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NOTE

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Trehalose decreases blood clotting in the cerebral space after experimental subarachnoid hemorrhage

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ABSTRACT. Subarachnoid hemorrhage (SAH) frequently results in several serious complications, such as cerebral vasospasm. We previously reported the effect of trehalose on vasospasm, inflammatory responses, and lipid peroxidation induced by blood exposure. Herein, to further elucidate the mechanism of action of trehalose, we investigated whether or not post-administration of trehalose can directly influence blood clotting in the cistern. As a result of trehalose injection after the onset of experimental SAH, blood clotting around the basilar artery was clearly inhibited. We also found that trehalose positively impacted coagulation and fibrinolysis parameters in rat, rabbit and human plasma *in vitro*. These findings suggest that trehalose has suppressive effects on blood clotting in addition to vasospasm, inflammatory responses, and lipid peroxidation after SAH.

KEY WORDS: blood clotting, coagulation, fibrinolysis, subarachnoid hemorrhage, trehalose

Aneurysmal subarachnoid hemorrhage (SAH) involves bleeding into the subarachnoid space and results in high mortality and morbidity [27]. Ischemic injury secondary to cerebral vasospasm is a major cause of brain damage after SAH [13]. In addition, a number of reports suggest an association between cerebral vasospasm and inflammatory or oxidative stress after SAH [2, 4, 15, 24]. However, cerebral vasospasm remains to be understood from both pathogenic and therapeutic perspectives. SAH occurs after the breakdown of an aneurysm on the cerebral artery wall, after which erythrocyte components are released into the subarachnoid space through hemolysis. Oxyhemoglobin, a major constituent in erythrocyte cytosol, has been suggested to elicit cerebral vasospasm and extensive stress responses after SAH, including inflammatory responses and oxidative stress [1, 24].

Many clinical and experimental studies have reported that the volume and duration of subarachnoid blood clotting are directly associated with the development and severity of cerebral vasospasm [8, 28]. Indeed, the removal of blood clots through irrigation of the subarachnoid space reportedly reduced the risk of cerebral vasospasm [11, 14, 21]. In addition, as causes of cerebral vasospasm are multifactorial, its preventive treatment would require a combination of approaches, including anti-oxidant and anti-inflammatory reagents, or a reagent with pleiotropic effects.

Trehalose, a non-reducing disaccharide in which the two glucose units are linked by an α,α-1,1-glycosidic bond, has multiple functions including protection against stresses such as desiccation, reactive oxygen species (ROS), cold, and endotoxic shock [6, 12, 17, 18]. Therefore, trehalose is considered to be a potentially powerful treatment for various diseases involving oxidative stress,

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desiccating conditions, protein misfolding, and/or chronic inflammation. Indeed, medical use of trehalose has previously been attempted for the cryopreservation of platelets, dry eye syndrome, oral dryness caused by dental treatment, and organ preservation solution for clinical lung transplantation [3, 16, 19, 26].

In our previous report, we investigated the effect of trehalose on cerebral vasospasm, inflammatory responses, and oxidative stress induced by blood exposure [5]. As a result, trehalose inhibited hemolysate-induced production of several inflammatory mediators, as well as blood- and ROS-induced lipid peroxidation *in vitro*. In addition, *in vivo* analysis using rabbit and rat models showed that trehalose suppressed vasospasm, inflammatory responses, and lipid peroxidation in blood-exposed vessels. Therefore, trehalose may provide a new therapeutic modality for complications after SAH.

To further investigate the effect of trehalose on harmful events in blood-exposed vessels in the present study, we examined whether or not trehalose directly acted to suppress blood clotting around the blood vessel in the arachnoid space after experimental SAH *in vivo*. We also evaluated the effect of trehalose on blood coagulation and fibrinolysis activity *in vitro*.

Trehalose and maltose were purchased from Wako (Osaka, Japan). Male Japanese White rabbits and male Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu, Japan). The design of the animal study was approved by the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo (Tokyo, Japan).

