

*Full Paper***Therapeutic Potentials of an Artificial Oxygen-Carrier, Liposome-Encapsulated Hemoglobin, for Ischemia/Reperfusion-Induced Cerebral Dysfunction in Rats**

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**Abstract.** We performed this study to elucidate whether a newly developed liposome-encapsulated hemoglobin, TRM-645 (TRM), can prevent cerebral dysfunction resulting from acute ischemic stroke when used as an oxygen carrier. Hippocampal long-term potentiation (LTP) in the perforant path–dentate gyrus synapses and anxiety-related behaviors in the elevated plus-maze test were evaluated as indices of cerebral functional outcomes in the rat with two-vessel occlusion (2VO), which was induced by 10-min clamping of bilateral common carotid arteries. Saline or TRM (hemoglobin concentration of 6 g/dl: 2.5 or 5 ml/kg) was administered via the tail vein immediately after ischemic insult. Hippocampal LTP formation was markedly impaired and the open arm durations in the elevated plus-maze decreased significantly 4 days after 2VO, compared to those of sham-operated (control) rats, suggesting the hippocampal synaptic dysfunction and anxiogenic properties in 2VO rats. TRM (5 ml/kg) restored the hippocampal LTP formation and normalized the anxiety-related behavior. TRM also improved the decreased tissue oxygen partial pressure in the 2VO rat hippocampus, possibly due to oxygen delivery to ischemic regions. Liposome-encapsulated hemoglobin TRM might have therapeutic potentials for protecting the brain from neurological complications associated with acute ischemic stroke, as a promising blood substitute for oxygen therapy.

**Keywords:** transient cerebral ischemia, long-term potentiation, artificial oxygen carrier, liposome-encapsulated hemoglobin, elevated-plus-maze

**Introduction**

Blood transfusion has become an increasingly important therapy because of development of highly advanced medical technologies such as organ transplantation and because of increasing frequent serious injuries from accidents/disasters (including natural and man-made). Moreover, the World Health Organization has reported that most countries fall short of ensuring the safety and sustainability of blood supplies because of the use of

untested blood units and poor blood-donor recruitment. Various types of red blood cell substitutes, including the acellular-type and the cellular-type hemoglobin (Hb)-based oxygen carriers, have been developed for clinical applications (1) to enable long-term storage and subsequent transfusion in emergency situations without concern for blood type and virus infections. However, clinical trials for acellular-type Hb derivatives have revealed some undesirable effects based on vasoconstriction and platelet aggregatory properties related to nitric monoxide scavenging action (2 – 5) and based on their rapid disappearance from circulating blood due to renal glomerular filtration (6, 7).

A stroma-free and liposome-encapsulated Hb, TRM-

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645 (TRM), which is an advanced formulation of Neo Red Cell to inactive blood-borne virus (8), is a promising Hb-based oxygen carrier. Particularly, TRM has a vesicular structure with a lipid bilayer membrane coating its surface with polyethylene glycol as a surface modifier, in which concentrated Hb molecules are encapsulated. This cellular-type Hb possesses some advantages as an artificial oxygen carrier: longer half-life in circulating blood and low vasoconstrictive activity (9, 10).

Cerebrovascular diseases such as focal or global cerebral ischemia reduce regional blood flow to affected regions, engendering hypoxia/ischemia, which might activate pathogenic cascades and eventual neuronal deficit/cell damage. Few therapies that are effective for cerebral ischemia are available because the therapeutic window for ischemia is narrow. Recently, artificial blood cell substitutes have received attention for their possible use in oxygen therapeutic methods for acute ischemic cerebrovascular diseases. Several studies have demonstrated that Neo Red Cell has sufficient oxygen transport capacity to tissues (11, 12). Moreover, liposome-encapsulated Hb can be beneficial for oxygenation of ischemic tissue because of its small particles (ca. 230-nm diameter) (13, 14). For those reasons, in addition to providing a red blood substitute for transfusion, TRM might be a candidate for use in oxygen therapy for treating ischemic diseases.

