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Experimental paper

Hemoglobin vesicles improve neurological outcomes after cardiac arrest in rats

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ABSTRACT

Aim: To investigate the effects of hemoglobin vesicles (HbVs) in preventing hypoxic brain injury after cardiac arrest in a rat model of asphyxia-related cardiac arrest.
Methods: Male Wistar rats were divided into three groups: HbVs (n = 18), control (n = 29), and sham (n = 7). Respiratory arrest was induced using muscle relaxants under ventilation. Cardiac arrest occurred 3–4 min later. After 8 min, HbVs or saline (5 ml/kg), adrenaline, and sodium bicarbonate were administered, followed by chest compressions and ventilation. Resuscitation was deemed successful with a mean arterial pressure > 60 mmHg sustained for at least 5 min. Behavioral and histopathological evaluations were performed 7 days later.
Results: Survival rates were 39 % and 24 % in the HbVs and control groups, respectively (P = 0.308). Motor activity scores and spatial memory were significantly higher in the HbVs group (P < 0.001). Hippocampal CA1 region staining indicated significantly less neuropathy in the HbVs group (P < 0.001).
Conclusion: The administration of HbVs during resuscitation was effective in mitigating brain damage after whole-brain ischemia in rats, as demonstrated by improved histopathological and neurological outcomes. This suggests potential neurological benefits for patients during resuscitation, although further research in larger animal models is required to validate these findings.

Introduction

In the United States, over 600,000 cardiac arrests occur annually, and the discharge rates for out-of-hospital and in-hospital cardiac arrests are low, at 9 % and 23 %, respectively.[1] After out-of-hospital cardiac arrest, only 4.8 % of patients achieve a Cerebral Performance Category Scale score of 1 or 2, which is considered to indicate a good neurological prognosis.[2] Even if these patients survive, up to 40 % of them do not reintegrate into society.[3,4].

Diffuse hypoxic brain damage results in a clinically persistent disturbance of consciousness.[5] Cardiac arrest induces global ischemia, disrupts overall cerebral circulation, and causes neurological damage to many brain regions, including the hippocampus, cortex, cerebellum, thalamus, prefrontal cortex, and putamen.[6] Despite various efforts to provide neuroprotection during post-resuscitation care, no useful approach has yet been established.

Rat experiments have demonstrated that neurological outcomes are worse during resuscitation if high concentrations of oxygen are not administered.[7] Moreover, these experiments have shown that administering high-concentration oxygen (100 % oxygen) can improve both neurological function and survival rates up to 3 h after resuscitation.[8] Notably, studies in humans indicate that achieving high oxygen concentrations (>150 mmHg) during resuscitation is difficult.[9] Even when 100 % oxygen is administered, only a few cases reach such levels, making it challenging to ensure proper oxygen delivery to the brain during resuscitation.[9].

High hemoglobin (Hb) concentrations are necessary to benefit from the administration of high volumes of oxygen because the amount of oxygen in the blood (CaO_2) depends on Hb levels and SaO_2. The equation is CaO_2 (mL/dL) = Hb × SaO_2 × 1.34, where 1.34 is a constant factor.[10] Neurological prognoses have been reported to vary with hemoglobin concentrations in patients after resuscitation.[11] The SOS-KANTO group reported that the median hemoglobin level on hospital arrival of patients who achieved good neurological outcomes after resuscitation was 14.4 g/dL, compared with 12.8 g/dL in those with poor outcomes.[12] Eleven other similar studies have been reported, all of which revealed that neurological prognoses were better in patients with higher Hb levels. However, early blood transfusion after cardiac arrest

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did not shorten the time to return of spontaneous circulation (ROSC) in pig experiments.[13] Moreover, hypoxic encephalopathy after resuscitation using blood transfusions has not been sufficiently investigated to date.

Hb-vesicles (HbVs) are cellular-type Hb-based oxygen carriers containing purified and concentrated Hb of human origin and are encapsulated in liposomes. The safety and efficacy of HbVs as an alternative to blood transfusion are being studied,[14,15] and a phase 1 study in healthy men has been completed.[16] The Hb purification process for the production of HbVs removes erythrocyte membrane components and inactivates and removes viruses; therefore, it is not affected by blood type and carries no risk of infection. In the present study, we investigated the cerebroprotective effects of HbVs against hypoxic encephalopathy during resuscitation in a rat model of asphyxia-related cardiac arrest.

