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Recombinant human soluble thrombomodulin ameliorates cerebral ischemic injury through a high-mobility group box1 inhibitory mechanism without hemorrhagic complications in mice

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Abstract

Background: It has been reported that recombinant human soluble thrombomodulin (rhsTM) has a high-mobility group box (HMGB)1 inhibitory effect. Some investigators reported that HMGB1 is associated with ischemic stroke. However, there have been no previous studies to determine whether rhsTM can ameliorate cerebral ischemic injury through its HMGB1 inhibitory mechanism in ischemic stroke. We investigated the effects of rhsTM on cerebral ischemic injury in a 4-hour middle cerebral artery occlusion (MCAO) murine model.

Methods: rhsTM (1 or 5 mg/kg, i.v.) was administered immediately after 4-hour MCAO. Infarct volume, motor coordination, plasma HMGB1 level, and hemorrhage volume were evaluated 24 hours after 4-hour MCAO.

Results: The infarct volume ($P < 0.05$) was reduced by rhsTM in mice subjected to 4-hour MCAO in a dose-dependent manner. Moreover, rhsTM (5 mg/kg) significantly improved motor coordination determined by the rotarod test ($P < 0.05$), and significantly decreased plasma HMGB1 level compared with vehicle-treated controls ($P < 0.001$). In addition, there was no difference in hemorrhage volume between vehicle-treated controls and the rhsTM treatment group.

Conclusions: This represents the first report that rhsTM ameliorates cerebral ischemic injury through an HMGB1 inhibitory mechanism without hemorrhagic complications in mice. Taken together, these observations indicate a palliative effect of rhsTM and suggest new therapeutic possibilities for treatment of ischemic stroke via inhibition of HMGB1.

Keywords: thrombomodulin; high-mobility group box 1(HMGB1); cerebral ischemia; stroke

1. Introduction

Stroke is the leading cause of morbidity and the third leading cause of mortality in the USA [1]. Approximately 80% of acute strokes are ischemic, with the rest being hemorrhagic (20% are caused by intracerebral or subarachnoid hemorrhage) [2]. About 25% – 35% of stroke cases present with large vessel occlusion [3].

High-mobility group box (HMGB)1 is widely expressed in various tissues, including the brain. The level of HMGB1 is elevated in the plasma of stroke patients, and is released from ischemic brain tissue in a mouse model of cerebral ischemia [4]. In addition, HMGB1, a non-histone DNA-binding protein, has been reported to be released in large quantities into the extracellular space immediately after ischemic insult and to induce neuroinflammation and microglial activation in the postischemic brain [5]. These results suggest that HMGB1 may be a clinically useful biochemical marker for ischemic stroke as well as a target for therapeutic interventions.

Thrombomodulin (TM) is a cell-surface glycoprotein that is widely expressed in a variety of cell types. TM acts as a thrombin receptor on the surface of vascular endothelial cells; binding of TM to the thrombin receptor significantly decreases the effect of thrombin in conversion of fibrinogen to fibrin, activation of coagulation factors V and VIII, and platelets, and its D1 (lectin-like) domain has potent antiinflammatory effects through a variety of molecular mechanisms [6]. It has been reported that the D1 domain of TM bound to HMGB1 has anti-inflammatory properties [7] as one of the antiinflammatory mechanisms of action of TM. In addition, recombinant human soluble TM (rhsTM) was reported to associate with HMGB1 in some animal models, such as acute lung distress syndrome, sepsis, heatstroke, and hyperalgesia [8-12]. In addition, the commercially developed rhsTM preparation, Recomodulin, was approved for the treatment of disseminated intravascular coagulation (DIC) resulting from infection and cancer in 2008 in Japan [13-17]. rhsTM is widely used for septic DIC in Japan. Moreover, Solulin [18], another rhsTM preparation, has been reported to reduce infarct volume by promoting reperfusion in mice subjected to middle cerebral artery occlusion (MCAO) induced by photothrombosis [19, 20]. However, there have been no investigations to evaluate the therapeutic usefulness of rhsTM in ischemic stroke through mechanisms involving HMGB1 in mice subjected to 4-hour MCAO. It remains unclear whether rhsTM can improve neurological impairment in this murine ischemic stroke model. The present study was performed to investigate whether rhsTM can ameliorate cerebral ischemic injury and neurological impairment through its inhibitory effect on HMGB1 in mice subjected to 4-hour MCAO.

2. Materials and Methods

2.1.Animals

Male ddY mice (25 – 35 g; Kiwa Experimental Animal Laboratory, Wakayama, Japan) were kept under a 12-hour light/dark cycle (lights on from 07:00 to 19:00) in an air-conditioned (23°C ± 2°C) room with food (CE-2; Clea Japan, Tokyo, Japan) and water available ad libitum. All procedures regarding animal care and use were performed in compliance with the regulations established by the Experimental Animal Care and Use Committee of Fukuoka University.