The hippocampus is known to be an extremely vulnerable brain region to ischemic attack. From the time that long-term potentiation (LTP) in the dentate gyrus of the rabbit hippocampus (15) was reported, LTP has been studied widely as an electrophysiological basis of learning and memory. We previously reported that hippocampal LTP formation is impaired by two-vessel occlusion (2VO) of the rat common carotid arteries for 10 min, without any marked tissue damages in the hippocampus (16). However, the pathophysiological significance of the impaired LTP formation after ischemia/reperfusion remains unclear because learning and memory in 2VO rats is unlikely to be degraded when evaluated using the Y-maze test. The hippocampus is a critical region for expression of emotion-related behaviors, along with the amygdala and other limbic/paralimbic systems. Furthermore, emotional stress reportedly suppresses hippocampal LTP formation (17, 18). Therefore, ischemia-induced alteration of hippocampal synaptic transmission might be related to changes in emotional traits such as anxiety and irritation.

We performed this study to clarify whether TRM, a newly developed liposome-encapsulated Hb, when used as an oxygen therapeutic agent, can prevent cerebral dysfunction caused by acute ischemic stroke. Thus, we evaluated the effects of TRM on hippocampal LTP in the

perforant path–dentate gyrus synapses and anxiety-related behaviors in the elevated plus-maze test as indices of cerebral functional outcomes in 2VO rats.

## Materials and Methods

### *Animals and materials*

Experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee at the Hokkaido University Graduate School of Medicine. Male Wistar/ST rats weighing 280–390 g were used (SLC, Inc., Hamamatsu). Animals were housed in a room with a 12-h light/dark cycle (lights on at 19:00) and were given free access to food and water.

A nanocapsule-type artificial oxygen carrier in which human concentrated Hb is encapsulated and suspended in saline at a Hb concentration of 6 g/dl, TRM, was kindly donated to us for these experiments (Terumo Corp., Tokyo).

### *Surgical procedures for 2VO*

All rats were divided into the following four groups: sham-operated (control) rats and saline-treated (5 ml/kg) or TRM-treated (2.5 or 5 ml/kg) 2VO rats. After induction of anesthesia, the trachea was intubated and the lung was mechanically ventilated. The halothane concentration was adjusted to 1% in 30% O<sub>2</sub> and N<sub>2</sub> and maintained throughout the experiment. After subcutaneous injection of 1% lidocaine, bilateral common carotid arteries were exposed and occluded with vessel clips for 10 min (2VO). Saline or TRM was administered via the tail vein at a volume of 2.5 or 5 ml/kg over 5 min immediately after the onset of a 10-min ischemic insult. Sham-operated rats without 2VO were used as control animals. Each animal's rectal temperature was maintained at  $37.5 \pm 0.5^\circ\text{C}$  throughout the experiment using an electric heating pad. After the animals recovered from anesthesia, tracheal tube extubation was performed. Each rat was returned to its home cage.

### *Recordings of regional cerebral blood flow (rCBF)*

For rCBF, the guide cannula for the probe was implanted to make contact with the dura mater surface on the right prefrontal cortex (coordinates: 3.2 mm anterior, 0.7 mm lateral to the bregma) 24 h before measurements. The probe was inserted into the guide cannula and then rCBF was measured pre-ischemia, during ischemia, and 60 min, 1 day, and 4 days after reperfusion using a laser tissue oxygen flowmeter with an optical fiber probe (FLO-C1; Omegawave, Inc., Tokyo). The obtained data were analyzed using a data recording and analysis system (PowerLab 2/20; AD Instruments Pty., Ltd., Castle Hill,

NSW, Australia).

#### *Recordings of tissue partial oxygen pressure (PtO<sub>2</sub>)*

PtO<sub>2</sub> was measured by an oxygen electrode method (POG-203; Unique Medical, Tokyo) before and 1 h after 2VO with a digital partial oxygen monitor. The oxygen electrode was calibrated at 150 mmHg in water aerated with air. The guide cannula was placed into the left hippocampal dentate gyrus (coordinates: 3.5-mm posterior, 2.0-mm lateral, 3.4-mm ventral to the bregma). Then the electrode was inserted into the guide cannula. The obtained data were analyzed using the PowerLab 2/20 data recording and analysis system.