Methods

Preparation and characterization of HbVs

HbVs were prepared under aseptic conditions, as previously reported.[17,18] Briefly, The Hb that was used for the preparation of HbVs was first purified from outdated human blood donations provided by the Japanese Red Cross Society (Tokyo, Japan). Carbonylation was performed for purification, the extracted Hb solution was heated for virus inactivation, and nanofiltration was then performed for virus removal. Next, the Hb solution was ultrafiltered to 40 g/dL before being co-encapsulated with equimolar pyridoxal 5′-phosphate (PLP) within liposomes using a kneading method.[18] The liposomal membrane comprised four lipids, namely: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate, and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-PEG_{5000}, at a molar ratio of 5, 4, 0.9, and 0.03, respectively. Finally, HbVs was suspended in isotonic saline to adjust the Hb concentration to approximately 10 g/dL. The average particle size of the HbVs was determined to be 251 ± 80 nm using dynamic light scattering. The methemoglobin content in the HbVs was < 10 %. The oxygen affinity of the HbVs, indicated by the P_{50} value, was measured with the Hemox Analyzer (TCS Scientific Co., New Hope, PA) and determined to be 15 Torr.

Animal preparation

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Animal Ethics Committee of Nara Medical University (approval number: 12873). The experimental procedures were performed according to the ARRIVE guidelines (National Center for the Replacement, Improvement, and Reduction of Animals in Research, 2010) (Supplemental material. 1). The National Institute of Health guidelines (Institute of Laboratory Animal Resources) were followed for animal care and handling to minimize animal pain and suffering.

Fifty-four male Wistar rats weighing 370–400 g and aged 8–9 weeks were purchased from Oriental Bioservices Inc. (Kyoto, Japan). They were housed under 12-h light and dark cycles, with free access to water and food.

Cardiac arrest model

An 8-min cardiac arrest per a modified asphyxiation model used by Huang et al.[19] was implemented. Briefly, the rats were anesthetized with 2.5 % isoflurane, intubated with a 16-gauge catheter (16-G Surflo Flash Terumo, Tokyo, Japan), and ventilated with a ventilator (SN-480–7 Shinano, Tokyo, Japan) at FiO_2 0.21, respiratory rate (RR) 100, and tidal volume (TV) 0.65 mL/100 g body weight. Catheters (24-G) were inserted into the right internal jugular vein and the left femoral artery (24-G Supercath5 MEDIKIT, Amersfoort, The Netherlands). The cardiac arrest model used in this study was a pulseless electrical activity (PEA) cardiac arrest induced by asphyxiation. The arterial blood pressure (BP) in the left femoral artery was measured. Subsequently, 2 mg/kg vecuronium was administered, and the ventilator was stopped after 5 min. Cardiac arrest was defined when the systolic BP fell below 25 mmHg, which occurred approximately 3–4 min after the respirator was stopped. Precordial chest compressions were initiated 8 min after respiratory arrest. Chest compressions were performed by the researcher using fingers at a constant rate of 200/min using a metronome. The intervention (HbVs) group received 5 mL/kg HbVs, and the control group received 5 mL/kg saline. Both groups also received 0.01 mg/kg adrenaline and 1 mEq/kg sodium bicarbonate. Ventilatory control was resumed after 1 min (FiO_2 1.0, RR 100, TV 0.65 mL/100 g of body weight). Resuscitation was considered successful when the mean arterial BP was maintained above 60 mmHg for > 10 min. Rats that failed to achieve ROSC within 2 min were excluded. The rats were weaned from the ventilator and extubated when spontaneous breathing was observed. They were returned to their cages approximately 1 h after extubation (Fig. 1). The sham group received only anesthesia, muscle relaxants, and mechanical ventilation. During the experiment, the rats were maintained at a temperature of 37 °C using a thermal pad. They were kept