2.2.Focal cerebral ischemia

Focal cerebral ischemia was induced according to the method described in our previous reports [21-23]. The mice were re-anesthetized with isoflurane (Escain; Pfizer, Osaka, Japan) 4 hours after occlusion, and reperfusion was established by withdrawal of the filament. MCAO was confirmed by examining forelimb flexion after awakening from anesthesia.

2.3.Cerebral infarct volume and hemorrhage volume 24 hours after MCAO

The animals were sacrificed by decapitation 24 hours after MCAO. The brains were removed and cut into four coronal sections 2 mm thick using a mouse brain matrix. The hemorrhagic area was measured in each slice using an image analysis system (NIH Image, version 1.63; National Institutes of Health, Bethesda, MD), and the hemorrhage volume was calculated. Cerebral infarct volume was also measured by image analysis in slices stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO).

2.4.Neurological score

Neurological score [21] was measured 24 hours after cerebral ischemia, and divided into five groups: 0 = normal motor function, 1 = flexion of the torso and of the contralateral forelimb on lifting of the animal by the tail, 2 = circling to the ipsilateral side but normal posture at rest, 3 = circling to the ipsilateral side, 4 = rolling to the ipsilateral side, and 5 = leaning to the ipsilateral side at rest (no spontaneous motor activity).

2.5.Rotarod test in MCAO Mice

Motor coordination was measured by the rotarod test as described previously [21, 22]. Mice were placed on a rod 3 cm in diameter with a nonskid surface rotated at a speed of 10 rpm (Neuroscience Inc., Tokyo, Japan), and the latency to fall was measured for up to 2 minutes.

2.6.HMGB1 measurements

Blood samples were collected 24 hours after MCAO in 4-hour MCAO mice. Plasma was obtained after centrifugation (1200 rpm for 10 minutes at 4°C). Plasma HMGB1 levels were measured by enzyme-linked immunoadsorbent assay (ELISA; Shino-Test Corporation, Kanagawa, Japan).

2.7.Drug preparation and administration

rhsTM, also known as ART-123 (Recomodulin), was provided by Asahi Kasei Pharma (Tokyo, Japan). rhsTM was dissolved in distilled water, and administered after 4-hour MCA occlusion (1 or 5 mg/kg i.v.).

2.8.Statistical analysis

Data are presented as means ± standard error of the mean (SEM). The data were analyzed by one-way analysis of variance (ANOVA) followed by Turkey's post hoc test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. All statistical analyses were performed using JMP® version 10 (SAS Institute, Cary, NC).

3. Results

3.1.Effects of rhsTM on brain infarct volume 24 hours after 4-hour MCAO

Infarct volume was measured 24 hours after 4-hour MCAO cerebral ischemia by triphenyltetrazolium chloride staining. The mean infarct volumes were 93.1 ± 7.0 mm^3 in the vehicle-treated group, 76.7 ± 7.3 mm^3 in the rhsTM (1 mg/kg)-treated MCAO group, 64.8 ± 6.4 mm^3 in the rhsTM (5 mg/kg)-treated MCAO group. The cerebral infarct volume was reduced by rhsTM in a dose-dependent manner ($F(2,32) = 4.804$, $P < 0.05$, one-way ANOVA), and the infarct volume was significantly improved at a dose of 5 mg/kg ($P < 0.05$, Tukey's test) compared with the vehicle-treated group (Figure 1).

3.2.Effects of rhsTM on neurological score and motor coordination in 4-hour MCAO

The mean neurological scores were 3.6 ± 0.3 in the vehicle-treated group, 3.6 ± 0.3 in the rhsTM (1 mg/kg)-treated MCAO group, and 2.9 ± 0.3 in the rhsTM (5 mg/kg)-treated MCAO group. rhsTM at a dose of 5 mg/kg showed a tendency to improve the neurological score in comparison with the vehicle-treated controls, but the effect was not statistically significant.

Mean riding times in the rotarod test were 120.0 ± 7.7 s in the sham-treated group, 22.3 ± 12.2 s in the vehicle-treated group, and 66.2 ± 9.9 s in the rhsTM (5 mg/kg)-treated MCAO group. Motor coordination in the rotarod test was significantly impaired in the vehicle-treated group ($F(2,27) = 25.387$, $P < 0.001$, one-way ANOVA). rhsTM at a dose of 5 mg/kg ($P < 0.05$, Tukey's test) significantly improved motor coordination in comparison with the vehicle-treated group (Figure 2).

