A Novel Embolic Model of Cerebral Infarction and Evaluation of *Stachybotrys microspora* Triprenyl Phenol-7 (SMTP-7), a Novel Fungal Triprenyl Phenol Metabolite

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**Abstract.** The aim of the present study was to establish a novel embolic model of cerebral infarction and to evaluate the effect of *Stachybotrys microspora* triprenyl phenol-7 (SMTP-7), a novel fungal triprenyl phenol metabolite. Thrombotic occlusion was induced by transfer of acetic acid–induced embolus into the brain. The regional cerebral blood flow was measured by a laser Doppler flowmeter to check the ischemic condition. Infarction area was assessed by 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining. Neurological scores were determined by a modified version of the method described by Longa et al. Emboli were accumulated at the temporal or parietal region of the middle cerebral artery. Additionally, we found that this model showed decreased cerebral blood flow and increased infarction area and neurological scores. Treatment with tissue plasminogen activator (t-PA) reduced infarction area and the neurological scores in a dose-dependent manner; moreover, the decreased cerebral blood flow recovered. SMTP-7 also reduced these values. The therapeutic time window of SMTP-7 was longer than that of t-PA. These results indicate that this model may be useful for understanding the pathophysiological mechanisms of cerebral infarction and evaluating the effects of therapeutic agents. Additionally, SMTP-7 is a promising approach to extend the therapeutic time window. Therefore, this novel compound may represent a novel approach for the treatment of cerebral infarction.

**Keywords:** acetic acid, cerebral infarction, embolus, *Stachybotrys microspora* triprenyl phenol-7 (SMTP-7)

Introduction

Stroke is the second most common cause of mortality worldwide (1) and cerebrovascular occlusion by blood clots represents an important cause of morbidity or mortality (2). One strategy for treating stroke is to restore blood flow within the ischemic area using a thrombolytic agent such as tissue plasminogen activator (t-PA). The administration of t-PA can salvage brain function (3). However, the empirical therapeutic time window for t-PA administration is quite narrow and it has been reported that the administration of t-PA increases the risk of hemorrhagic transformation (4). Overall, only a small percentage of patients with stroke can benefit from t-PA–induced thrombolysis. Therefore, it is urgent to develop new strategies to combat this disease.

A number of animal models have been reported (5 – 7). However, the effects of thrombolytic agents cannot be evaluated in these models because animal species undergo mechanical occlusions using filaments or ligation. In other models, blood clots or rose Bengal were injected in order to induce cerebral ischemia (8 – 10). Although these models can be used to evaluate thrombolytic therapy, there are problems of non-uniformity in the infarction area and technical difficulties. Therefore, we theorized that a novel model that reflects the pathological state of embolic infarction in humans is needed to assess the effects of therapeutic agents. Ikoma et al. (11) re-
ported on the preparation of an animal model of arterial thrombosis by local application of acetic acid and the usefulness of the model for evaluation of antithrombotic agents. The emboli generated by this method were composed of networks of fibrin fibers with numerous aggregated platelets and entrapped erythrocytes. On the basis of this report, we hypothesized that cerebral infarction could be induced in order to transfer the embolus generated by this method into the brain and started the establishment of a novel embolic cerebral infarction model. A preliminary investigation has been reported previously (12, 13).

A novel triprenyl phenol, designated staplabin, has been isolated from a culture of Stachybotrys microspora IFO 30018 and is the first low molecular weight compound that stimulates plasminogen–fibrin binding (14). This compound also causes the susceptible activation of plasminogen by inducing a conformational change in plasminogen (15). Stachybotrys microspora IFO 30018 produces a variety of staplabin analogs and some of them are several times more potent than staplabin (16, 17). In particular, Stachybotrys microspora triprenyl phenol-7 (SMTP-7, Orniplabin; CAS registry No. 273379-50-9) is 5- to 10-times more potent. From these observations, we hypothesized that SMTP-7 may be effective against embolic stroke and represent new treatment strategies.

The aim of the present study was to establish a novel embolic model of cerebral infarction and to evaluate the effect of SMTP-7 using this model. We report here the establishment of a novel embolic infarction model. Additionally, this investigation demonstrated that SMTP-7 has an excellent therapeutic time window for the treatment of embolic infarction.