#### *Recordings of electroencephalogram (EEG)*

For EEG recordings, stainless steel screw electrodes were implanted on the bilateral parietal bones (2-mm lateral and 3-mm posterior to the bregma) 24 h before measurements. The EEG signals were recorded for 10 min at pre-ischemia; during ischemia; and 60 min, 1 day, and 4 days after reperfusion. The EEG signals were analyzed using fast Fourier transformation; the power spectrum was calculated using a frequency analysis system (PowerLab 2/25, AD Instruments Pty., Ltd.). Each wave was defined as follows: delta waves, 1–3 Hz; theta waves, 4–7 Hz; alpha waves 8–13 Hz; and beta waves, 14–30 Hz.

#### *Recordings of LTP*

Hippocampal LTPs were recorded 4 days after 2VO. Some rats were examined 1 day and 7 days after 2VO to characterize time-dependent effects of 2VO on hippocampal LTP formation. Under 1% halothane anesthesia, tracheostomy was done and lungs were mechanically ventilated. The rat was fixed in a stereotaxic frame with the bregma and lambda in the same horizontal plane. A stainless steel bipolar stimulating electrode was inserted into the perforant path (coordinates: 8.1-mm posterior and 1.5-mm lateral to the bregma, 2.5-mm ventral from the cortical surface), and a recording electrode was inserted into the granule cell body layer of the dentate gyrus (coordinates: 3.5-mm posterior and 2.0-mm lateral to the bregma, 3.3-mm ventral from the cortical surface). Constant current square pulses (frequency, 0.1 Hz; duration, 250  $\mu$ s) were used to evoke the field responses, which were amplified and monitored with an oscilloscope (VC-10; Nihon Kohden Corp., Tokyo). They were averaged by using the PowerLab 2/20 data analysis system. High-frequency tetanic stimulation (tetanus) consisting of 10 trains at 1 Hz, each composed of 8 pulses at 400 Hz, and at the baseline stimulation voltage. After a 15-min baseline recording, the population spike amplitude was measured for 60 min following tetanic stimulation.

The values were normalized relative to the 5-min mean value obtained immediately before tetanic stimulation. The time course changes and the area under the curve (AUC) of 0–60 min after tetanus were evaluated as an index of cerebral functional outcome.

#### *Measurement of anxiety-related behaviors*

Anxiety-related behaviors based on unconditioned fear were measured using the elevated plus-maze test (19, 20). The apparatus consisted of a central platform (10  $\times$  10 cm), two opposite open arms (50  $\times$  10 cm), and enclosed arms (50  $\times$  10  $\times$  40 cm), which were elevated 50-cm above the floor. The behavior of each rat was monitored using a CCD camera and was recorded/analyzed using a software package (LimeLight; Actimetrics, Wilmette, IL, USA); all measurements of rat movements were generated automatically by the software. Briefly, the rats were placed on a central platform and allowed to enter into the arms freely for 10 min. The total numbers of entries into all four arms (total arm entries) was recorded as an index of locomotive activity. The open arm entries and time spent in the open arms were calculated respectively as the percentage of the total arm entries and of the time spent in all four arms.

#### *Statistics*

The resultant values were expressed as the mean  $\pm$  S.E.M. Student's paired *t*-test was performed to test for significant differences within groups. One-way analysis of variance (ANOVA) or a Mann-Whitney U test was used to test to determine significant differences between the experimental groups. When applicable, a Tukey-Kramer or Dunnett test was used as a post hoc test. Statistically significant differences were inferred for *P* < 0.05. Statistical analyses were performed by computer software (StatView ver. 5.0; SAS Institute, Inc., Cary, NC, USA).

## **Results**

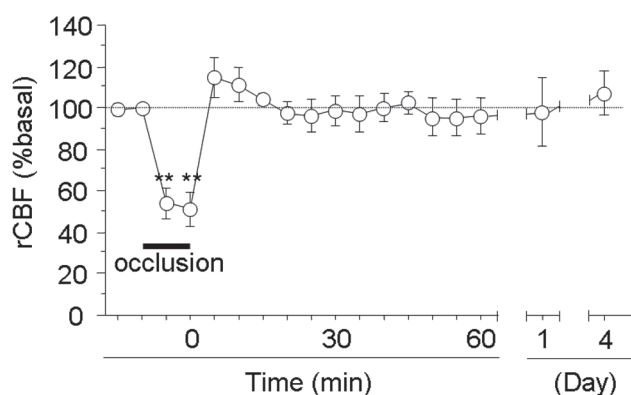
#### *Time-course changes of neurological outcome in 2VO rats*

*Time-course changes of rCBF:* Figure 1 shows that rCBF during 2VO in the prefrontal cortex rapidly reduced to approximately 40% of the pre-ischemic value. After reperfusion, a slight and transient increase in rCBF was observed. However, rCBF recovered to the pre-ischemic level in 1 day and was maintained until at least 4 days after 2VO.

