed detection of a 65% difference in the survival rate with an approximate power of 0.8 and alpha level of 0.05. The number of sham rats was minimized to save animals.

## RESULTS

### Hemodynamics

THE INITIAL VOLUME -controlled hemorrhage decreased MAP in all experimental rats from a baseline value of  $87 \pm 11$  mmHg (range, 70–102 mmHg) to  $35 \pm 5$  mmHg (range, 30–39 mmHg) at HST<sub>5</sub>, and then MAP was controlled at approximately 40 mmHg according to the protocol (Fig. 2). A similar volume of blood was additionally withdrawn from each group to maintain MAP at 40 mmHg (group 1,  $3.7 \pm 0.8$  mL versus group 2,



**Fig. 2.** Changes in mean arterial pressure during phases I (hemorrhagic shock) and II (resuscitation) of a three-phase hemorrhagic shock rat model. Values represent the mean  $\pm$  standard deviation. Group 1 (closed circles), phase I (60 min) and phase II (60 min) for a total of 120 min. Group 2 (open circles), phase I (75 min) and phase II (75 min) for a total of 150 min. Sham (closed squares), observation for 150 min. \* $P < 0.01$  between group 1 and 2.

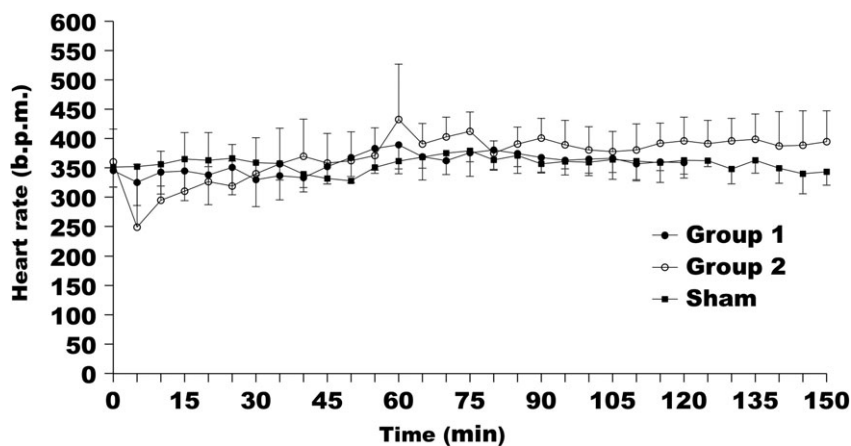
$3.7 \pm 1.2$  mL) during phase I. During phase II, the initial fluid resuscitation achieved mean MAPs of  $119 \pm 9$  mmHg in group 1 and  $97 \pm 21$  mmHg in group 2 ( $P < 0.05$ ). Two rats (33%) in group 1 and six rats (100%) in group 2 required the second resuscitation regimen to maintain a MAP of  $\geq 70$  mmHg ( $P < 0.01$ ). Mean arterial pressures in the sham group remained at  $\geq 85$  mmHg throughout the experiment.

Mean heart rates in all experimental rats decreased from a mean value of  $353 \pm 43$  b.p.m. (range, 316–455 b.p.m.) to  $287 \pm 87$  b.p.m. (range, 175–456 b.p.m.) at

HST<sub>5</sub> and gradually increased to  $389 \pm 40$  b.p.m. in group 1 at HST<sub>60</sub> and  $412 \pm 34$  b.p.m. in group 2 at HST<sub>75</sub> (at the end of phase I) without a significant difference (Fig. 3). During phase II, heart rates did not differ significantly between groups.

### Body weight and physiological variables

Body weight and physiological variables showed no significant differences among the groups at baseline (Table 1). Base excess decreased to the nadir at the end of phase I in

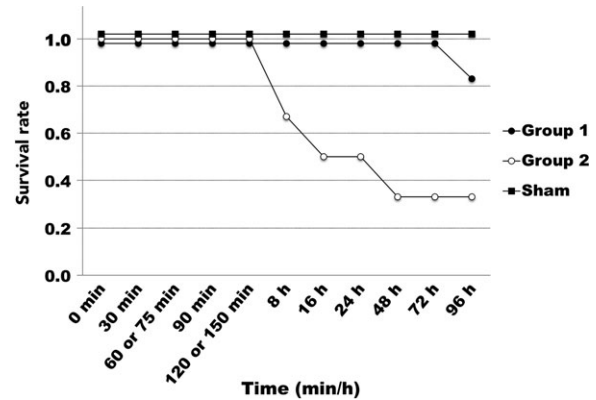


**Fig. 3.** Changes in heart rates during phases I (hemorrhagic shock) and II (resuscitation) in a three-phase hemorrhagic shock rat model. Values represent the mean  $\pm$  standard deviation. Group 1 (closed circles), phase I (60 min) and phase II (60 min) for a total of 120 min. Group 2 (open circles), phase I (75 min) and phase II (75 min) for a total of 150 min. Sham (closed squares), observation for 150 min.