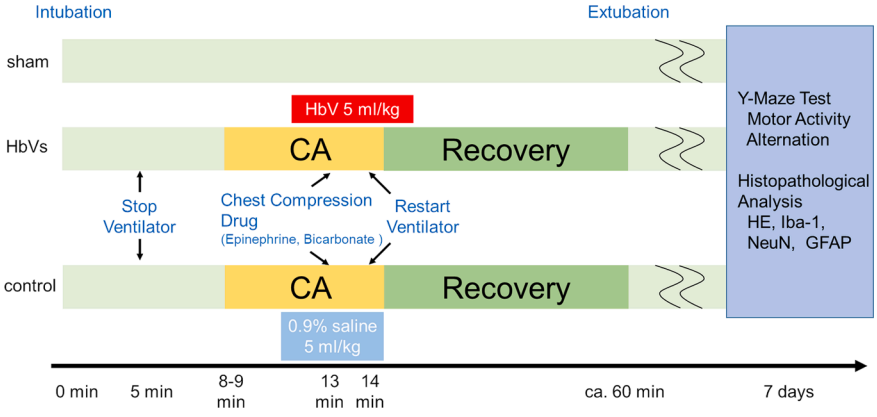

Fig. 1. Resuscitation protocol. The rats were placed in the prone position and anesthetized with isoflurane, followed by tracheal intubation and artificial ventilation. A venous line was secured via the right internal jugular vein, and an arterial pressure line was secured via the left femoral artery. After the administration of the muscle relaxant (vecuronium), the ventilator was stopped. Cardiac arrest (defined as systolic blood pressure < 25 mmHg) occurred approximately 3–4 min later. Eight min after the ventilator was stopped, 5 mL/kg of hemoglobin vesicles (HbVs – hemoglobin-based oxygen carriers) or 0.9 % saline was administered. At the same time, chest compressions (200 times/min) were started. One minute later, the ventilator was restarted. Resuscitation was considered successful if a systolic blood pressure of 60 mmHg or higher was achieved for 5 min. After spontaneous breathing was restored, the rats were extubated. Behavioral and pathological evaluations were performed 7 days later. The sham group received only artificial ventilation and administration of muscle relaxants.

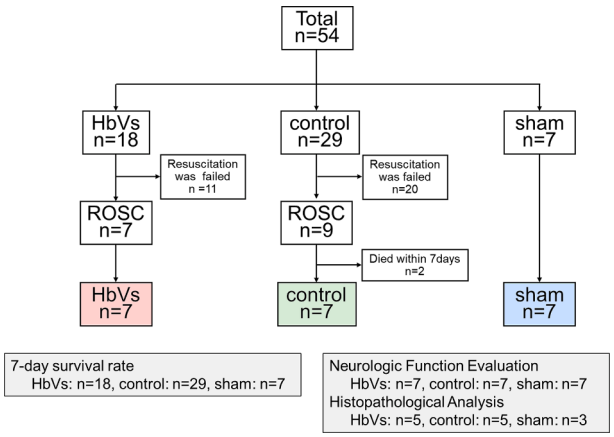

Fig. 2. Experimental group flowchart. Step 1: Group Assignment – Rats were divided into HbVs, control, and sham groups. Step 2: Asphyxia and Cardiac Arrest Induction – Standardized procedures were used to induce cardiac arrest through asphyxiation, ensuring consistent arrest conditions. Step 3: Resuscitation Protocol – Interventions, including HbVs administration, were initiated 8 min post-arrest, with epinephrine and chest compressions provided to all groups. Step 4: Outcome Measures – ROSC and survival rates were recorded immediately, with neurological and histopathological assessments following at 7 days. Overall, 54 rats were used, and an attempt was made to have a total of seven rats each in the HbVs, control, and sham groups. Eleven rats died in the HbVs group, and 20 and 2 rats died in the control group on the day of the procedure and within 7 days, respectively. HbVs, hemoglobin vesicles; ROSC, return of spontaneous circulation.

warm throughout the procedure, including after extubation and before being returned to their cages, to ensure their well-being and stability.

Study protocol

This study had predefined primary and secondary endpoints. The primary endpoint was the neurological function evaluation, which was assessed using a behavioral analysis performed 7 days post-resuscitation with a Y-maze apparatus. The secondary endpoints included the resuscitation rate, which was evaluated from the beginning of the experiment to day 7, and histopathological evaluation performed after 7 days post-resuscitation (Fig. 2). The study was observational, and randomization of groups was not fully achieved due to practical constraints, as detailed in the limitations section.