3.3.Effects of rhsTM on HMGB1 in the plasma

The mean plasma level of HMGB1 was significantly increased in the vehicle-treated group compared with the sham-treated group (37.0 ± 3.11 ng/mL and 18.2 ± 3.81 ng/mL, respectively, $P < 0.01$, Tukey's test). The mean plasma levels of HMGB1 were 20.1 ± 3.81 ng/mL in the rhsTM (1 mg/kg)-treated MCAO group and 14.9 ± 3.11 ng/mL in the rhsTM (5 mg/kg)-treated MCAO group. These observations indicated that rhsTM dose-dependently suppressed the plasma HMGB1 level in comparison with the vehicle-treated group ($F(3,26) = 9.682$, $P < 0.001$, one way ANOVA). rhsTM at doses of 1 mg/kg ($P < 0.01$, Tukey's test) and 5 mg/kg ($P < 0.001$, Tukey's test) significantly decreased the plasma HMGB1 level (Figure 3).

3.4.Effects of rhsTM on hemorrhage volume 24 hours after 4-hour MCAO

The mean hemorrhage volumes were 3.47 ± 2.42 mm^3 in the vehicle-treated group, 6.91 ± 3.25 mm^3 in the rhsTM 1mg/kg-treated MCAO group, and 5.08 ± 2.57 mm^3 in the rhsTM (5 mg/kg)-treated MCAO group. The differences in hemorrhage volume between these three groups were not significant (Figure 4).

4. Discussion

The present study was performed to evaluate the effects of rhsTM against ischemic brain injury and neurological impairment through reductions in HMGB1 levels in mice subjected to 4-hour MCAO. The results presented here indicated that delayed treatment

with rhsTM reduced the infarct volume, neurological impairment, and plasma HMGB1 level without intracerebral hemorrhage in this 4-hour MCAO model. This represents the first report demonstrating that rhsTM can ameliorate cerebral ischemic injury through an HMGB1 inhibitory mechanism without hemorrhagic complications. Thus, rhsTM may have a wide therapeutic time window in patients with ischemic stroke.

rhsTM significantly decreased plasma level of HMGB1 and neurological impairment induced by cerebral ischemia in comparison with vehicle-treated controls (see Figure 3), suggesting that rhsTM inhibits plasma expression of HMGB1 in this 4-hour MCAO model. We reported previously that both minocycline [21] and cannabidiol [24] significantly reduced plasma HMGB1 levels and improved motor coordination in comparison with vehicle-treated controls. Kim et al.[5] reported that anti-HMGB1 antibody inhibited inflammation and microglial activation induced by cerebral ischemia, and improved motor coordination on the rotarod test. In addition, previous studies have shown that TM binds and sequesters HMGB1 directly via the D1 domain [7]. These findings suggested that rhsTM may improve ischemic stroke by inhibiting HMGB1 activity. Solulin was reported to reduce infarct volume in animal models of ischemic stroke due to its anticoagulant and antiinflammatory effects [19, 20]. These findings were supported by those of the present study. Ryang et al. [19] reported that solulin downregulated the expression of inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6)] in the penumbra, and significantly decreased the expression of CD11B, a marker of microglia/macrophage activation, in rats subjected to 2-hour MCAO. HMGB1 was previously recognized as a proinflammatory molecule secreted by monocytes and macrophages in response to TNF-α, IL-1β, or lipopolysaccharide (LPS) [25]. These results suggested that rhsTM has an antiinflammatory effect on HMGB1 in cerebral ischemia.

In previous animal studies regarding HMGB1, rhsTM was administered at doses of 1 – 10 mg/kg [8, 10, 12, 26]. We reported previously that recombinant tissue plasminogen activator (rtPA) at a dose of 10 mg/kg had no effect on infarct volume and mice showed massive intracerebral hemorrhage after 4-hour MCAO [27]. In a preliminary study, we confirmed that rhsTM at a dose of 20 mg/kg tended to increase hemorrhage volume in comparison with the 5 mg/kg-treated group (data not shown). Therefore, in this study, we selected rhsTM doses of 1 and 5 mg/kg. The present study demonstrated that rhsTM dose-dependently reduced infarct volume without intracerebral hemorrhage. Thus, our data indicate that rhsTM is a safe and effective anticoagulant, unlike other agents, such as rtPA.