*Time-course changes of electrophysiological parameters:* As Fig. 2 shows, the EEG during 2VO had a decreased fast-wave component and an increased slow-wave component. That is, the alpha and beta waves

decreased to approximately 50% and the delta waves increased to approximately 140% of the pre-ischemic values. The decreased alpha waves recovered at 30 min after reperfusion, but the decreased beta waves and the increased delta waves changed inversely 1 day after 2VO. All waves had reverted to their pre-ischemic EEG patterns by 4 days after 2VO.

Figure 3 shows that the amplitude of evoked potentials was increased significantly after high-frequency tetanic stimulation as compared to the pre-stimulation value, signaling LTP formation in the perforant path–dentate gyrus synapses in control rats. Meanwhile, LTP formation was impaired significantly in 2VO rats. In contrast,



**Fig. 1.** Time-course changes of regional cerebral blood flow (rCBF) in the right prefrontal cortex on 2-vessel occlusion (2VO). During 2VO, the value of rCBF was significantly lower than the basal value. However, rCBF reverted to the basal value on Day 1 and Day 4. Values indicate the mean  $\pm$  S.E.M. \*\* $P < 0.01$  vs. the basal value.

the 2VO-induced LTP impairment was more markedly observed at 4 days than at 1 day after 2VO. The impaired LTP at 7 days was comparable to that at 4 days after 2VO.

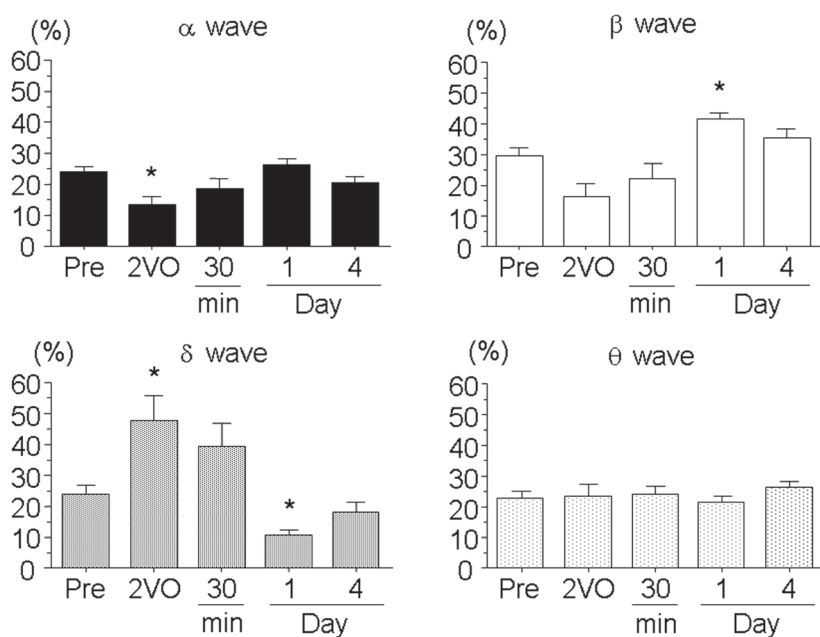
#### Effects of TRM in 2VO occlusion rats

**Effects on rCBF and  $PtO_2$ :** As shown in Fig. 4A,  $PtO_2$  during 2VO decreased to approximately 70% of the pre-occlusion value in the DG of the 2VO rat hippocampus. This decreased  $PtO_2$  was inhibited significantly by 5.0 ml/kg TRM compared to control rats [TRM vs. control (time after occlusion): 93.9% vs. 72.4% (0–5 min), 88.6% vs. 66.6% (5–10 min), respectively].

On the other hand, as shown in Fig. 4B, the decreased rCBF during 2VO in the prefrontal cortex was not affected by administration of 5.0 ml/kg TRM when compared to control rats.