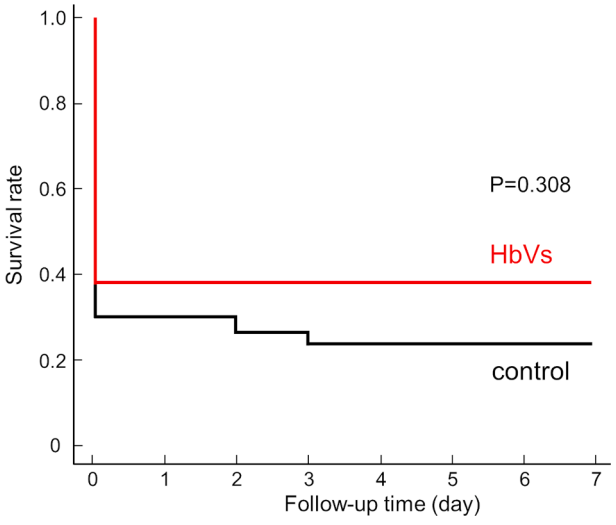

Fig. 3. Survival rate after cardiac arrest. Kaplan–Meier curves of the cumulative survival rate by 7 days after cardiac arrest. Thirty-one animals, 11 in the HbVs and 20 in the control group, were difficult to resuscitate. Additionally, in the control group, one rat died on each of days 2 and 3 after resuscitation. The survival rate was slightly higher in the HbVs group than in the control group, but the difference was not statistically significant (P = 0.308). HbVs, hemoglobin vesicles.

Neurological function evaluation

The Y-maze apparatus used three equally spaced arms (120°; 80 cm long × 30 cm high × 15 cm wide) to measure spontaneous activity and spatial memory over 8 min.[20] The amount of motor activity was measured as the extent to which the subject moved to a different arm during an 8-min period in the Y-maze. The rate at which the rats entered a different arm from the arm they had entered immediately before (alternation) was measured as an indicator of spatial working memory.

Histopathological analysis

The rats were euthanized under deep anesthesia using isoflurane. Their brains were quickly removed and fixed in 4 % paraformaldehyde. Coronal sections (3-μm thick) were prepared and used for histopathological evaluation. The hippocampus plays an important role in learning and memory and is selectively vulnerable to ischemic injury.[21] In this brain structure, the hippocampal ammonis angle 1 (CA1) region is particularly sensitive to cerebral ischemia and is commonly investigated to assess neuronal cell death after hypoxia.[22] Therefore, hematoxylin and eosin (HE), neuronal nuclear antigen (NeuN), ionized calcium-binding adapter molecule 1 (Iba-1), and glial fibrillary protein (GFAP) stainings were performed on the CA1 region. HE and Neu-N stainings were used to evaluate the normality or degeneration of neuronal nuclei. Microglial expression due to cerebral ischemia was assessed using Iba-1 staining. GFAP staining was performed to evaluate astrocyte accumulation during nerve injury and repair in the cerebral cortex. Areas positive for Iba-1, NeuN, and GFAP stainings were analyzed using a BZ-X Analyzer (BZ-X_Analyzer.exe 1.3.1.1, KEYENCE, Osaka, Japan).

Statistical analyses

The resuscitation rate was evaluated from Kaplan–Meier curves and the log-rank test using R, version 4.31 (https://www.r-project.org/). One-way analysis of variance and *t*-test were used to analyze behavioral and histopathological outcomes. P-values < 0.05 were considered statistically significant. With a significance level of 0.05, a statistical power of 0.8, and an effect size of 0.75 from the preliminary experiment (Neurological function evaluation), the required sample size was 7 (using R, version 4.31).

Results

The goal was to prepare seven animals each for the HbVs, control, and sham groups to conduct behavioral evaluation. Consequently, 54 rats were used in the present study. Thirty-one animals — 11 in the HbVs and 20 in the control groups — were difficult to resuscitate. Additionally, in the control group, one rat died on day 2 and one on day 3 after resuscitation

Survival rates were generated using Kaplan–Meier curves. The ROSC rates, defined as BP maintained above 60 mmHg for more than 10 min, were 39 % in the HbVs group and 24 % in the control group (Fig. 2). During the 7-day observation period, two animals in the control group that initially achieved ROSC died. Analysis using the log-rank test showed no significant differences in survival rates (P = 0.308; Fig. 3).