Although rhsTM has an anticoagulant effect due to its binding to thrombin [28], rhsTM at a dose of 5 mg/kg did not increase hemorrhage volume in comparison with vehicle-treated controls (Figure 4). Mohri et al. [29] reported that, in rat models, rhsTM acted as a direct thrombin inhibitor, and therefore its dose dependency curve is steep and linear like that of heparin. In addition, they reported that the anticoagulant effect of rhsTM was not the same in primate and rat models [29]. In our preliminarily study, rhsTM at a dose of 20 mg/kg tended to increase hemorrhage volume in comparison with 5 mg/kg-treated mice (data not shown). A high dose of rhsTM may be associated with a risk of bleeding. However, rhsTM reduced the rate of clot growth without delaying the start of coagulation as determined on thromboelastography [30]. These results suggested that rhsTM may have a wider safety margin than other anticoagulants. Indeed, the Japanese rhsTM (Recomodulin) clinical phase III trial [15] demonstrated that the incidence of bleeding-related adverse events up to 7 days after the start of infusion was lower in the rhsTM-treated group than in the heparin-treated group [50/116 patients (43.1%) vs. 65/115 patients (56.5%); $P = 0.0487$]. These results suggest that rhsTM may be a safer anticoagulant treatment option for ischemic stroke.

Previously solulin was reported to reduce infarct volume in animal models of ischemic stroke due to its anticoagulant and antiinflammatory effects [19, 20]; these MCAO times in these studies were 30 or 60 minutes [20] and 120 minutes [19]. In addition, our study demonstrated that rhsTM significantly improved ischemic stroke in 4-hour MCAO. The only drug approved for lytic therapy in clinical cases of ischemic stroke is rtPA, which has shown significant benefit in patient outcome when given up to 4.5 hours after onset [31]. However, less than 10% of all acute stroke patients are eligible for rtPA [2]. In addition, only 2% – 5% of patients with stroke receive rtPA, mainly due to delay in reaching the hospital [32]. This study suggested that rhsTM may be useful in ischemic stroke even in cases in which rtPA would not be indicated due to delayed hospital admission.

5. Conclusions

Our results suggest that rhsTM inhibits plasma expression of HMGB1 and decreases neurological impairment induced by cerebral ischemia without hemorrhagic complications in mice. These observations indicate a palliative action of rhsTM and suggest new therapeutic possibilities for treatment of ischemic stroke via inhibition of HMGB1. Further studies are required to determine the mechanism of action of rhsTM in ischemic stroke.

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Conflict of interest

There are no conflicts of interest.

Sources of Funding

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Figure legends

Figure 1. Effects of rhsTM on brain infarct volume 24 hours after 4-hour MCAO.
Values are expressed as means ± SEM. The infarct volume was measured by 2% 2,3,5-triphenyltetrazolium chloride staining. rhsTM was administered i.v. immediately after 4-hour MCAO.
*P < 0.05 vs. vehicle-treated group (Tukey's test).

Figure 2. Effects of rhsTM on motor coordination 24 hours after 4-hour MCAO.
Values are expressed as means ± SEM. The motor coordination was measured by the rotarod test with a rotation speed of 10 rpm. rhsTM was administered i.v. immediately after 4-hour MCAO.
*P < 0.05, ***P < 0.0001 vs. vehicle; **P < 0.001 vs. sham (Tukey's test).

Figure 3. Effects of rhsTM on plasma HMGB1 level.
Values are expressed as means ± SEM. Plasma HMGB1 levels were measured by enzyme-linked immunoadsorbent assay 24 hours after 4-hour MCAO.
*P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle (Tukey's test).

Figure 4. Effects of rhsTM on hemorrhage volume 24 hours after 4-hour MCAO.
Values are expressed as means ± SEM. The hemorrhagic area was measured in each slice using an image analysis system.

References

[1] Writing Group M, Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, et al. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation. 2010;121:e46-e215.