Materials and Methods

Animals

All experiments were conducted in accordance with the regulations of the Committee of Animal Care and Welfare of Showa University.

Male Mongolian gerbils weighing 55 – 80 g (Saitama Jikken, Saitama) were used. Mongolian gerbils were maintained in an air-conditioned animal room at 20 ± 2°C, with 50 ± 20% relative humidity, and a 12-h light–dark cycle (lights on 8:00 to 20:00). Animals received a standard laboratory diet; additionally, water was provided ad libitum.

Embolic infarction model

Animals were anesthetized with 5% isoflurane (Escain®, Mylan, Tokyo), and anesthesia was maintained with 1.0% – 1.5% isoflurane. Body temperature was maintained by using a heating lamp throughout surgery and during the recovery period. Under anesthesia, the right common carotid artery (RCCA) was isolated via a midline incision and the surrounding tissue was carefully removed. The RCCA was temporarily clamped using an aneurysm clip and acetic acid was introduced in accordance with the method of Ikoma et al. (11) in order to generate the embolus. Acetic acid was applied to the RCCA with a cotton swab. The cotton swab was soaked sufficiently with acetic acid and then acetic acid was applied with 30 strokes to the RCCA. After 10 min, the clip was removed, and the embolus was transferred to the brain for embolization by the bloodstream. After closure of the surgical site, the animals were allowed to awaken from anesthesia and subsequently transferred into the cage. To evaluate the effect of the surgical preparation, the same surgically operated animals without administration of acetic acid served as a sham control group (n = 6 in each group).

Monitoring of cerebral blood flow (CBF)

CBF was determined by laser Doppler flowmetry (MoorFLPI laser Doppler blood flow assessment; Moor Instruments, Ltd., Axminster, Devon, UK). Under the anesthesia, the scalp of each animal was carefully denuded. Subsequently, images of the blood flow were acquired by scanning the head region with a laser beam. After measurement of the baseline value, the animal was subjected to ischemia as described above. Then, CBF was again determined until 24 h after ischemia in accordance with the manufacturer’s instructions (n = 3 in each group). The data were stored on a computer and analyzed with MoorFLPI software (version 2.1). This software enables analysis of perfusion value profiles within a region of interest in circular areas of the same size containing the same number of pixels. Blood flow values were expressed as percentages of the baseline values.

Evaluation of neurological scores

Neurological scores were determined on the 5-point scale described by Longa et al. (18): no deficit as 0; failure to extend right hindlimb fully as 1; circling to the right as 2; falling to the left as 3; no spontaneous walking with a depressed level of consciousness as 4.

Analysis of infarction area

The animals were sacrificed with diethylether. Subsequently, brains were removed, sectioned coronally into four 2-mm sections with a brain matrix (RBM 2000C; ASI Instruments, Warren, MI, USA) and incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Wako Pure Chemical, Osaka) in saline for 30 min at 37°C. After TTC staining, brain slices were photographed on the posterior surface of each section and areas of infarc-
tion were delineated on the basis of relative lack of staining in the ischemic slice. The infarction areas were measured utilizing Image J software (version 1.31; NIH, Bethesda, MD, USA) and numerically integrated across the thickness of the slice to obtain an estimate of the infarction area and whole brain area, respectively. The areas from all slices were summed to calculate the total infarction area and were expressed as percentage of the whole brain area.

Treatment protocol
Each drug was administered as follows: t-PA (Activacin®; Kyowa Hakko Kirin, Tokyo) was intravenously infused at a dose of 0.01, 0.1, or 10 mg/kg (n = 6 in each group) as a 10% bolus and the remainder continuously infused over a 30-min interval by using a syringe pump (KDS200P; Muromachi Kikai, Tokyo) at 1 h after embolization. A dose of 3 mg/kg of edaravone (Radicut®; Mitsubishi Tanabe, Tokyo), 10 mg/kg of argatroban (Novastan®; Mitsubishi Tanabe), or ticlopidine (Sigma, St. Louis, MO, USA) was continuously infused over a 30-min interval by using a syringe pump at 1 h after embolization (n = 6 in each group). SMTP-7 (T.M.S. Co., Ltd., Tokyo) was intravenously infused at a dose of 1 or 10 mg/kg (n = 6 in each group), by the same procedure as used for t-PA. In experiments for studying the therapeutic time window, 10 mg/kg of t-PA and SMTP-7 were infused 1, 3, and 6 h (n = 6 in each group) after the embolization described above.

