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# Early hemostatic therapy using recombinant factor VIIa in a collagenase-induced intracerebral hemorrhage model in rats

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## Summary

Neurological deterioration during the first day after intracerebral hemorrhage (ICH) is associated with early hematoma growth in 18 to 38% of patients. While clinical studies continue to evaluate efficacy of activated recombinant factor VII (rFVIIa) for reducing frequency of early hematoma growth, there have been no studies investigating the effect of rFVIIa on early hematoma growth. We used a collagenase-induced ICH model in the rat to evaluate the effects of rFVIIa on early hematoma growth.

Two hours after injection of 0.14 U of type IV bacterial collagenase in 10 μL of saline into the basal ganglia, a small amount of blood collected in the striatum. The ICH gradually increased in size, extending posteriorly to the thalamus by 24 hours after injection. Intravenous administration of rFVIIa immediately after collagenase injection decreased average hematoma volume at 24 hours compared with vehicle-treated group ($168.1 \pm 13.4$ mm$^3$ vs. $118.3 \pm 23.0$ mm$^3$, $p < 0.01$). There was also a decrease in total hemoglobin content in rats treated with rFVIIa compared with vehicle-treated rats (optical density at 550 nm: $0.87 \pm 0.08$ vs. $0.71 \pm 0.09$, $p < 0.05$). There was no difference in cortical brain water content overlying the hematoma between the rFVIIa- and vehicle-treated groups ($81.4 \pm 0.7$% vs. $81.7 \pm 0.4$%). Our study indicates that treatment with rFVIIa may be useful in reducing the frequency of early hematoma growth in ICH patients.

*Keywords:* Factor VII; brain edema; hematoma; intracerebral hemorrhage.

## Introduction

Intracerebral hemorrhage (ICH) represents approximately 15% of stroke cases in the United States and 20% to 30% in Asian populations [30]. The 30-day mortality rate is 35% to 50%, and most survivors are neurologically disabled, with only 20% of patients becoming fully independent at 6 months [3]. In contrast to the successful therapeutic advances for ischemic stroke and subarachnoid hemorrhage, there remains a lack of effective treatment for ICH. Neurological deterioration frequently begins during the first day and is attributed to the development of brain edema and mass effect surrounding the hematoma [16]. Deterioration during the first day after bleeding is also strongly associated with early hematoma growth, and hematoma volume is an important predictor of 30-day mortality [2, 4, 10, 11, 17]. Studies involving patients scanned within 3 hours of the onset of ICH have shown that early hematoma growth, documented by subsequent CT scans, occurs in 18% to 38% of patients [4, 9, 12, 17].

The mechanism by which hematomas enlarge is unclear. Bleeding has been thought to be completed within minutes of onset and hematoma growth has been assumed to be the result of rebleeding from the initial site of arterial or arteriolar rupture [14]. Although this may be true in many cases, several lines of recent evidence have suggested that hematoma growth is due to bleeding into the congested and damaged tissue in the regions around the hematoma [24, 25]. As hematoma growth is a dynamic process in acute ICH, intervention with ultra-early hemostatic therapy could minimize, and possibly even prevent, early hematoma growth. The rapid action of recombinant activated factor VII (rFVIIa) at the local bleeding site, coupled with its low risk of systemic adverse effects, makes this agent a potentially valuable hemostatic treatment during the high-risk stage of ICH [8].

One of the key prerequisites for identifying an effective therapy for ICH is the development of an animal model that accurately mimics the dynamic processes involved in human ICH. Infusion of bacterial collagenase into the striatum disrupts vasculature and causes bleeding in the surrounding brain tissue [29]. Magnetic

resonance imaging and histopathological examination have shown that hematoma enlargement occurs for up to 4 hours after the collagenase injection in this model of ICH [6]. We slightly modified the procedure reported by Rosenberg *et al.* [29] by increasing the amount of solution and decreasing the concentration of collagenase in the solution. This allowed slower hematoma development in animals, and enabled evaluation of the effects of rFVIIa on early hematoma enlargement. Although no experimental studies investigating the effect of rFVIIa on ICH have been published, a clinical study is currently underway to evaluate its efficacy in reducing the frequency of early hematoma growth in patients with acute ICH [8, 23, 24].

## Materials and methods

The Ethics Committees for Animal Experiments at Kagawa University approved the experimental protocols used in this study. Animals were allowed free access to food and water before the experiment.

### *Experimental model*

A total of 66 male Sprague-Dawley rats were used in this study. The rats were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg). A polyethylene (PE-50) catheter was introduced into the femoral artery to obtain blood samples for analysis of blood gases, blood pH, hematocrit, and blood glucose concentration. Rectal temperature was maintained at 37.5 °C during the surgery using a feedback-controlled heating system.