[2] Moretti A, Ferrari F, Villa RF. Pharmacological therapy of acute ischaemic stroke: Achievements and problems. Pharmacol Ther. 2015.
[3] El-Koussy M, Schroth G, Brekenfeld C, Arnold M. Imaging of acute ischemic stroke. Eur Neurol. 2014;72:309-16.
[4] Muhammad S, Barakat W, Stoyanov S, Murikinati S, Yang H, Tracey KJ, et al. The HMGB1 receptor RAGE mediates ischemic brain damage. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008;28:12023-31.
[5] Kim JB, Sig Choi J, Yu YM, Nam K, Piao CS, Kim SW, et al. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006;26:6413-21.
[6] Li YH, Kuo CH, Shi GY, Wu HL. The role of thrombomodulin lectin-like domain in inflammation. J Biomed Sci. 2012;19:34.
[7] Abeyama K, Stern DM, Ito Y, Kawahara K, Yoshimoto Y, Tanaka M, et al. The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. J Clin Invest. 2005;115:1267-74.
[8] Kudo D, Toyama M, Aoyagi T, Akahori Y, Yamamoto H, Ishii K, et al. Involvement of high mobility group box 1 and the therapeutic effect of recombinant thrombomodulin in a mouse model of severe acute respiratory distress syndrome. Clinical and experimental immunology. 2013;173:276-87.
[9] Iba T, Nakarai E, Takayama T, Nakajima K, Sasaoka T, Ohno Y. Combination effect of antithrombin and recombinant human soluble thrombomodulin in a lipopolysaccharide induced rat sepsis model. Critical care. 2009;13:R203.
[10] Hagiwara S, Iwasaka H, Goto K, Ochi Y, Mizunaga S, Saikawa T, et al. Recombinant thrombomodulin prevents heatstroke by inhibition of high-mobility group box 1 protein in sera of rats. Shock. 2010;34:402-6.
[11] Tanaka J, Seki Y, Ishikura H, Tsubota M, Sekiguchi F, Yamaguchi K, et al. Recombinant human soluble thrombomodulin prevents peripheral HMGB1-dependent hyperalgesia in rats. Br J Pharmacol. 2013;170:1233-41.
[12] Tanaka J, Yamaguchi K, Ishikura H, Tsubota M, Sekiguchi F, Seki Y, et al. Bladder pain relief by HMGB1 neutralization and soluble thrombomodulin in mice with cyclophosphamide-induced cystitis. Neuropharmacology. 2014;79:112-8.
[13] Aikawa N, Shimazaki S, Yamamoto Y, Saito H, Maruyama I, Ohno R, et al. Thrombomodulin alfa in the treatment of infectious patients complicated by disseminated intravascular coagulation: subanalysis from the phase 3 trial. Shock. 2011;35:349-54.

[14] Yamakawa K, Fujimi S, Mohri T, Matsuda H, Nakamori Y, Hirose T, et al. Treatment effects of recombinant human soluble thrombomodulin in patients with severe sepsis: a historical control study. Critical care. 2011;15:R123.
[15] Saito H, Maruyama I, Shimazaki S, Yamamoto Y, Aikawa N, Ohno R, et al. Efficacy and safety of recombinant human soluble thrombomodulin (ART-123) in disseminated intravascular coagulation: results of a phase III, randomized, double-blind clinical trial. Journal of thrombosis and haemostasis : JTH. 2007;5:31-41.
[16] Moll S, Lindley C, Pescatore S, Morrison D, Tsuruta K, Mohri M, et al. Phase I study of a novel recombinant human soluble thrombomodulin, ART-123. Journal of thrombosis and haemostasis : JTH. 2004;2:1745-51.
[17] Kearon C, Comp P, Douketis J, Royds R, Yamada K, Gent M. Dose-response study of recombinant human soluble thrombomodulin (ART-123) in the prevention of venous thromboembolism after total hip replacement. Journal of thrombosis and haemostasis : JTH. 2005;3:962-8.
[18] van Iersel T, Stroissnig H, Giesen P, Wemer J, Wilhelm-Ogunbiyi K. Phase I study of Solulin, a novel recombinant soluble human thrombomodulin analogue. Thrombosis and haemostasis. 2011;105:302-12.
[19] Ryang YM, Dang J, Kipp M, Petersen KU, Fahlenkamp AV, Gempt J, et al. Solulin reduces infarct volume and regulates gene-expression in transient middle cerebral artery occlusion in rats. BMC Neurosci. 2011;12:113.
[20] Su EJ, Geyer M, Wahl M, Mann K, Ginsburg D, Brohmann H, et al. The thrombomodulin analog Solulin promotes reperfusion and reduces infarct volume in a thrombotic stroke model. Journal of thrombosis and haemostasis : JTH. 2011;9:1174-82.
[21] Hayakawa K, Mishima K, Nozako M, Hazekawa M, Mishima S, Fujioka M, et al. Delayed treatment with minocycline ameliorates neurologic impairment through activated microglia expressing a high-mobility group box1-inhibiting mechanism. Stroke. 2008;39:951-8.
[22] Egashira N, Hayakawa K, Mishima K, Kimura H, Iwasaki K, Fujiwara M. Neuroprotective effect of gamma-glutamylethylamide (theanine) on cerebral infarction in mice. Neurosci Lett. 2004;363:58-61.
[23] Mishima K, Hayakawa K, Abe K, Ikeda T, Egashira N, Iwasaki K, et al. Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. Stroke. 2005;36:1077-82.
[24] Hayakawa K, Irie K, Sano K, Watanabe T, Higuchi S, Enoki M, et al. Therapeutic time window of cannabidiol treatment on delayed ischemic damage via high-mobility group box1-inhibiting mechanism. Biol Pharm Bull. 2009;32:1538-44.