The amount of motor activity was rated as 10 (interquartile range [IQR]: 10–10.5) in the HbVs group and 8 (IQR: 7.5–8) in the control group. Spatial memory was rated as 60 % (IQR: 58 %–65) in the HbVs group and 43 % (IQR: 35 %–46) in the control group. Both behavioral parameters differed significantly between the HbVs and control groups

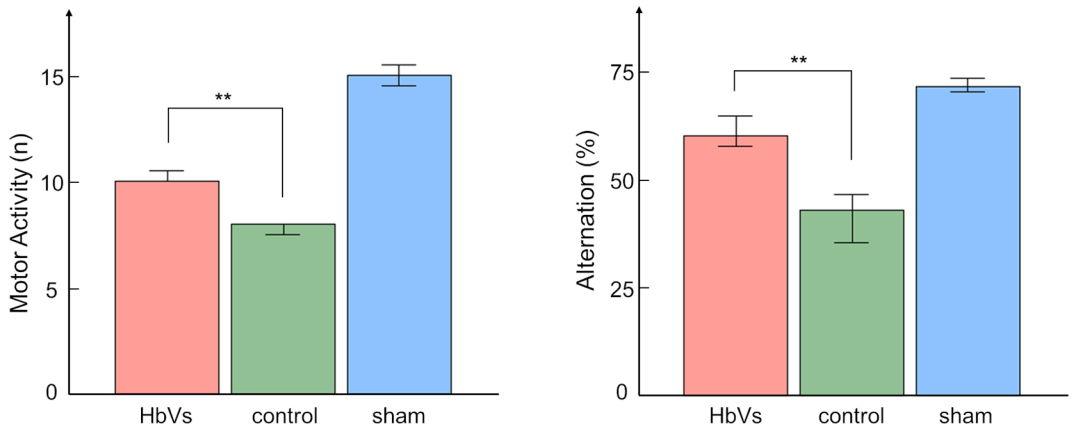

Fig. 4. HbVs effects on motor activity and spatial memory 7 days after cardiac arrest. One-way analysis of variance *t*-test were used to analyze motor activity and spatial memory. Significant differences ($P < 0.001$) were noted between the HbVs and control groups in terms of both motor activity and spatial memory. The graphs below the bars indicate interquartile ranges. There were seven rats in each group. HbVs, hemoglobin vesicles.

(both $P < 0.001$; Fig. 4).

Histopathological evaluation showed neuronal degeneration in both the HbVs and control groups after resuscitation; however, more cells were degenerated in the control group. Iba-1 staining revealed fewer stained areas in the HbVs group ($P < 0.001$), suggesting reduced microglial activation in the HbVs group. On the other hand, NeuN staining showed more stained areas in the HbVs group, suggesting more normal neurons ($P < 0.001$) in this group. HE staining revealed many normal-appearing neurons in the HbVs group (Fig. 5). GFAP staining showed fewer areas stained in the HbVs group in the cortical and CA-1 regions ($P < 0.001$), suggesting less nerve damage and a reduced increase in astrocytes after injury. This difference was more pronounced in the CA-1 region, which is particularly vulnerable to ischemia (Fig. 6). Detailed experimental results are summarized in the Supplemental material (Supplemental material. 2).

Discussion

In the present study including a rat asphyxiation model, the HbVs-treated group showed better motor activity and spatial recognition than did the untreated control group. Furthermore, HbVs administration suppressed reactive astrocytes and microglia in the CA-1 hippocampal region and in the cerebral cortex and demonstrated reduced necrosis and degeneration of normal neurons. These results indicated the neuroprotective effects of HbVs.

Various animal studies have investigated neuroprotection after cardiac arrest.[23–35] These studies aimed to identify methods to reduce neuronal damage after ischemia/hypoxemia by ameliorating the decrease in ATP levels. Unlike previous studies, the present study sought to administer oxygen to the ischemic neurons more rapidly. Many previous studies have demonstrated the usefulness of HbVs, given their oxygen-carrying capacity. Similar to red blood cells (RBCs), HbVs carry oxygen. In addition, although the concentration of Hb in the HbVs suspension is 10 g/dL, which is half that of a packed RBC concentrate for transfusion (20 g/dL)[36], HbVs are much smaller (250 nm) than RBCs (8 μm), and it is expected that HbVs would pass promptly through vessels with thrombus formation or constricted vessels. Aggravated clot formation is indeed often seen after cardiac arrest. [37,38] In a tracheal graft transplantation model, HbVs led to faster perfusion to the graft than RBCs, suggesting that HbVs may be favored over RBCs for achieving perfusion into ischemic areas.[39] The P_{50} of HbVs was originally 28 Torr; however, oxygen affinity could be altered by modifying the content of the allosteric effector PLP. In this study, the P_{50} of HbV was adjusted to a higher oxygen affinity – 15 Torr. Under these conditions, oxygen binds more easily than under normal conditions but is more likely to release oxygen to other tissues under ischemic conditions. In a rat model of hemorrhage after pneumonectomy, renal tissue oxygen pressures were higher in the group that received HbVs with a lower P_{50} value. These results suggest that the use of HbVs with lower P_{50} values may increase arterial blood oxygen content and improve the oxygenation of organ tissues after pneumonectomy[40] and that of other ischemic tissues[41–43]; moreover, it may be more suitable than RBCs for treating severe tissue hypoxia during cardiac arrest.

