Stepdown Infusion of Barbiturate improves Neurological Function in a New Rat Model of Rebleeding Subarachnoid Hemorrhage.

Sosho Kajiwara
Kurume University Hospital: Kurume Daigaku Byoin

Yu Hasegawa (✉ fpmhasse@yahoo.co.jp)
International University of Health and Welfare - Okawa Campus: Kokusai Iryo Fukushi Daigaku - Okawa Campus
https://orcid.org/0000-0001-6285-3920

Kana Fujimori
Kurume University Hospital: Kurume Daigaku Byoin

Motohiro Morioka
Kurume University Hospital: Kurume Daigaku Byoin

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Abstract

The manuscript complies with all instructions to authors. Furthermore, authorship requirements have been met and the manuscript has been approved by all the authors. The manuscript has not been published elsewhere, nor is it under consideration by another journal.

All experiments were approved by the Institutional Animal Care and Use Committee of Kurume University and all applicable institutional guidelines for the care and use of animals were followed.

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Full Text

Rebleeding from a ruptured aneurysm is seen in 17% and has been recognized as the main risk factors for fatal prognosis in subarachnoid hemorrhage (SAH) patients.1 Although there have been provided some medical treatments to prevent rebleeding, it is theoretically difficult to prevent rebleeding because no effective drugs were found so far.2 Therefore, available protective posttreatments against the rebleeding-induced brain injuries is important to improve the prognosis for SAH patients.

As increased intracranial pressure (ICP) after SAH and subsequent global cerebral ischemia is a major cause of the poor prognosis,3 reduction of increased ICP is candidate for the posttreatment target. Barbiturate is known as a sedative and anesthetic drug and also exert reduction of ICP. We previously developed a novel treatment method of barbiturate using thiamylal administrating 3.0mg/kg/h at 0-24h, 2.0mg/kg/h at 24-48h, 1.5mg/kg/h at 48-72h, and 1.0mg/kg/h at 72-96h.4 The “step-down infusion of barbiturate therapy (sd-B)” reduced composite death of the patients with severe traumatic brain injuries at discharge in the intensive care unit and the effect was associated with inhibition of increased ICP.5 The significant results suggest that sd-B exert favorable effects against brain injury caused by rebleeding in SAH.

In this study, we hypothesized that sd-B using thiamylal improved prognosis in rebleeding SAH model in rats. To address the hypothesis, we newly established a rebleeding SAH model and evaluate the posttreatment beneficial effects on the model.

All experiments were approved by the Institutional Animal Care and Use Committee of Kurume University and performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Fifty male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighting 304.4-397 g (9-10 weeks) were divided randomly into the following groups: (1) sham-operated with distilled water (Sham+W), (2) sham-operated with barbiturate (Sham+B), (3) SAH-rebleeding with distilled water (SAH+W), (4) SAH-rebleeding with barbiturate (SAH+B). We monitored neurological function and case fatality as the primary endpoints and our evaluated brain injuries including brain edema and cortical
neuronal cell death as secondary endpoints. We calculated the sample size based on the result of the rotarod test between sham and SAH groups.\textsuperscript{6} To produce a new model of rebleeding SAH model, we firstly made a SAH model using a prechiasmatic single-blood injection method as previously described.\textsuperscript{6, 7} Five minutes after 1\textsuperscript{st} injection with 200µL arterial blood over 12 seconds, additional 100µL over 12 seconds was injected at the same place again. Then, the operative lesion was disinfected with iodine, and meloxicam (1 mg/kg; Caymen Chemical, Ann Arbor, MI, USA) was administered subcutaneously for appropriate analgesia.\textsuperscript{7, 8}

Thiamylal (Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan) was made using distilled water and filled into an osmotic minipump (Model 2001, Durect Co. Cuperito, CA, USA) to administered at a rate of 1mg/kg/hr. Three pumps were implanted intraperitoneally immediately after rebleeding SAH surgery, and each pump was removed every 24 hours to complete sd-B for 3 days. Study protocol was shown in Suppl Figure 1a.