A previously reported rabbit single-hemorrhage model was used [5]. Briefly, twelve rabbits were anesthetized, and fresh autologous arterial blood was drawn from the ear artery. Immediately after removal of 1.0 m*l* of cerebrospinal fluid (CSF), 1.0 m*l* of blood was injected into the cisterna magna in 2 min with the animal in a tilted, head-down, prone position. The animal was maintained in the same position for 60 min to allow the injected blood to keep the injected blood around the basilar artery. Three hr after the blood injection, animals in Saline, Trehalose, or Maltose groups received a 1.0-m*l* injection of saline, 7.5% trehalose, or 7.5% maltose, respectively, into the cisterna magna.

Forty-eight hr after the induction of experimental SAH, the brain and basilar artery were sampled. Relative areas of the blood clot on the rabbit brain were quantified using the densitometer function of Photoshop (Adobe, San Jose, CA, USA). Paraffin sections of the basilar artery were prepared and stained with hematoxylin and eosin (H&E).

Rabbit plasma containing 0.38% sodium citrate (Charles River Laboratories Japan, Yokohama, Japan), commercial human plasma "Daiichi" (Sekisui Medical, Tokyo, Japan) or 0.32% sodium citrate-containing venous plasma from rats was mixed with saline, 20% trehalose (final concentration, 5%), or 20% maltose (final concentration, 5%). A hepaplastin test was conducted using a hepaplastin test kit (Eisai, Tokyo, Japan) for plasma samples according to the manufacturer's instructions with slight modifications. In brief, a plasma sample was added to the hepaplastin test reagent, and the coagulation time was measured at 37°C. A thrombotest was conducted using a Thrombotest kit (Eisai) for rat and human plasma samples or Fukugouinshi T "KOKUSAI" (Sysmex Corp., Kobe, Japan) for rabbit plasma samples according to the manufacturer's instructions, with slight modifications. In brief, a plasma sample was added to each thrombotest reagent and coagulation time was measured at 37°C.

Plasminogen activities in rabbit and commercial human plasma were assayed by Testzym S PLG (Sekisui Medical) according to the manufacturer's instructions, with slight modifications. In brief, the plasma samples containing saline, 5% trehalose, or 5% maltose were incubated at 37°C, and were collected. Collected plasma mixed with streptokinase solution was pre-incubated at 37°C for 5 min and then mixed with the substrate solution. This mixture was incubated at 37°C for 2 min and absorbances were measured at 405 nm and 505 nm with a spectrometer. Plasminogen activities were calculated by subtracting the absorbance at 405 nm from that at 505 nm.

Data are expressed as means ± standard deviation and were analyzed for significance by ANOVA with Dunnett's or Tukey–Kramer's multiple comparison test. Values of $P<0.05$ were considered to indicate statistical significance.

We administered trehalose or maltose (a structural isomer of trehalose with an α,α-1,4-glycosidic bond) at a final concentration of 7.5%, or saline into the cisterna magna in a single dose 3 hr after blood injection. Histological analysis revealed the characteristic features of vasospasm in the basilar arteries of saline- or maltose-injected animals, whereas vasospasm was attenuated in trehalose-injected animals (Supplementary Fig. 1), as previously observed [5]. Blood in the cisterna magna was considered to be clotted, to some extent, at 3 hr after blood injection. To investigate whether or not trehalose administration could influence blood clotting in the cistern, brains were collected 48 hr after experimental SAH and investigated. As expected, thick blood clots accumulated and entirely covered the basal surface of the brain stem in saline-injected animals (Fig. 1A, left panels). Blood clots were very slightly smaller in maltose-injected animals than in saline-injected animals (Fig. 1A, right panels). In contrast, blood clots had almost disappeared in trehalose-injected animals, and the quantified area of blood clots was significantly smaller than in saline- or maltose-injected animals (Fig. 1A, middle panels and 1B). These data suggest that trehalose injection may directly reduce blood clotting in the cistern after experimental SAH.