**Table 1.** Mean ( $\pm$  standard deviation) values for body weight and physiological variables at baseline, the end of phase I (60 min after baseline in group 1 and 75 min after baseline in sham and group 2), and the end of phase II (120 min after baseline in group 1 and 150 min after baseline in sham and group 2) in a hemorrhagic shock (HS) rat model

	Baseline			End of phase I			End of phase II		
	Sham (n = 3)	Group 1 (n = 6)	Group 2 (n = 6)	Sham (n = 3)	Group 1 (n = 6)	Group 2 (n = 6)	Sham (n = 3)	Group 1 (n = 6)	Group 2 (n = 6)
Body weight (g)	377 $\pm$ 25	390 $\pm$ 27	370 $\pm$ 27	N/A	N/A	N/A	N/A	N/A	N/A
pH	7.49 $\pm$ 0.02	7.49 $\pm$ 0.05	7.49 $\pm$ 0.08	7.49 $\pm$ 0.02	7.4 $\pm$ 0.05**	7.29 $\pm$ 0.19***	7.46 $\pm$ 0.01	7.44 $\pm$ 0.02	7.32 $\pm$ 0.02*, ***
PaO <sub>2</sub> (mmHg)	208 $\pm$ 27	241 $\pm$ 39	208 $\pm$ 30	254 $\pm$ 69	248 $\pm$ 128	219 $\pm$ 18	243 $\pm$ 56	223 $\pm$ 34	236 $\pm$ 12
PaCO <sub>2</sub> (mmHg)	32 $\pm$ 3	30 $\pm$ 4	32 $\pm$ 7	34 $\pm$ 3	24 $\pm$ 3**	28 $\pm$ 7***	35 $\pm$ 2	32 $\pm$ 4	37 $\pm$ 3*
Base excess (mmol/L)	1.1 $\pm$ 3.3	-0.1 $\pm$ 3.2	0.5 $\pm$ 2.0	1.9 $\pm$ 1.3	-9.0 $\pm$ 2.8**	-14.0 $\pm$ 6.0***	0.6 $\pm$ 0.8	-3.0 $\pm$ 2.0	-6.3 $\pm$ 3.4***
Hematocrit (%)	36 $\pm$ 6	32 $\pm$ 4	36 $\pm$ 5	37 $\pm$ 5	27 $\pm$ 2**	28 $\pm$ 7***	37 $\pm$ 3	32 $\pm$ 3	31 $\pm$ 8

Group 1, 60 min of phase I (HS at a mean arterial pressure of 40 mmHg) and phase II (fluid resuscitation to normalize blood pressure). Group 2, 75 min of phases I and II. Sham, anesthetized then observed for 150 min.  
 \* $P < 0.05$  versus group 1 at the end of phase II.  
 \*\* $P < 0.05$  versus baseline in group 1.  
 \*\*\* $P < 0.05$  versus baseline in group 2.  
 N/A, not available.



**Fig. 4.** Cumulative survival following phases I (hemorrhagic shock) and II (resuscitation) in a hemorrhagic shock rat model. Group 1 (closed circles), phase I (60 min) and phase II (60 min) for a total of 120 min. Group 2 (open circles), phase I (75 min) and phase II (75 min) for a total of 150 min. Sham (closed squares), observation for 150 min. Cumulative survival (Kaplan–Meier) was significantly better in group 1 than group 2 ( $P < 0.05$ ).

both experimental groups ( $P < 0.05$  compared with values at baseline) and remained at a significant negative value after resuscitation at the end of phase II in group 2 ( $P < 0.05$  compared with the value at baseline). We found significant differences between the groups in pH and PaCO<sub>2</sub> values at the end of phase II.