[25] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science (New York, NY). 1999;285:248-51.
[26] Kawasaki T, Okamoto K, Kawasaki C, Sata T. Thrombomodulin improved liver injury, coagulopathy, and mortality in an experimental heatstroke model in mice. Anesthesia and analgesia. 2014;118:956-63.
[27] Nakano T, Irie K, Hayakawa K, Sano K, Nakamura Y, Tanaka M, et al. Delayed treatment with ADAMTS13 ameliorates cerebaral ischemic injury without hemorrhagic complication. Brain Res. 2015.
[28] Okamoto T, Tanigami H, Suzuki K, Shimaoka M. Thrombomodulin: a bifunctional modulator of inflammation and coagulation in sepsis. Crit Care Res Pract. 2012;2012:614545.
[29] Mohri M, Gonda Y, Oka M, Aoki Y, Gomi K, Kiyota T, et al. The antithrombotic effects of recombinant human soluble thrombomodulin (rhsTM) on tissue factor-induced disseminated intravascular coagulation in crab-eating monkeys (Macaca fascicularis). Blood Coagul Fibrinolysis. 1997;8:274-83.
[30] Mohri M, Sugimoto E, Sata M, Asano T. The inhibitory effect of recombinant human soluble thrombomodulin on initiation and extension of coagulation--a comparison with other anticoagulants. Thrombosis and haemostasis. 1999;82:1687-93.
[31] Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. The New England journal of medicine. 2008;359:1317-29.
[32] Kikuchi K, Miura N, Kawahara KI, Murai Y, Morioka M, Lapchak PA, et al. Edaravone (Radicut), a free radical scavenger, is a potentially useful addition to thrombolytic therapy in patients with acute ischemic stroke. Biomed Rep. 2013;1:7-12.

Figure 1. Effects of rhsTM on brain infarct volume 24 hours after 4-hour MCAO.