The rat was positioned in a stereotactic frame and the scalp was incised along the midline. Using sterile technique, a 1-mm burr hole was opened in the skull near the left coronal suture 3-mm lateral to the midline. A blunt 26-gauge needle was inserted into the left caudate putamen (striatum) under stereotactic guidance (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3 mm lateral to the bregma). Solutions containing 0.14 U of bacterial collagenase (Type IV, Sigma-Aldrich, St. Louis, MO) in 10 μL of saline were infused into the brain over a period of 10 minutes using a microinfusion pump (Eicom EPS-26, Kyoto, Japan). The stereotactic needle was removed 5 minutes after completion of infusion. The burr hole was sealed with cyanoacrylate glue and the incision was closed with sutures. The rats were placed in a warm box and allowed to recover from the anesthesia and given free access to food and water.

### *Experimental protocols*

This study was divided into 3 parts. The first part examined hematoma growth after the collagenase infusion. Animals received an infusion of 10 μL of collagenase solution (0.14 U) into the left basal ganglia. Hematoma volume was evaluated at 2, 4, 6, 12, and 24 hours after collagenase infusion (n = 5 for each time point). Control animals received 10 μL of saline into the basal ganglia and were sacrificed at 24 hours (n = 5).

The second part of the study investigated whether general administration of rFVIIa reduces hematoma enlargement. Treated-animals received an intravenous injection of 120 μg/kg of rFVIIa (NovoSeven, Novo Nordisk, Denmark) in 200 μL water immediately after collagenase infusion. Control animals received vehicle (200 μL of water) immediately after collagenase infusion. Recombinant human VIIa was reported to inhibit the bleeding tendency induced by warfarin treatment in rats [7]. The dose of rFVIIa used in this study (120 μg/kg) was chosen based on a previously determined range used in an experimental study (50 and 250 μg/kg) [7] and in a randomized clinical trial for patients with ICH (10- to 120-μg/kg bolus dose) [8]. At 24 hours after collagenase infusion, animals were sacrificed for measurements of hematoma volume (n = 6 in each group) or brain hemoglobin content (n = 6 in each group).

The third part of the study evaluated the effect of general administration of rFVIIa on brain edema formation 24 hours after collagenase infusion. Treated-animals (n = 6) received an intravenous injection of 120 μg/kg of rFVIIa in 200 μL water immediately after collagenase infusion. Control animals (n = 6) received vehicle (200 μL of water) immediately after collagenase infusion.

### *Analytical methods*

#### Morphometric measurement of hematoma volume

Animals were sacrificed by decapitation under deep sodium pentobarbital anesthesia (100 mg/kg), and the brains were rapidly removed and sectioned coronally at 2-mm intervals. After taking photographs using a digital camera, the hemorrhage area for each slice was measured with the use of a computerized image analysis system (ImageJ, version 1.32, National Institutes of Health, Bethesda, MD). Total hematoma volume was calculated by summing the clot area in each section and multiplying the distance (2 mm) between sections.

#### Spectrophotometric hemoglobin assay

A modified spectrophotometric assay was used to determine blood volume (hemoglobin) in the brain after ICH [5]. The ipsilateral cerebral hemisphere was collected from each animal. Distilled water (3 mL) was added to each hemisphere, followed by homogenization for 30 seconds, sonication on ice with an ultrasonicator for 1 minute, and centrifugation at 13 000 rpm for 30 minutes. The hemoglobin containing supernatant was collected, and 400 μL of Drabkin's solution was added to a 100-μL aliquot. Fifteen minutes later, the optical density of the solution at 550-nm wavelength was measured to assess hemoglobin content.

#### Measurement of brain water content

After sacrificing the rats by decapitation, the brains were rapidly removed and 2 coronal slices of 4-mm thickness were cut 4 mm from the frontal pole. The brain slices were divided along the midline and the cortex was separated from the basal ganglia bilaterally. Tissue samples were immediately weighed on an electronic analytical balance to the nearest 0.1 mg to obtain the wet weight (WW). Tissue samples were then dried in an oven at 110 °C for 24 hours and weighed again to obtain the dry weight (DW). The formula $(WW - DW)/WW \times 100$ was used to calculate the brain water content and expressed as a percentage of WW.

#### Data analysis

All data in this study are presented as mean ± SD. Data were analyzed using Student *t* test or analysis of variance (ANOVA) with Fisher PLSD test using StatView version 5.0 (SAS Institute, Chicago, IL). A 2-tailed probability value of less than 0.05 was used to indicate a significant difference.