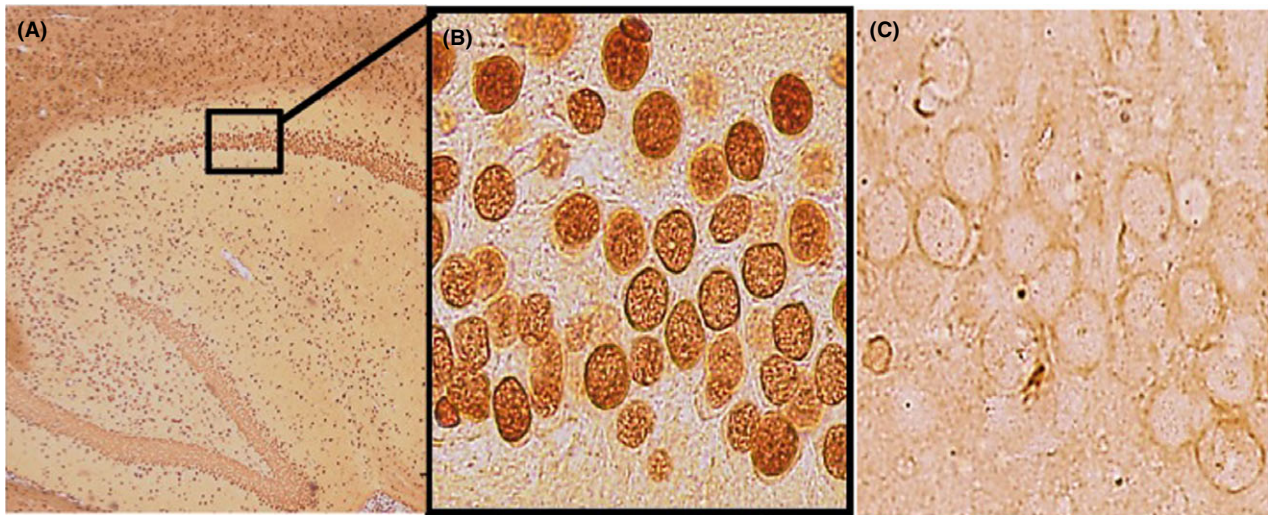
### Survival and neurological findings

All rats survived beyond phases I and II. The three sham rats as well as five (83%) and two (33%) of the six rats in groups 1 and 2, respectively, survived for up to 96 h. Three (60%) of five non-survivors died within 24 h after resuscitation phase II. Group 1 rats showed better survival than group 2 rats ( $P < 0.05$ ; Fig. 4). All these survivors were functionally normal with OPC = 1 and NDC = 0 at 96 h.

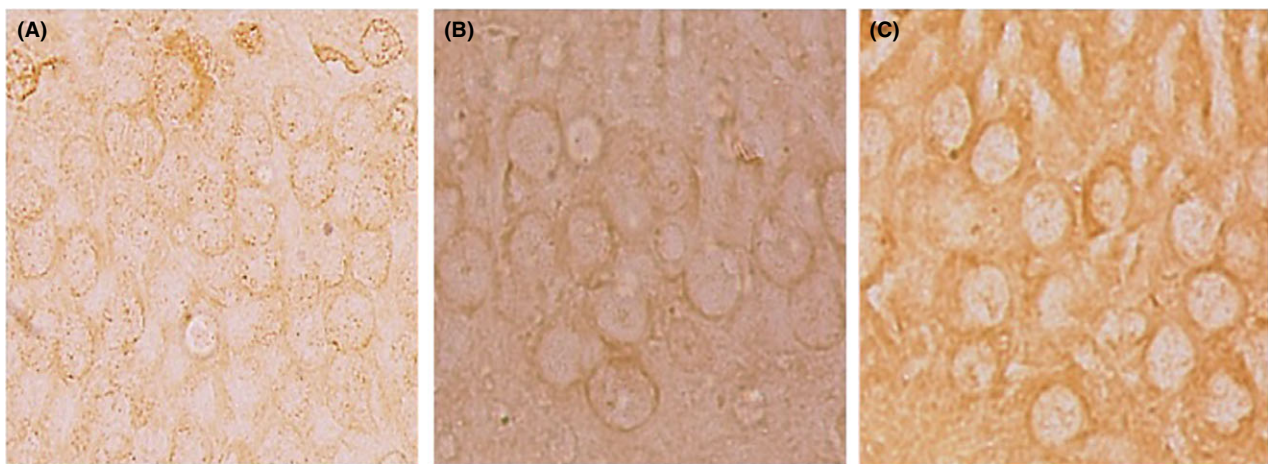
### Apoptosis evaluations

Damage to neuronal cells was evaluated in the hippocampus by detection of cellular apoptosis with the TUNEL method. Chromatin condensation was observed in hippocampal neurons treated with DNase I in the positive control specimen (Fig. 5A, B), whereas no apoptotic cells were observed in the negative control without treatment with terminal transferase (Fig. 5C). Representative morphologic findings of the hippocampal CA1 region in each group are shown in Figure 6. The TUNEL staining did not show apoptotic cells in the hippocampus of any surviving rats.





**Fig. 5.** Photomicrographs of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining in the hippocampus of rats after hemorrhagic shock. A, Positive control tissue (+DNase I). Magnification,  $\times 40$ . B, Positive control (+DNase I) in the CA1 region. Magnification,  $\times 400$ . C, Negative control (– terminal transferase) in the CA1 region. Magnification,  $\times 400$ .



**Fig. 6.** Representative photomicrographs of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining of the CA1 region of the hippocampus in each group of rats after hemorrhagic shock. A, Group 1, phase I (hemorrhagic shock) 60 min and phase II (resuscitation) 60 min. B, Group 2, phase I 75 min and phase II 75 min. C, Sham tissues, anesthetized then observed for 150 min.