Trehalose may have inhibitory effects on blood clot formation or stimulating effects on fibrinolytic action, or both. To analyze the effects of trehalose on coagulation activity, a thrombotest and hepaplastin test, two assays for coagulation factor activities (factors II, VII, and X), were carried out *in vitro* using rabbit plasma, commercial human plasma and venous plasma from rats. In the thrombotest, coagulation times of all the plasma samples containing trehalose were significantly longer than that of plasma containing saline (Fig. 2A). Similar effects of trehalose on prolonging coagulation time of rat and human plasma were observed using the hepaplastin test (Data not shown). These results suggest that trehalose reduces the activity of blood-clotting factors in plasma *in vitro*.

Finally, to examine the influence of trehalose on fibrinolytic activity, plasminogen (PLG) activity was measured *in vitro* in rabbit plasma incubated at 37°C for 6 days. On day 0, there was no difference in PLG activity between saline-, trehalose-, or maltose-containing plasma (Fig. 3). However, while PLG activity in saline- or maltose-containing plasma significantly decreased

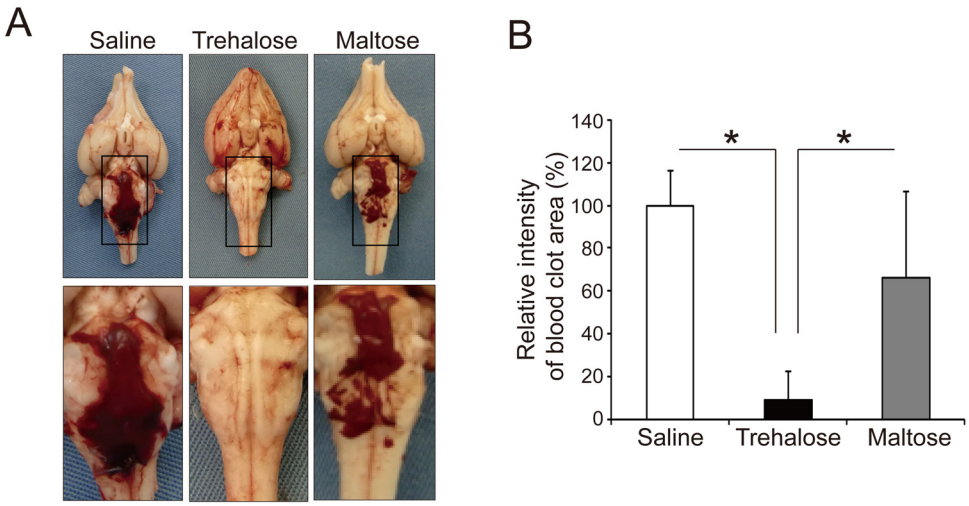

Fig. 1. Effects of trehalose on blood clotting in experimental subarachnoid hemorrhage (SAH). Saline, 7.5% trehalose, or 7.5% maltose was injected into the cisterna magna 3 hr after experimental SAH induction. Forty-eight hr after the induction of experimental SAH, the brain (A) Upper panels, representative gross specimens of rabbits. Lower panels, higher magnifications of squares in upper panels. (B) Ratio of blood clot areas in trehalose-injected or maltose-injected groups to that in the saline-injected group (**P*<0.05, ANOVA). All data are means ± standard deviation representative of six independent experiments.