Statistical analyses
All data are expressed as the mean ± S.E.M. Multiple comparisons were analyzed by ANOVA followed by the Student-Newman-Keuls (SNK) test or the Bonferroni test; A P-value less than 0.05 was considered significant.

Results

Brain distribution of embolus and change in CBF
Figure 1 shows representative photographs of an embolus in the case of local application of 100% acetic acid (glacial acetic acid was regarded as having a concentration of 100%). The major region of the embolus was localized in the temporal and parietal regions of the middle cerebral artery (MCA). There were a few cases where the embolus was located in a different region of the brain. The proportions of cases of temporal MCA and parietal MCA were about 60% and 40%, respectively.

In the control group, CBF of the ipsilateral hemisphere markedly decreased (decreased by 70%) immediately after embolization and remained at that level until 24 h later, while it changed little (decreased by <20%) in the sham group. There were significant differences between the sham and control groups at all points. Although CBF of the contralateral hemisphere also slightly decreased (decreased by 30% – 35%), no significant differences were observed between the groups (Fig. 2).

Clinical observation and cerebral infarction area
Twenty-four hours after the application of acetic acid, various clinical manifestations such as narrowing eye fissure on the side of the embolization, tonic failing, and decreased spontaneous walking appeared individually or in combination. Some animals exhibited severe damage such as a depressed level of consciousness. More severe clinical manifestations tended to be observed in the case of embolization at the origin of MCA. As shown in Fig. 3A, the two sides of the cerebral hemisphere were symmetrical and no damage was observed in the sham group. In the control group, obvious infarction by ischemia was observed at the ipsilateral hemisphere at 24 h after embolization. In addition, massive ipsilateral hemispheric edema was observed. The infarction area was restricted to the cortex.
Fig. 2. Change in cerebral blood flow in embolic infarction model. A) Pseudocolor images of cerebral tissue. Color bar indicates cerebral blood flow. B) Change in cerebral blood flow over time. Each point represents the mean ± S.E.M. of 3 experiments. **P < 0.01: Statistically significant difference from sham group. Closed circle: Sham. Open circle: Control. Pre: Baseline value. Post: Post value just after embolization.

Fig. 3. Changes in neurological scores and cerebral infarction area. A) Representative photograph of TTC-stained cerebral sections. B) Changes in neurological scores and infarction area over time after embolization. Closed circle: Infarction area. Open circle: Neurological score. C) Concentration dependence of acetic acid. Each value represents the mean ± S.E.M. of 6 experiments. *P < 0.05, **P < 0.01: Statistically significant difference from the sham group.
Figure 3B shows the changes in neurological scores and cerebral infarction area over time. Clinical manifestations were observed from 6 h after embolization and peaked at 24 h. These manifestations were rapidly improved in most animals by the 2nd day after embolization. In contrast, no significant changes in infarction area were observed at 6 and 12 h after embolization. However, the infarction area markedly increased at 24 h and this increase was sustained until at least 120 h after embolization. Consequently, the drug effects were evaluated at 24 h after embolization. Next, the dependence on the concentration of acetic acid used for this model was examined. Although a slight neurological deficit was observed, a clear infarction area was not observed by the application of 60% acetic acid. However, 80% and 100% acetic acid led to increased infarction area and neurological scores in a concentration-dependent manner. Significant differences were observed in comparison to the sham group. The highest infarction area and neurological scores were induced at 100%. Thus, additional studies were implemented utilizing 100% acetic acid (Fig. 3C).