To assess motor function, we measured 18-point composite scoring test (Modified Garcia test), 4-point beam walking test, and rotarod test at 1 day and 3 days after SAH induction as previously described.\textsuperscript{7}

Three days after SAH induction, the rats were euthanized under deep anesthesia, and their brains were quickly collected, and cut at the point of the bregma. Then, the left hemisphere of the rostral side, cerebellum, and brain stem were used to measure brain water content (BWC, n=10 in each group) using a previously described method.\textsuperscript{8}

The caudal side was kept in 4% paraformaldehyde solution, embedded in paraffin, and cut into 5µm section. Histological evaluation (n=6 in each group) using Nissl staining and Ionized calcium binding adaptor molecule-1 (Iba-1; 1:2000; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) staining was performed based on our previous methods.\textsuperscript{7, 9} The number of positive cells was counted using images taken from 2 fields of the both left and right somatosensory cortex at 200× magnification. The mean number of cells was expressed as cells/field.

Two animals died from anesthesia complications. In primary endpoints, the case fatality rates were 33% (10 out of 15 rats) in SAH+W and 23% (3 out of 13 rats) in SAH+B. According to neurological function (Figure 1), in comparison with the rats in Sham+W group, the rats in SAH+W group showed significant deterioration of neurological function at 1 day (17.1±0.2 in modified Garcia test; 1.3±0.3 in beam waking test; 47.7±11.7sec in rotarod test) and 3 day (15.3±0.6 in modified Garcia test; 67.7±12.9 sec in rotarod test). Although the rats in SAH+B also showed significant reduction in rotarod test at 1 day (51.0±8.2sec), significant improvement in modified Garcia test was observed at 3 days (17.5±0.3) in comparison with the score of SAH+W group.

In secondary endpoints, as shown in Figure 2a-c, increase in brain water content of left hemisphere in SAH+W group (80.7±0.3%) was significant in comparison with Sham+W group (79.7±0.2%). On the other hand, significant reduction of the values was observed in Sham+B (79.2±0.1% in cerebrum, 78.2±0.1% in
cerebellum, and 73.5±0.1% in brain stem) and SAH+B (79.6±0.2% in cerebrum, 78.1±0.1% in cerebellum, and 73.2±0.1% in brain stem) groups in comparison with SAH+W group (78.8±0.2% in cerebellum and 74.2±0.2% in brain stem).

As shown in Figure 3a, cortical neuronal cell death was significantly observed in SAH+W group (89.8±13.4 cells), whereas sd-B inhibited the loss of neuronal cells (139.0±5.0 cells). As show in Figure 3b, increase in number of reactive microglia in SAH+W group (9.3±2.4 cells) was marginally significant in comparison with Sham+W (2.2±0.5 cells) and the number of total microglia in SAH+W group (17.5±1.6 cells) was significantly higher than the groups in Sham+W (11.3±1.3 cells), Sham+B (7.7±0.5 cells), and SAH+B (11.1±1.9 cells).

Our current study provided the following significant findings of the sd-B therapy; 1) sd-B improved neurological function as the primary endpoint in a new rat model of SAH rebleeding. 2) sd-B significantly reduced brain edema and neuronal cell death in the model as the secondary endpoint.

Barbiturate exerts protective effects for brain injuries by way of 1) reduction of ICP via vasoconstriction in normal brain area 2) decrease of metabolic oxygen demand 3) stabilization of lysosomal membrane 4) reduction of intracellular calcium concentration 5) modification of amino acid and neurotransmitter release, 6) scavenging of free radical 7) reduction in cerebrospinal fluid production, 8) anti-inflammatory response via suppression of substance P and 9) γ -aminobutyric acid receptor activation. Among them, we selected thiamylal regarding the ultra-short acting and fewer cardiovascular complication, and stronger potentiation than thiopental.5,13 Then, we previously explore a novel administration method, sd-B, which could keep a stable concentration of thiamylal, reduction of side effects such as pneumonia and arrhythmia and provided good prognosis in the patients with severe traumatic brain injuries.4,5 Rebleeding is supposed to produce repeated ICP elevation and subsequent severe CBF reduction, and also additive subarachnoid blood clot enhances oxidative stress and inflammatory response. Those additional cytotoxic responses induce neuronal cell death, microglial activation, brain edema, resulting in neurological deficit and death. Our present study revealed that sd-B significantly decreased BWC in whole brains and microglia. Although the effects might be secondary to other undetermined protective effects, we thought our novel method reduced cerebrospinal fluid production and oxidative stress, resulting in rescue the neuronal death and improvement of neurological function.