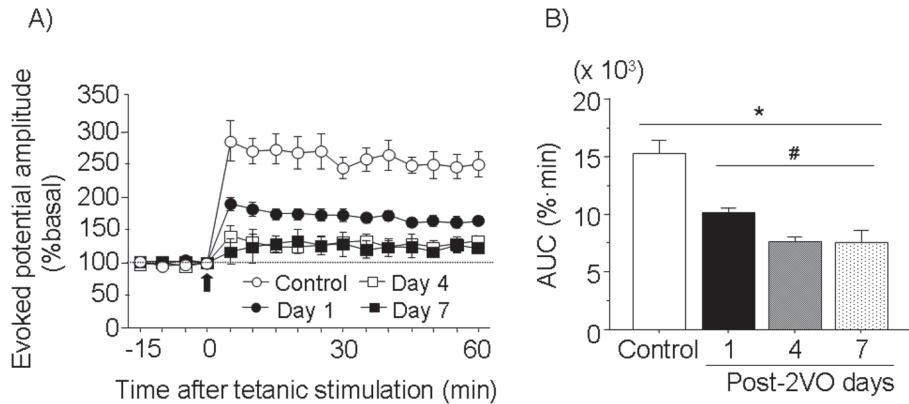
**Effects on hippocampal LTP impairment in 2VO rats:** Impaired hippocampal LTP formation (approximately 120% of the pre-tetanic value), which was observed 4 days after reperfusion, was restored by TRM (2.5–5 ml/kg) in a volume-dependent manner. The 2.5 ml/kg TRM restored LTP formation slightly but insufficiently (ca. 130% of pre-tetanic value), whereas 5.0 ml/kg TRM ameliorated LTP formation to the same degree as that of the control rats (Fig. 5A). A significant effect of 5.0 ml/kg TRM was also recorded, as evaluated by AUC, reflecting the ensemble effect of time-course changes in LTP (Fig. 5B).

**Effects on the anxiety-related behavior:** In the elevated plus-maze test performed 4 days after 2VO, time spent in the open arm was significantly less for saline-treated

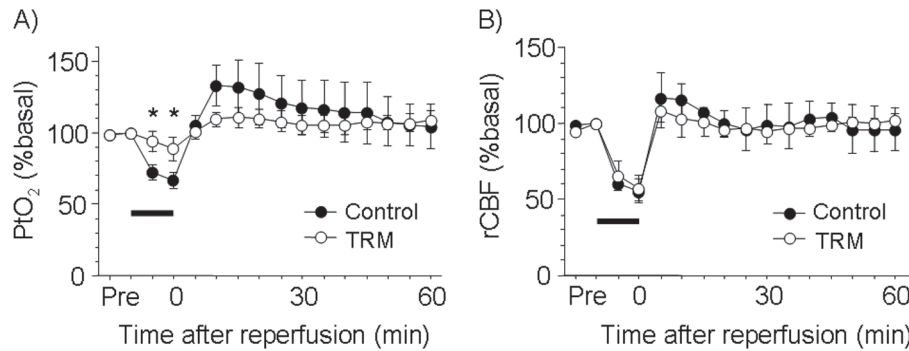


**Fig. 2.** Time-course changes of the electroencephalogram (EEG) in 2VO rats. EEG showed reduced frequency of fast waves (alpha and beta) with predominant delta waves during 2VO. However, the EEG wave pattern reverted to the pre-2VO pattern on Day 4. Values indicate the mean  $\pm$  S.E.M. \* $P < 0.05$  vs. the respective basal values.