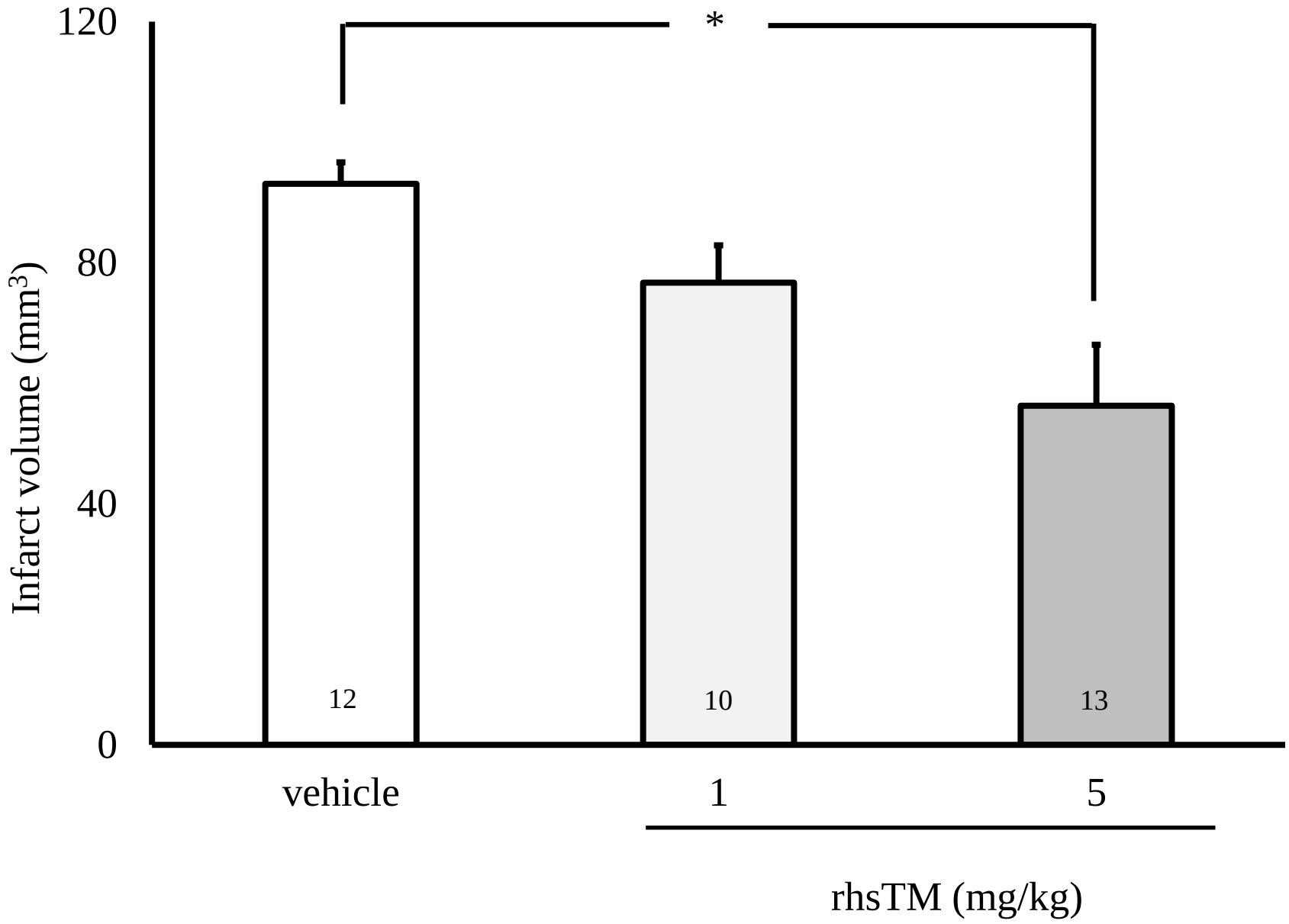

Figure 2. Effects of rhsTM on motor coordination 24 hours after 4-hour MCAO.

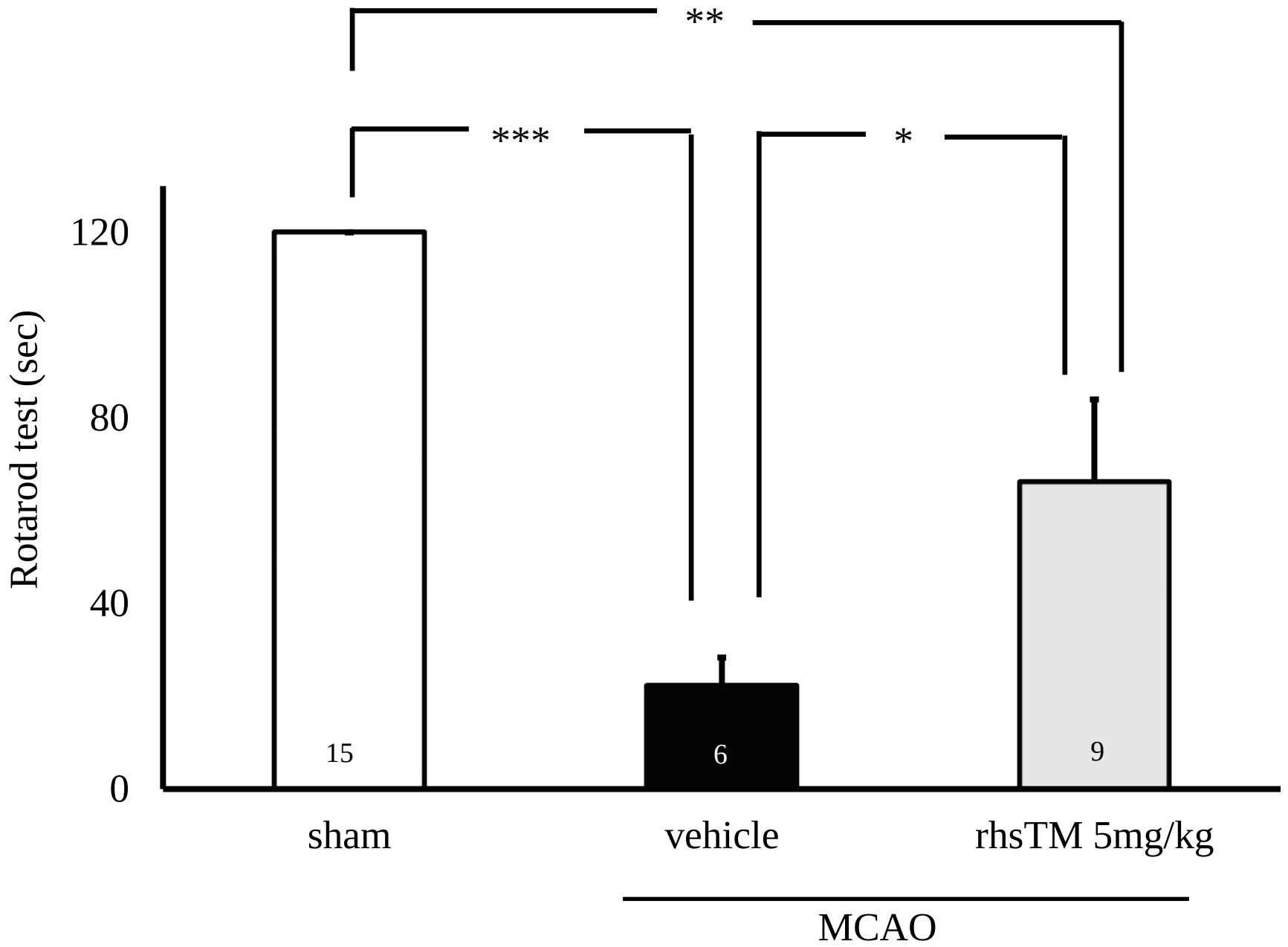

Figure 3. Effects of rhsTM on plasma HMGB1 level.