SAH patients suffer high mortality and morbidity and the determinant factors are early brain injury, delayed cerebral ischemia, and rebleeding of the ruptured aneurysm.2 To explore SAH pathophysiology, there are 3 available experimental SAH models.14,15 In consideration with strength/weakness of each model regarding a rebleeding model, although the endovascular perforation model is thought to mimic clinical SAH phenotypes, preservation of monofilament nylon suture or tungsten rod for second puncture should induce significant cerebral ischemia and accelerate high mortality. Blood injection model to cisterna magna is introduced as a model for delayed effects of SAH by the single or double blood injection (time of second injection are 24-48hr after first injection),16 it should not be suitable for the model because rebleeding is mostly occurred within the first hours after the initial bleeding.2 In this study,
we newly established rebleeding SAH model which modified prechiasmatic blood injection model by Prunell et al.\textsuperscript{14,17} After the blood injection, ICP abruptly increased more than 100mmHg and subsequent reduction was seen around 20mmHg within a few minutes.\textsuperscript{17} Regarding rebleeding SAH model, we could perform second blood injection at the same place to produce rebleeding (re-rupture from a ruptured aneurysm). Therefore, we thought this model was most suitable to produce rebleeding SAH based on the simplicity and reproducibility.

Although we did not evaluate the changes of ICP and CBF which could provide detailed mechanism on the effect of sd-B and accuracy and validity of our new rebleeding model, our present study provided the first evidences that posttreatment sd-B ameliorated neurological function and brain injuries on rebleeding SAH in rats. Clinically, anesthetic drug is used to SAH patients to sedation and control of blood pressure. Therefore, sd-B is one of the good candidate treatments for favorable outcome for the SAH patients with rebleeding.

**Declarations**

**Author Contributions**

SK and YH contributed to the study's conception and design. SK, KF and YH performed the experiments. MM helped with the interpretations. SK wrote the first draft of the manuscript. YH and MM revised the manuscript. All authors reviewed and approved the manuscript.

**Data Availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Conflicts of Interest**

There are no conflicts of interest.

**References**


Modiﬁed Garcia test (a), Beam walking test (b), and Rotarod test (c) among the 4 groups at 1 and 3 days after rebleeding SAH. Values are presented as mean±SEM. Abbreviations: B, barbiturate; W, water. * means p<0.05.

All data are presented as the mean ± SEM and parametric and non-parametric evaluations were performed using a one-way ANOVA with the Tukey-Kramer test and Kruskal-Wallis test followed by the Steel-Dwass test in four groups, respectively.
Figure 2

Brain water content at 3 days after rebleeding SAH. Values are presented as mean±SEM. Abbreviations: B, barbiturate; W, water. * means p<0.05.

All data are presented as the mean ± SEM and parametric and non-parametric evaluations were performed using a one-way ANOVA with the Tukey-Kramer test and Kruskal-Wallis test followed by the Steel-Dwass test in four groups, respectively.
Figure 3

Nissl stain (a) and Iba-1 positive cells (b) at 3 days after rebleeding SAH. Values are presented as mean±SEM. Abbreviations: B, barbiturate; W, water. * means p<0.05. Bar indicates 200 µm.

All data are presented as the mean ± SEM and parametric and non-parametric evaluations were performed using a one-way ANOVA with the Tukey-Kramer test and Kruskal-Wallis test followed by the Steel-Dwass test in four groups, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVE.pdf
- SuppleFigure.pptx