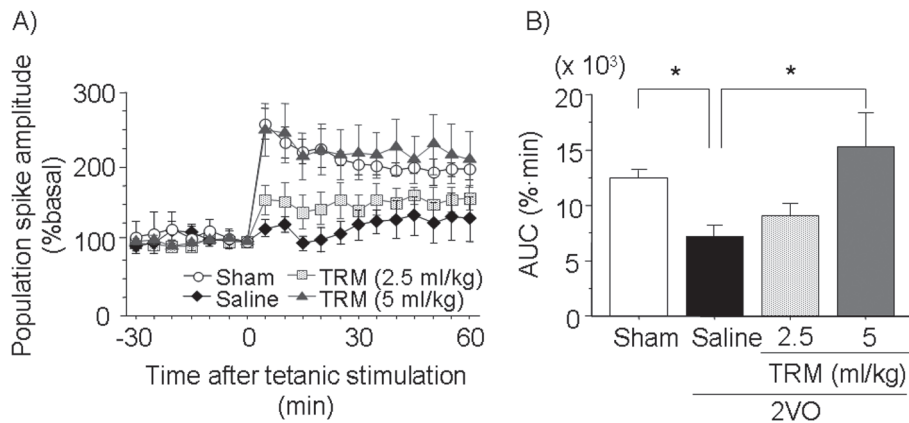




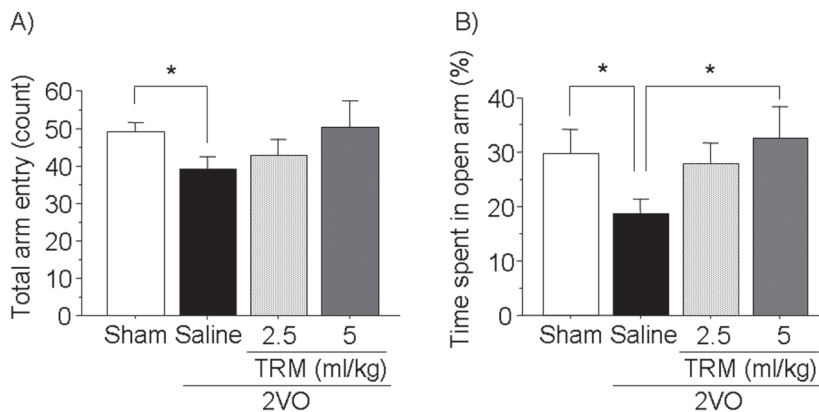
**Fig. 3.** Changes over time of hippocampal long-term potentiation in the perforant path–dentate gyrus synapses after 2VO. Time-course changes (A) and the area under the curve (AUC) (B) of the population spike amplitude during 60 min after tetanic stimulation. The values are expressed as % means  $\pm$  S.E.M. of the respective pre-tetanic stimulation amplitude ( $n = 5 - 10$ ). \* $P < 0.05$  intact vs. 2VO rats. # $P < 0.05$  Day 1 vs. Day 4 and Day 7.



**Fig. 4.** Time-course changes of partial oxygen pressure in tissue (PtO<sub>2</sub>) (A) and regional cerebral blood flow (rCBF) (B) in 2VO rats. The PtO<sub>2</sub> changes during 2VO were significantly lower than the respective basal values, except for those in TRM-treated rats. Decreased PtO<sub>2</sub> in TRM-treated rats was inhibited significantly compared to control and saline-treated rats. The value of rCBF was significantly lower than the respective basal values; however, no significant differences were detected among the three groups. Values indicate the mean  $\pm$  S.E.M. \* $P < 0.05$ , differences among the three groups.



**Fig. 5.** Effect of TRM-645 on impairment of hippocampal LTP formation in 2VO rats. Time-course changes of LTP (A) and the area under the curve (AUC) (B) of the population spike amplitude during 60 min after tetanic stimulation. TRM (5 ml/kg)-treated rats showed ameliorated LTP formation on day 4 after 2VO. Values indicate the mean  $\pm$  S.E.M. \* $P < 0.05$ : saline-treated rats vs. TRM-treated and sham-operated rats.



**Fig. 6.** Effect of TRM-645 on anxiety-related behaviors of 2VO rats in the elevated plus-maze test. Saline-treated rats exhibited significantly less open-arm time than sham-operated and TRM (5 ml/kg)-treated rats. No significant differences were found between sham-operated and TRM-treated rats. Values indicate the mean  $\pm$  S.E.M. \* $P$  < 0.05; saline-treated rats vs. TRM-treated and sham-operated rats.

2VO rats than for control rats. This 2VO-induced anxiogenic-like behavior was alleviated significantly in a volume-dependent manner by TRM administered during 2VO (Fig. 6B). Total arm entries in saline-treated 2VO rats decreased slightly but significantly in comparison to the data of control rats. However, TRM did not impart significant effects on total arm entries, indicating that TRM-induced effects on emotional behaviors did not result from effects on motor activity (Fig. 6A).