Effect of t-PA on acetic acid–induced cerebral infarction

One hour after embolization, animals were intravenously administered either saline or 10 mg/kg t-PA. As illustrated in Fig. 4, A and B, CBF of the ipsilateral hemisphere already recovered up to 65% of the baseline at the end of the t-PA administration. Statistically significant differences were observed in comparison with later values. The cerebral infarction area and neurological scores from control and t-PA–treated animals were also compared. The administration of t-PA ameliorated the infarction area and neurological scores dose-dependently compared with control animals. There were significant differences between the control and t-PA–treated animals at 10 mg/kg (Fig. 4C).

Effect of edaravone, argatroban, and ticlopidine on acetic acid–induced cerebral infarction

As shown in Fig. 5, 3 mg/kg of edaravone had little effect on cerebral infarction area and neurological scores induced by acetic acid. There were significant differences between the control and edaravone-treated animals. In contrast, argatroban and ticlopidine hardly had any effect at a dose of 10 mg/kg.

Comparative study of SMTP-7 and t-PA on acetic acid–induced cerebral infarction

Figure 6A shows the effects of SMTP-7 on cerebral
infarction area and neurological scores. SMTP-7 dose-dependently ameliorated the infarction area and neurological scores. At 10 mg/kg, statistically significant differences were apparent between the SMTP-7–treated and control groups.

In the next investigation, we compared the therapeutic time window for SMTP-7 with that for t-PA. Representative photographs of brain tissues taken from animals subjected to treatment with SMTP-7 and t-PA are shown in Fig. 6B. In the t-PA–treated group, 3 or 6 h after embolization, a clear infarction area was observed at the cerebral hemisphere affected by embolization. In contrast, no infarction area was visible at not only 3 h but also 6 h after embolization in the SMTP-7–treated group. The changes in infarction area and neurological scores over time are shown in Fig. 6C. Although t-PA ameliorated each value at administration at 1 h after embolization, its effect gradually decreased time-dependently. However, the effect of SMTP-7 was maintained until 6 h after administration. There were significant differences between the SMTP-7–treated and control groups at all points.

**Discussion**

In the present study, we have presented a new embolic cerebral infarction model and evaluated the effect of SMTP-7 using it. Mongolian gerbils were used in present experiments. The reason why we used gerbils is that cerebral ischemia is more easily achieved in gerbils than rats (19). Additionally, the gerbil lacks a circle of Willis (20). The posterior communicating arteries are absent in all gerbils and an anterior anastomosis is also absent in

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**Fig. 5.** Effect of edaravone, argatroban, and ticlopidine on neurological scores and cerebral infarction. Each column represents the mean ± S.E.M. of 6 experiments. *P < 0.05: Statistically significant difference from the control group.