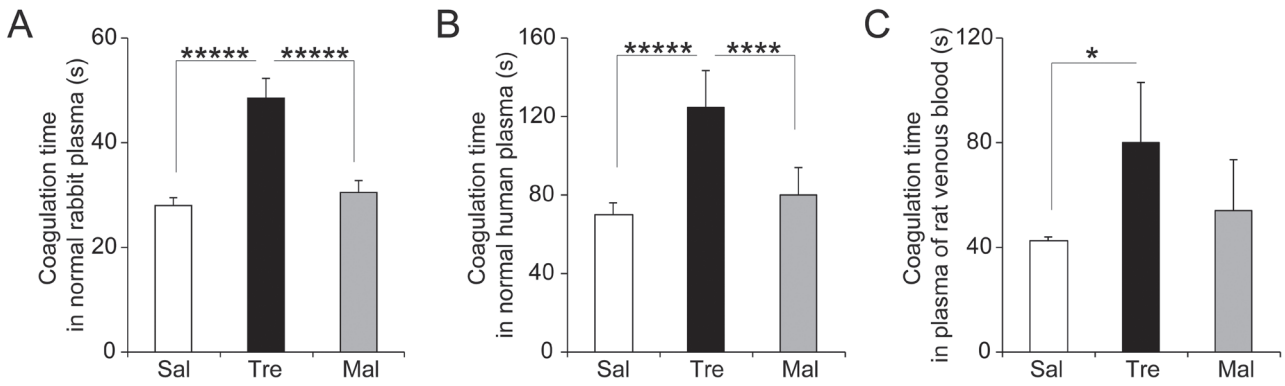

Fig. 2. Effects of trehalose on blood coagulation *in vitro*. (A–C) Coagulation time *in vitro* as assayed using a thrombotest in rabbit plasma (A), commercial human plasma (B) or plasma from rat venous blood (C) containing saline, 5% trehalose (Tre), or 5% maltose (Mal). **P*<0.05, *****P*<0.001, and ******P*<0.0005 (ANOVA). All data are means ± standard deviation representative of two to three independent experiments with n=6 per group.

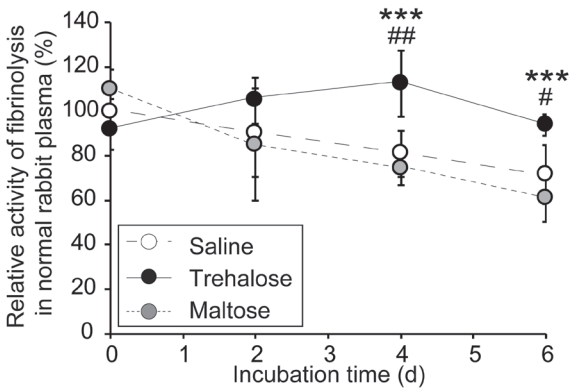

Fig. 3. Effects of trehalose on fibrinolysis *in vitro*. Changes in plasminogen (PLG) activities *in vitro* in rabbit plasma containing saline, 5% trehalose, or 5% maltose for 6 days. Ratio of PLG activity in trehalose- or maltose-containing plasma groups to PLG activity in saline-containing plasma at 0 day. ****P*<0.005 compared with saline, and $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared with maltose (ANOVA). All data are means ± standard deviation representative of two independent experiments with n=4 per group.

in a time-dependent manner, PLG activity in trehalose-containing plasma was relatively maintained (Fig. 3). We also confirmed the same effect of trehalose using human plasma (data not shown). These data suggest that trehalose does not accelerate, but rather preserves fibrinolytic activity in plasma *in vitro*.

In the present study, we demonstrated that: (a) a single administration of trehalose after the onset of experimental SAH reduced blood clotting around the brain stem in a rabbit single-hemorrhage model; (b) trehalose suppressed coagulation activity in rabbit, human and rat plasma samples *in vitro*; and (c) trehalose also preserved fibrinolytic activity in rabbit and human plasma samples *in vitro*.

To date, the best-known way to prevent complications of SAH, mainly cerebral vasospasm, is to remove as much blood clot as possible and as early as possible by surgical means. Indeed, cisternal irrigation therapy with urokinase and intracisternal administration of tissue plasminogen activator (tPA) have been performed to prevent symptomatic vasospasm after clipping or coil embolization of aneurysms [7, 23]. A recent clinical report showed removal of blood from the subarachnoid space with a lumbar drainage decreased the development of cerebral vasospasm [21].