## Discussion

In this study, we evaluated the effects of a newly developed artificial oxygen carrier, TRM, on cerebral dysfunction induced by transient global ischemia, as indices of hippocampal LTP and anxiety-related behaviors in 2VO rats. TRM ameliorated the impaired LTP formation observed 4 days after 2VO, when injected immediately after the onset of a 10-min ischemic insult. In addition, TRM normalized the emotional disturbance, as measured by the increased time spent in the open arm in the elevated plus-maze test, indicating the anxiogenic property in 2VO rats. Our findings suggest that a liposome-encapsulated Hb, TRM, might have therapeutic use in oxygen therapy by minimizing neurological complications associated with ischemic stroke.

In this study, we characterized the 2VO-induced cerebral dysfunction from the viewpoint of temporal changes in rCBF, EEG, and synaptic plasticity after ischemic insult since our previous study showed no pathological changes in the 2VO rat hippocampus (21, 22). Any marked pathological changes in the hippocampus were not noted in the 2VO group compared to the sham-operated groups, using the staining method with neutral red, a marker of irreversible damage after ischemia (22). Furthermore, we also confirmed the same evidence using 2,3-triphenyltetrazolium chloride (TTC) staining, a marker of intact cellular metabolism (data not shown).

These histological findings indicated that microscopic changes, such as neuronal cell death, were not evident in the 2VO rat hippocampus.

During a 10-min clamping of bilateral common carotid arteries, rCBF was reduced significantly to ca. 40% of the basal value; after releasing the clamps that value reverted to the pre-ischemic level and remained at that level until 4 days after 2VO. The EEG data show that delta wave activities increased, whereas those of alpha and beta waves decreased during occlusion, which might reflect cerebral ischemic changes resulting from carotid artery occlusion (23, 24). The EEG profiles inversely changed just after reperfusion: that is, the activities of delta waves decreased and those of beta waves increased, which caused the reversion to the pre-ischemic profiles by 4 days after 2VO. Thus, it can be inferred that cerebral circulation and spontaneous neurological activity can recover to normal by 4 days after 2VO. It is generally accepted that EEG as a cerebral functional measure closely correlates with CBF. Our group previously reported increases in slow-wave components and concomitant decreases in fast-wave components in the ischemic rat with multifocal infarction, accompanied with a decrease in cortical CBF (25). Thus, 2VO-induced EEG changes, that is, increases in the delta waves and decreases in the beta waves, plausibly reflect global cerebral hypofunction related to transient hemodynamic changes after ischemia.

In contrast to the apparent recovery of rCBF and EEG 4 days after 2VO, hippocampal LTP formation in 2VO rats was progressively impaired with time after ischemic insult; 4 days after 2VO, LTP was more impaired than that observed 1 day after 2VO. These findings reveal the delayed deficit of synaptic function in the perforant path–dentate gyrus synapses and its vulnerability to ischemia. In other words, hippocampal LTP is a sensitive index for evaluating cerebral neuronal outcomes after ischemia/reperfusion. The 2VO-induced LTP impair-

ment without pathological deficit in the hippocampus (21, 22) may be due to synaptic dysfunction, that is, abnormal neurotransmission, receptor dysfunction, aberrant biosynthesis of neurotransmitter, and exaggerated intracellular signaling pathway(s), as a consequence of ischemic attack. Indeed, an ischemic episode induces a profound structural remodeling of synaptic networks in the hippocampus through AMPA- and NMDA-receptor activation, which can lead to abnormal neuronal activities (26).

TRM (2.5 and 5 ml/kg, intravenously) improved the LTP formation of the 2VO rat hippocampus in a volume-dependent manner. In rats treated with 5 ml/kg TRM, hippocampal LTP was induced and maintained to the same extent as that in control rats, presumably indicating the protective effect of TRM on hippocampal synaptic function against ischemic attack. Pathophysiological significance of 2VO-induced synaptic dysfunction remains unclear because LTP impairment did not accompany learning and memory deficits as examined using the Y-maze test (data not shown). On the other hand, the elevated plus-maze test revealed that saline-treated 2VO rats showed significant decrease in time spent on the open arms, as compared with control rats, reflecting anxiogenic effects of ischemic/reperfusion. Indeed, clinical studies have shown that the prevalence of depressive illness and anxiety disorders is 20% – 50% following a stroke (27). The hippocampus is a pivotal region for emotional expression, along with limbic and prefrontal areas, including the amygdala and frontal cortex (28, 29). Psychiatric stress is known to alter emotional expression and suppress hippocampal LTP formation (17, 18). In the present study, rCBF in the frontal cortex decreased in the 2VO rat, which plausibly reflects those in the limbic and prefrontal area including the hippocampus. We supposed therefore that anxiety-like behavior observed in the 2VO rat is a behavioral consequence of transient cerebral ischemia, which could be closely correlated with synaptic dysfunction in the neural circuits, such as hippocampal LTP impairment. Taken together, it is presumed that the altered hippocampal synaptic transmission in 2VO rats might be associated with emotional disturbances such as anxiety and irritation after ischemic stroke. Our findings that TRM normalized anxiety-related behaviors as well as LTP impairment in 2VO rats possibly indicates a critical role of the hippocampus in expression of appropriate emotional behavior and further supports the beneficial effects of this liposome-encapsulated Hb on ischemia-induced neurological deficit.