Post-resuscitation neuropathy is caused by hypoxia. Hypoxia leads to mitochondrial damage, generation of reactive oxygen species, disturbance of the electron transport chain, disturbance of the tricarboxylic acid cycle, mitochondrial swelling and collapse, cytochrome *C* release, apoptosis, degeneration of adenosine monophosphate-activated protein kinase, excitotoxicity, degeneration of cellular metabolism, lipids, proteins, and neurochemicals, endoplasmic reticulum damage, and inflammation,[44] primarily because ischemia decreases ATP production.[45] We propose that HbVs administration prevented neuropathy in our rat model by increasing the rapidity of oxygen carrier provision to the brain.

In the present study, an asphyxia model with pronounced hypoxemia was used to assess neuroprotection following resuscitation. Ventricular fibrillation and asphyxia are commonly used models of cardiac arrest in rats. We chose the asphyxiation model in this study because we believe that the effect of the oxygen-carrying capacity of HbVs would be more apparent. Among the different types of cardiac arrest, those associated with asphyxiation have a particularly poor prognosis because of the length of time that neurons are exposed to hypoxemia, which results in increased neuronal damage.[46] In previous asphyxiation models, resuscitation resumed 8 min after respiratory arrest; in this study, resuscitative actions were initiated 8 min after respiratory arrest. However, we considered the possibility that if oxygen administration was initiated immediately by artificial respiration, it would not have been possible to determine whether HbVs-induced ischemic brain oxygenation had been achieved. In clinical practice, many cases of asphyxia cannot be treated immediately because of the presence of foreign objects or ventilation problems. HbVs can be administered within approximately 10 s by establishing a venous or bone marrow tract. Therefore, we modified the model from the study by Huang et al. to include a 1-min interval between the start of chest compressions and restarting of the ventilator.

Although the possibility of a neuroprotective effect of HbVs has been suggested, no significant difference in the resuscitation rates was observed in the present study. The brain is vulnerable to ischemia: complete blockage of blood flow to the brain for as little as 5 min kills vulnerable neurons in several areas of the brain, whereas it takes 20–40 min of ischemia before cardiomyocytes and renal cells die.[47].