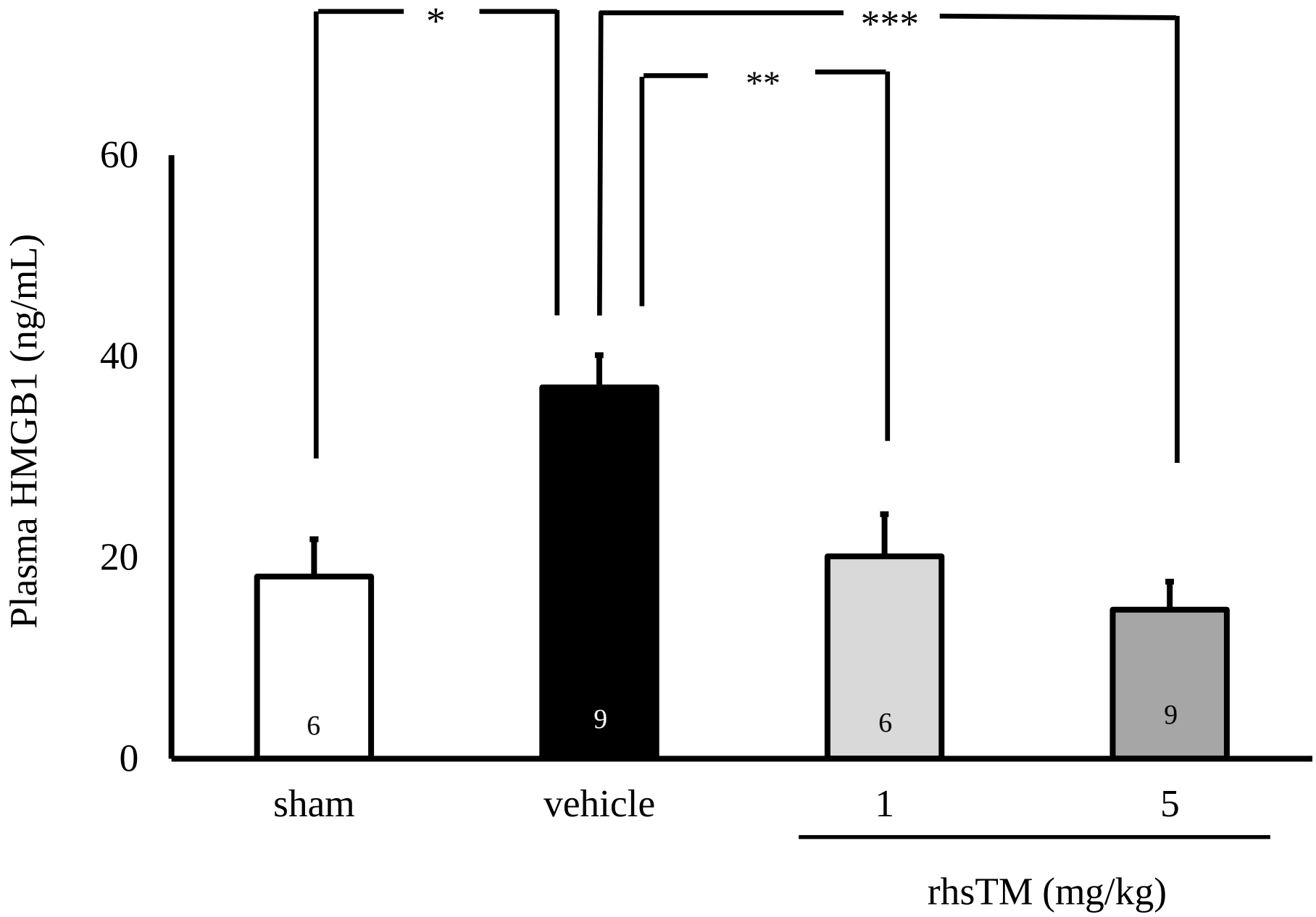

Figure 4. Effects of rhsTM on hemorrhage volume 24 hours after 4-hour MCAO.