A possible mechanism, by which TRM improved 2VO-induced neurological outcomes, might be its oxygen delivery ability to the affected ischemic regions via collaterals, through which red blood cells can not pass

because of their large size. The speculation is supported by results of PtO<sub>2</sub> profiles that PtO<sub>2</sub> reduction during a 10-min 2VO was attenuated significantly in TRM-treated 2VO rats compared to saline-treated 2VO rats. Furthermore, 2VO rats showed reactive hyperoxic hyperemia immediately after reperfusion, which presumably resulted from neuronal and endothelial nitric oxide action (30), whereas TRM-treated 2VO rats showed no hyperoxic state. Takeoka et al. (31) reported that cellular Hb vesicle dispersion, ferryl Hb, and ferric ion are generated by the reaction of Hb with H<sub>2</sub>O<sub>2</sub> permeated through the bilayer membrane of the vesicle; however, they are stably encapsulated within the vesicle and do not cause lipid peroxidation. These results lead us to speculate that TRM might protect cerebral tissues from excessive oxygen supply to the cerebral tissue affected, which consequently generates reactive oxygen species and free radicals.

Ischemic stroke is a leading cause of mortality and the leading cause of disability in the world (32). Ischemic strokes include focal and global cerebral ischemia, which reduce regional blood flow to affected regions and cause hypoxia-ischemia, with consequent activation of pathogenic cascades, with eventual neuronal deficit and cell damage (33). Many drugs have been evaluated for ameliorating neurological outcomes associated with cerebral ischemic strokes. However, few effective therapies are available for cerebral ischemia because the therapeutic time window for ischemia is narrow (34, 35). Indeed, pharmacological therapies in the acute ischemic stage are confined to thrombolysis and free-radical scavenging (36). Chalela et al. (37) described that tissue plasminogen activator (t-PA) is the only approved therapy for acute ischemic stroke. However, serious side effects, such as intracerebral hemorrhage, are reported in approximately 6% of patients given intravenous t-PA (38). Edaravone, a free-radical scavenger, is well known as a potential therapeutic agent for global ischemic stroke (39 – 42). Otani et al. (43) reported that edaravone ameliorated rat hippocampal LTP formation after transient incomplete ischemia, 2VO, with a therapeutic time window. For oxygen supply to the ischemic region, hyperbaric oxygenation has been used as primary or adjunctive therapy in the acute or subacute stage (32). The present findings that PtO<sub>2</sub> reduction during the ischemic period for 10 min in TRM-treated 2VO rats was significantly less potent than that in saline-treated 2VO rats suggests that TRM-induced oxygenation of ischemic regions can alleviate LTP formation and anxiety-like behaviors after acute cerebral ischemia/reperfusion. Although multidisciplinary approaches for clinical practice will be needed for possible treatment in the acute ischemic stage, it is expected that TRM could be useful as one therapeutic measure for oxygenation in the ischemic penumbra and

might thereby contribute to minimize brain neurological complications associated with acute ischemic stroke.

In conclusion, the present results demonstrated that cellular-type Hb, TRM, alleviated the ischemic complications, that is, hippocampal synaptic dysfunction and emotional disturbance in transient global ischemic model rats. Our findings indicate that the liposome-encapsulated Hb might be a promising candidate, not only as an artificial blood cell substitute for transfusion, but also as an oxygen therapeutic agent for acute ischemic stroke.

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