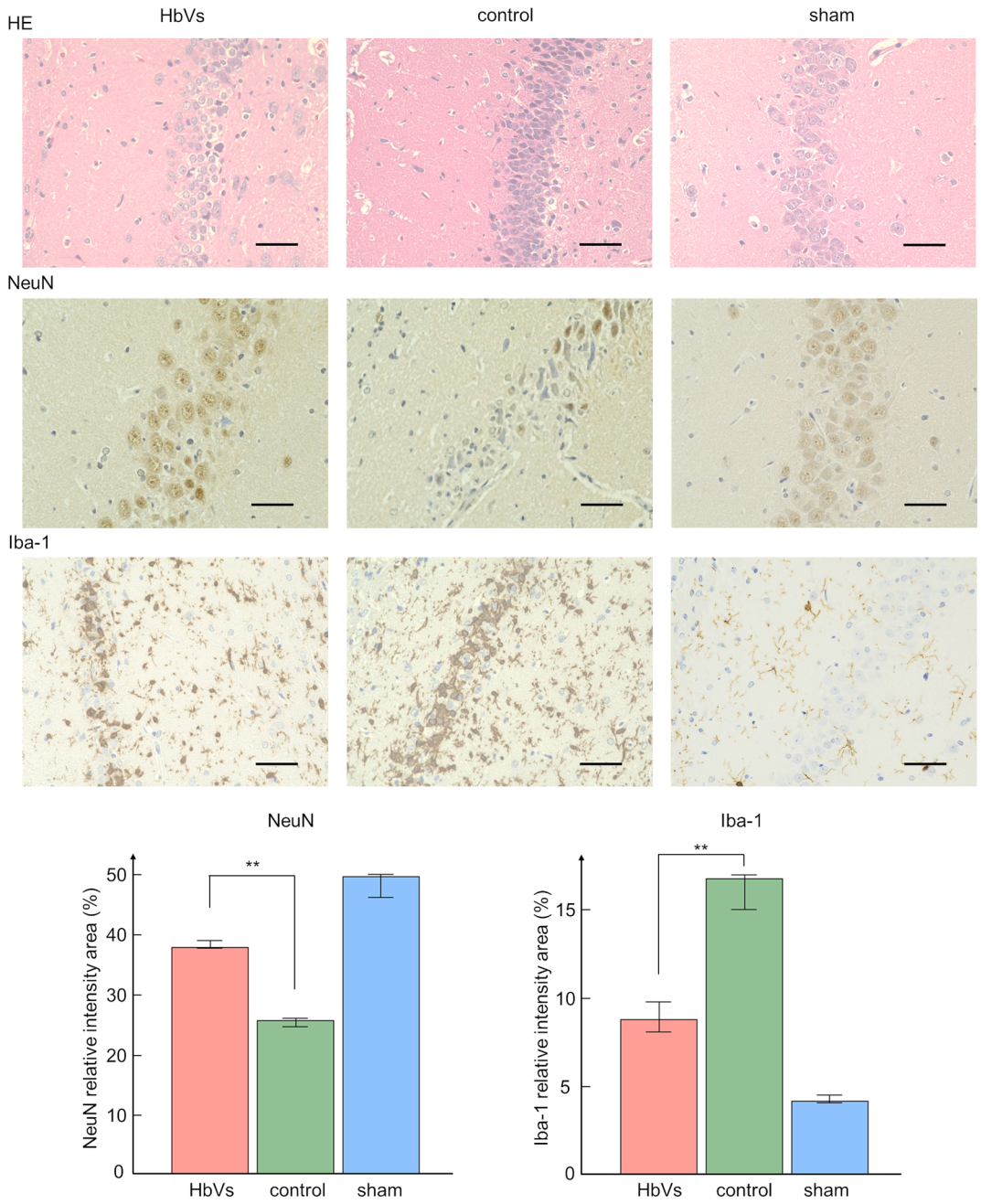

Fig. 5. HbVs effect on neuropathy and microglial activity in the hippocampal CA1 region 7 days after cardiac arrest Representative micrograph of hematoxylin and eosin staining, NeuN staining, and Iba-1 staining in the hippocampal CA1 region. Bar, 50 μm. NeuN-stained and Iba-1-stained areas in the CA1 region were analyzed using a one-way analysis of variance and *t*-test. ** Significant differences ($P < 0.001$) vs. the control group. The graphs below the bar indicate interquartile ranges. HbVs and control groups, n = 5 rats; sham group, n = 3. HbVs, hemoglobin vesicles.

Limitations

This study has several limitations. First, the sample size for quantitative pathology analysis was initially determined based on the evaluation of spatial memory. However, we concluded that a smaller sample size would be sufficient for the histopathological evaluation after observing the results. Therefore, we reduced the sample size for the HbVs and control groups to 5 animals each. This reduction in sample size may have potentially decreased the statistical power to detect smaller differences. Additionally, the cardiac arrest model used in this study was based on asphyxiation-induced PEA, which, while relevant to certain clinical scenarios, may not fully represent other types of cardiac arrest, such as ventricular fibrillation. This study was not fully randomized due to practical constraints, which may have introduced selection bias. The observational nature of the study should be considered when interpreting the results. Furthermore, we initially planned to measure oxygen content and lactate levels following HbVs administration. In the preliminary experiment, we used five rats to collect blood gas samples; unfortunately, all of them died during the process, which prevented us from measuring oxygen content and lactate levels. Lastly, while our findings suggest that HbVs may improve neurological outcomes and resuscitation rates in a rat model, it remains uncertain whether these results are directly applicable to humans. Larger animal studies and further research will be necessary to address these limitations and to evaluate the long-term effects of HbVs beyond the 7-day observation period.