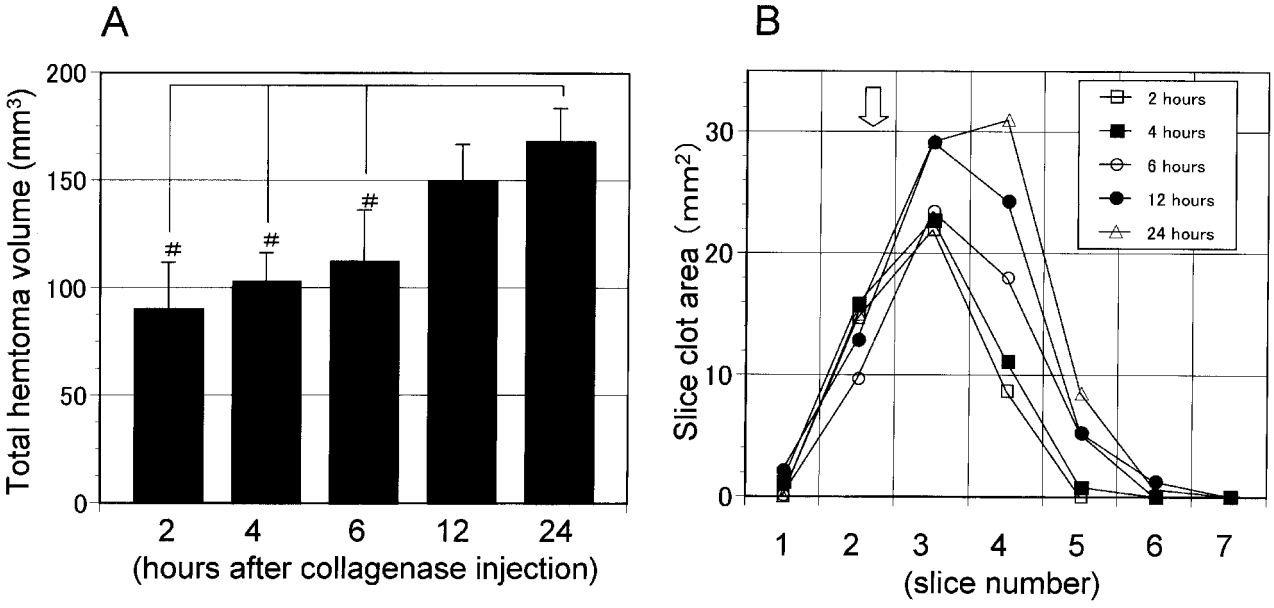


Fig. 1. Time course of hematoma growth after intracerebral infusion of type IV bacterial collagenase solution into the striatum. The average hematoma volume at 24 hours after collagenase injection was significantly larger compared with the values at 2, 4, and 6 hours after the collagenase injection (A) (#$p < 0.001$). The hematoma mainly increased in size in slices posterior to the collagenase injection site (arrow) (B)

## Results

There were no significant differences in the values of arterial blood pressure, blood gas tension, blood pH, hematocrit level, body temperature, or blood glucose concentration between the 2 groups.

### *Hematoma enlargement*

Two hours after collagenase injection, a small amount of blood had already collected in the striatum extending along with the external capsule, and the average hematoma volume was $90.5 \pm 21.3$ mm$^3$ (Fig. 1A). At 4 hours after collagenase injection, the amount of blood that had collected in the striatum had become larger and roughly spherical in shape; the average hematoma volume was $103.3 \pm 12.9$ mm$^3$ (Fig. 1A). The clot occupied almost the entire area of the striatum, extending to the ventricular wall medially and corpus callosum superiorly. The amount of blood gradually increased in size and extended to the thalamus posteriorly by 24 hours after collagenase injection (Fig. 1B); the average hematoma volume was $112.7 \pm 23.5$ mm$^3$ at 6 hours, $149.8 \pm 17.0$ mm$^3$ at 12 hours, and $168.4 \pm 14.9$ mm$^3$ at 24 hours (Fig. 1A). When comparison was made with the value at 2 hours after collagenase injection, the average hematoma volume was larger at 12 hours ($p < 0.001$) and 24 hours ($p < 0.001$). When comparison was made with the value at 4 hours, the average hematoma volume was also larger at 12 hours ($p < 0.01$) and at 24 hours ($p < 0.001$). When comparison was made with the value at 6 hours, the average hematoma volume was larger only at 24 hours ($p < 0.001$).