**Fig. 6.** Effects of SMTP-7 on neurological scores and cerebral infarction area. A) Dose dependence of SMTP-7. B) Comparison of TTC-stained cerebral sections. C) Comparative study of therapeutic time window. Each value represents the mean ± S.E.M. of 6 experiments. *P < 0.05, **P < 0.01: Statistically significant difference from the control group. Closed circle: t-PA. Open circle: SMTP-7.
20% – 30% of the gerbil. Therefore, we hypothesized that brain distribution of embolus is more reproducible and that an internal control is included in the contralateral hemisphere. This model is distinct from other blood clot embolic models in terms of the induction of ischemia. The blood clot embolic models previously reported are induced by injection of clots through the common carotid artery (9) or through a catheter attached to the external carotid artery (10). Although these models are closer than suture models to what actually happens in patients with stroke, they exhibit problems of non-uniformity in the infarction area and technical difficulties. Additionally, the site of injection can be occluded in these models; therefore, changes in cerebral microcirculation may occur. We overcame this limitation by delivering an embolus into the MCA using the bloodstream. Emboli were lodged at the sites of MCA in all animals at 24 h after embolization. After embolization, CBF was decreased to 30% of initial levels in the ipsilateral hemisphere and this decreased CBF persisted for at least 24 h after embolization. This decrease in CBF is comparable to the values obtained from the conventional blood clot embolic model (10, 21). Almost equal decreases in CBF were obtained. Neurological scores and cerebral infarction area are also consistent with those obtained from the suture and conventional blood clot embolic model (22, 23). All animals exhibited moderate to severe neurological scores and infarction areas 24 h after embolization and a clear infarction area was observed at the cerebral hemisphere affected by embolization. Clinical manifestations were improved in most animals by 48 h after embolization, but the infarction area was observed until 120 h later. A previous report also showed that clinical signs were much improved by the 5th day after embolization (9). The reason for this discrepancy is not clear from the present study. Recent study has revealed that the contralateral hemisphere contributes to functional recovery after stroke (24). Functional recovery may begin 48 h after embolization. Next, we examined whether t-PA ameliorates the effects of the cerebral infarction in this model by analyzing the CBF, infarction area, and neurological scores. These data suggest that the embolus is dissolved by treatment with t-PA. Our model is in agreement with the data reported from animal models (25, 26) and clinical trials (27). More importantly, in our model, the embolus is formed in an artery, although it is formed outside the body in the conventional models. Niessen et al. (28) described that differences in blood clots caused different outcomes of t-PA treatment. It is possible that drug effects may be influenced by embolus structure. Therefore, the use of emboli formed in arteries is more suitable and simulates cerebral infarction in humans better than the use of emboli formed outside the body. In addition, we examined that effect of edaravone, argatroban, and ticlopidine. Postischemic treatment with edaravone decreased cortical infarction in focal embolization of rats at a dose of 3 mg/kg, i.v. (29). The present result was also agreement with this report. Moreover, post ischemic administration of argatroban and ticlopidine did not show any inhibitory effects. From these findings, we theorized that this model can form the basis for further progress in understanding the mechanisms of cerebral infarction and evaluating the therapeutic effects of various drugs.

Cerebral hemorrhage is associated with complications of t-PA–induced reperfusion after infarction. The risk of cerebral hemorrhage limits the therapeutic time window. This is why the application of t-PA remains limited. Earlier reports documented that combined administration with other drugs may extend the therapeutic time window of t-PA in cerebral infarction (25, 30, 31). We have demonstrated evidence for the excellent therapeutic time window of the first low molecular weight thrombolytic agent, SMTP-7. In addition, the administration of SMTP-7 did not exacerbate hemorrhage. This novel low molecular weight compound promotes the conversion to plasmin by conformational change of plasminogen under conditions with a plasminogen activator (15). Because plasminogen is activated on the surface of cells and the embolus, the effect of SMTP-7 is based on a physiological process. In other words, this compound enables the local amplification of plasmin at the sites at which its generation is needed. This is one reason why SMTP-7 appears to cause less hemorrhage. A previous report documented that inflammatory responses play a crucial role in the pathogenesis of cerebral infarction (32). Reactive oxygen is one of the inflammatory mediators causing injury to ischemic brain. Our preliminary experiment shows that SMTP-7 also has the ability to scavenge reactive oxygen. Therefore, SMTP-7 may limit the extent of cerebral infarction by combined effects. Additional studies are required to clarify this possibility. A large number of neuroprotective drugs have demonstrated varying degrees of effectiveness in preclinical models but failed to achieve positive results when brought forward into clinical trials. For preclinical drug development, the STAIR meeting recommended that putative stroke recovery drugs should be evaluated in both rodent and gyrencephalic species, that is, those similar to humans, for example, macaque monkeys (33). We are evaluating the effect of SMTP-7 utilizing a thrombotic MCA occlusion model in the cynomolgus monkey. Treatment with SMTP-7 reduced infarction area and the neurological scores at the same dose as used in the present study (P < 0.05). Additionally, no hemorrhagic region was observed (unpublished data). It is difficult to evaluate the potency of many compounds in
monkeys because of ethical concerns. Therefore, the selection of compounds using this model is efficient for drug development.

In summary, we have demonstrated that a novel embolic model induced by the application of acetic acid provides predictable cerebral infarction. This model is relevant to embolic stroke in humans and may be useful in determining the efficacy of therapeutic agents. In addition, SMTP-7 is a promising approach to extend the therapeutic time window. Therefore, this novel low molecular weight compound may represent a novel approach to the treatment of cerebral infarction.

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References