## DISCUSSION

THE CRITICAL MAP level during severe HS that can cause brain damage in selectively vulnerable regions is not unknown. This information is crucial when “hypotensive resuscitation” to control blood pressure at a lower level is applied during ongoing and uncontrolled HS, to prevent increased bleeding and cardiac arrest. This is of particular importance in the prehospital field. This rat model mimicked

prolonged HS under spontaneous breathing in such settings, but did not reproduce the clinical settings in intensive care, including artificial ventilation.

When the MAP is reduced to approximately 40 mmHg, as in this study, CBF decreases to 40% of the normal level and may critically impair electrophysiological brain activity, but may not cause permanent loss of neuronal viability.<sup>4–6</sup> Carrillo *et al.*<sup>9</sup> reported that HS at a MAP of 40 mmHg for 60 min or MAP of 30 mmHg for 45 min did not cause

subtle problems with brain function, as evaluated with the Morris water maze cognitive test, or histological brain damage, as examined with hematoxylin–eosin staining. Our study is the first examination of apoptosis to look for ischemic brain damage after prolonged HS. In this study, the base excess showed profound negative values during HS and the insult of HS might be sufficient to produce ischemic damage. However, apoptotic neurons were not found in the hippocampus, which is the region in the brain most vulnerable to ischemia. A higher mortality rate in rats with HS for 75 min suggested a more profound effect compared with HS for 60 min. However, HS at a MAP of 40 mmHg for both prolonged periods did not cause functional damage or neuronal apoptosis in the hippocampus. These findings suggest that the brain could tolerate a MAP of 40 mmHg for a prolonged period and not show signs of damage. However, the tolerable time period for severe HS that does not cause ischemic brain damage is still unknown.

Human studies have indicated that aggressive fluid administration for treatment of prehospital and intraoperative trauma results in increased blood loss and mortality, so there is a shift in the clinical paradigm toward “hypotensive resuscitation.”<sup>1,2</sup> In contrast to standard fluid resuscitation, this strategy requires administration of less fluids to maintain vital organ perfusion. However, minimum fluid resuscitation was not applied during HS in this study, because relatively low MAP control was strictly required. In the future, studies are required to assess the “hypotensive resuscitation” strategy including its effects on brain outcomes with fluid administration during HS.

Our study has several limitations. First, from the standpoint of the time course of apoptosis, we investigated only one point. Polytrauma combined with HS induces apoptosis at an early stage at 6 h after resuscitation in multiple visceral organs.<sup>7</sup> In addition, apoptosis in the brain seems to occur and reach a peak several days after the ischemia/reperfusion procedure.<sup>11,12</sup> In this study, examination with TUNEL staining was undertaken 4 days after the insult. At such a delayed stage, apoptosis may appear if the insults were significant enough to cause damage. Second, we used sevoflurane anesthesia because it is popularly used in clinical practice and has few side-effects. This study could not be carried out in awake rats. Sevoflurane has a neuroprotective effect against cerebral ischemic injury,<sup>13,14</sup> although the mechanisms have been poorly elucidated. Accordingly, the anesthetic effect should be taken into account when considering the results of this study. Third, this model did not include intensive care that might have affected prolonged outcomes for rats. Application of intensive care would be difficult in the rat HS model. We did not use a tracheal tube because this would require deeper anesthesia and cause

airway and secretion problems. Finally, the major limitation in this study was the experimental protocol, in which non-survivors were excluded from apoptosis evaluation because reliable TUNEL staining requires circulation and perfusion–fixation death, which would have been impossible if the rats had already died, and the cause of death was most likely cardiovascular dysfunction because these rats died shortly after resuscitation attempts. Any apoptosis found in these rats could have been caused by a secondary irreversible postresuscitative shock state. The question we wanted to ask involved brain damage in survivors after prolonged HS, but not brain damage after secondary cardiovascular dysfunction.

## CONCLUSIONS

**S**URVIVING RATS FOLLOWING HS with a MAP of 40 mmHg for 60 or 75 min, followed by resuscitation, maintained normal neurologic function. Apoptosis did not occur in the hippocampus, which is the most vulnerable region in the brain. These findings suggest that prolonged HS at a MAP of 40 mmHg does not lead to brain ischemic damage in anesthetized rats.

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## DISCLOSURES

Approval of the research protocol: The protocol of this study was approved by the Animal Ethics Committee of Osaka Medical College, Japan (approval no. 28043; date, 2016-4-15) and the experimental procedures followed the guidelines for proper conduct of animal experiments of the Science Council of Japan.

Informed consent: N/A.

Registry and registration no: N/A.

Animal Studies: All animal experiments were conducted following the national guidelines and the relevant national laws for the protection of animals.

Conflict of interest: None declared.

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