Our previous rabbit model studies showed that even after the onset of experimental SAH a single administration of trehalose suppressed cerebral vasospasm, oxidative stress, and inflammation [5]. We believe that a reduction in blood clots by trehalose injection in the cistern is strongly associated with the positive impact of trehalose on pathological events after experimental SAH. Blood clotting in the cistern after SAH is affected by complicated mechanism involved in the activity of coagulants, anticoagulants and fibrinolytic agents in bleeding blood as well as in CSF and brain tissue [9, 10, 22]. In this study, it is unclear when the blood clotting is completed. When the trehalose was injected at 3 hr after the onset of experimental SAH, the blood clot formation might be finished completely. Therefore, the effect of trehalose on coagulation factor activities is likely attributable to its suppressive effect on blood-clotting activity, as demonstrated by *in vitro* studies. Trehalose interacts with calcium ions, which are required for activation of blood-clotting factors [20]; i.e., trehalose may function as a chelating agent for calcium ions. Such function of trehalose may contribute to its suppressive effect on blood-clotting activity. In addition, we found the retentive effect of trehalose on fibrinolytic activity. *In vitro* fibrinolysis assay showed that the activity in the saline or maltose-containing plasma was decreased in time-dependent manner while that in trehalose-containing plasma was almost unchanged. Trehalose stabilizes the structures of various enzymes by preventing protein aggregation and denaturation under stress conditions [12]. This action may also partly contribute to reduction in blood clot. However, the suppressive effect of trehalose on coagulant was considered to be a primary action on reduction in blood clot because its effect on fibrinolytic agent was not direct but rather secondary action. The results of these *in vitro* studies also suggest that trehalose may be effective in suppressing blood clots regardless of animal species.

Our results showed that maltose had a slight tendency to reduce the blood clot. Unlike trehalose, maltose promoted only a slight protection of the thermal unfolding induced by exposure of enzyme to heat, and had no effect on calcium binding [20, 25]. This may be the cause of the differences in the effect on blood clotting between trehalose and maltose.

We acknowledge that trehalose has a weaker effect on reduction of blood clotting than urokinase and tPA because it is thought to does not directly accelerate fibrinolytic activity. However, because the complete removal of blood clots by a fibrinolytic agent is generally difficult in clinical conditions, it is possible that residual blood clots and unremoved spasmogens may induce cerebral vasospasm, inflammatory responses, and lipid peroxidation. As mentioned in our previous report, trehalose elicits beneficial effects including suppression of cerebral vasospasm, inflammatory responses, and lipid peroxidation, even though the blood vessel was constantly exposed to blood [5]. We also reported that trehalose directly functions to suppress inflammatory and oxidative stress responses induced by blood exposure in cultured cells and primary cells [5]. Trehalose might be accessible at the surface of blood vessels by reducing rigid blood clotting, whereby it can directly inhibit such harmful events. Therefore, by remaining in the subarachnoid space, trehalose may be able to suppress complications of SAH even if the blood clot cannot completely be removed.

Trehalose alone has pleiotropic actions and is, therefore, thought to have an advantage over other drug candidates with only a single action, such as radical scavenging or anti-inflammatory effects. Moreover, trehalose is expected to have additional or synergistic therapeutic effects on vasospasm when combined with existing therapeutic modalities; for example, trehalose in combination with triple-H therapy, systemic administration of fasudil hydrochloride, or transluminal angioplasty may be more effective in preventing cerebral vasospasm. Furthermore, trehalose has already been used as an additive agent in solutions for organ preservation [3], and has proven harmless in those contexts. Thus, trehalose may become a new therapeutic option for patients with SAH, either as monotherapy or in combination with another treatment modality.

CONFLICT OF INTEREST. The authors declare that they have no competing interests.

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