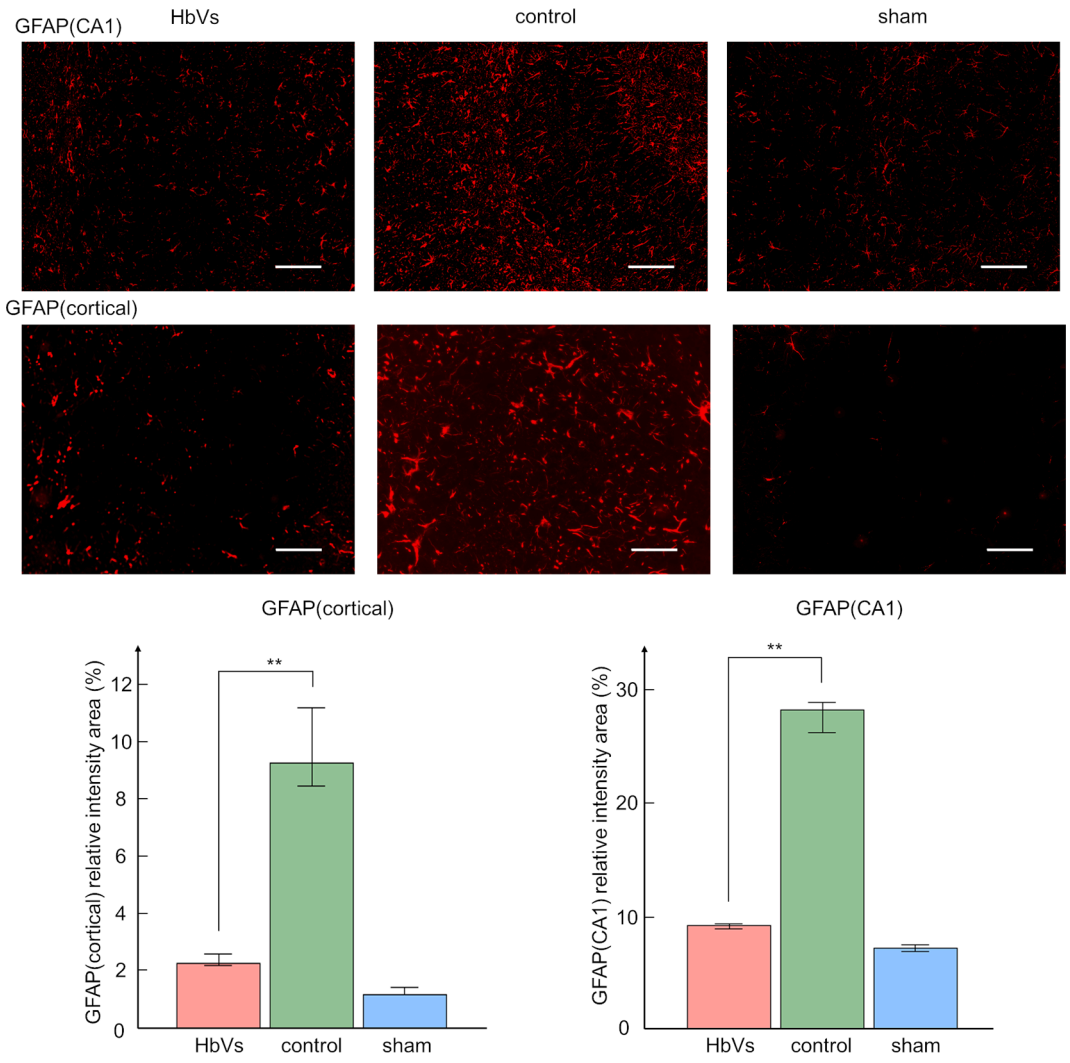

Fig. 6. Effect of HbVs on neuronal damage and astrocyte activation in the hippocampal CA1 region and cerebral cortex by 7 days after cardiac arrest. Representative micrographs of glial fibrillary acidic protein (GFAP) staining in the hippocampal CA1 region and cerebral cortex. Reactive astrocytes show GFAP labeling (red). Bar, 50 μm. The GFAP-stained areas in the hippocampal CA1 region and cerebral cortex were analyzed using a one-way analysis of variance and *t*-test. ** Significant differences ($P < 0.001$) vs. the control group. The graphs below the bar indicate interquartile ranges. HbVs and control groups, $n = 5$; sham group, $n = 3$. HbVs, hemoglobin vesicles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conclusions

The results of this animal study suggest that the administration of HbVs during resuscitation for cardiac arrest may improve neurological outcomes after resuscitation, as evidenced by improved behavioral function in rats. The survival rate was higher with no significant difference. Further studies in larger animal models are needed before considering human clinical trials.

Ethics approval statement

Approval number: 12,873.

Data Statement

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

CRediT authorship contribution statement

Keisuke Tsuruta: Writing – original draft, Visualization, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hidetada Fukushima:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Hiromi Sakai:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resplu.2024.100819.

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