### *Effects of rFVIIa on hematoma enlargement*

Blood collection in the basal ganglia was assessed morphometrically 24 hours after collagenase injection in rats receiving either rFVIIa or vehicle. Intravenous administration of rFVIIa immediately after collagenase injection significantly decreased the average hematoma volume compared with vehicle-treated rats ($168.1 \pm 13.4$ mm$^3$ vs. $118.3 \pm 23.0$ mm$^3$, $p < 0.01$) (Fig. 2A). Brain hemoglobin content was also used to assess hematoma mass. Again, there was a decrease in hemispheric total hemoglobin content in rats treated with rFVIIa compared with vehicle-treated rats (OD at 550 nm: $0.87 \pm 0.08$ vs. $0.71 \pm 0.09$, $p < 0.05$) (Fig. 2B). Since the hemoglobin concentration in the blood was the same in the rFVIIa and vehicle groups, this indicates that the clot mass was significantly smaller in the rFVIIa-treated rats.

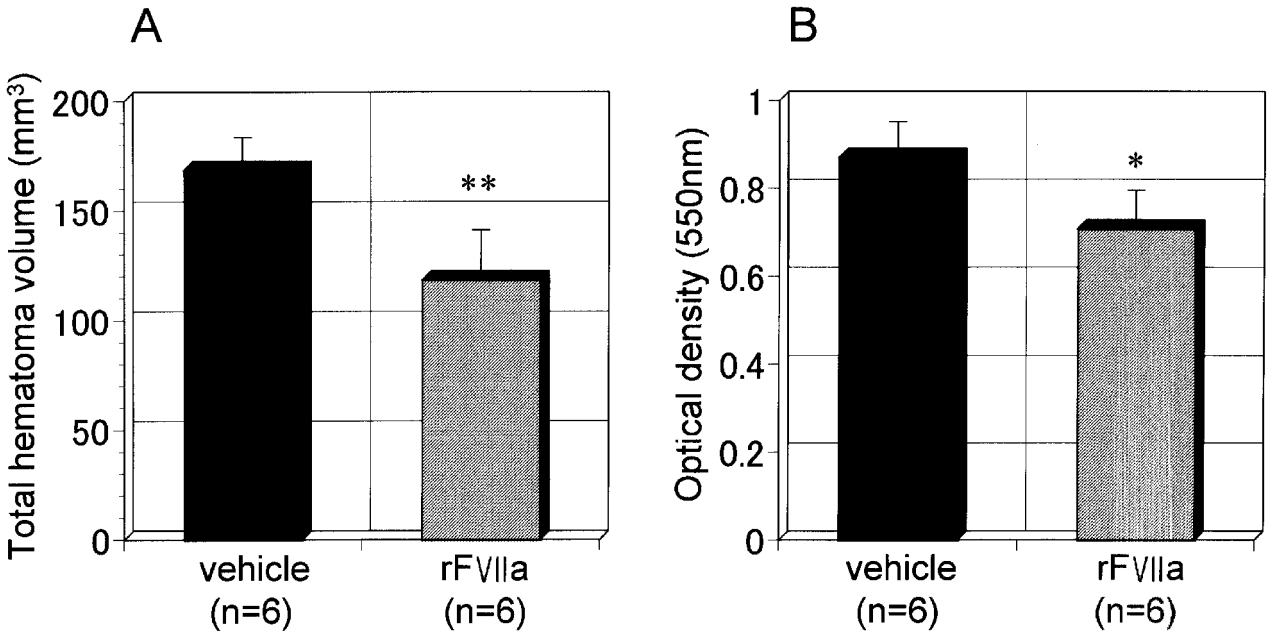


Fig. 2. Hematoma volume (A) and brain hemoglobin (optical density) (B) 24 hours after intracerebral infusion of collagenase solution. Immediately after collagenase injection, rats received an intravenous infusion of rFVIIa (120 μg/kg) or vehicle (water). Values are mean ± SD. **$p < 0.01$ and *$p < 0.05$ compared with vehicle

*Effects of rFVIIa on brain water content*

Brain water content in the cortex overlying the hematoma was determined 24 hours after collagenase injection using the drying/weighing method in rats receiving either rFVIIa or vehicle. In spite of reduced hematoma volume by administration of rFVIIa, there were no differences in ipsilateral cortical water content between rFVIIa-treated and vehicle-treated groups (anterior: 81.4 ± 0.7% vs. 81.7 ± 0.4%, posterior: 81.7 ± 0.6% vs. 81.7 ± 0.5%).

## Discussion

*Early hematoma growth after ICH*

Neurological deterioration during the first day after ICH is strongly associated with early hematoma growth, and the volume of the hematoma is an important predictor of 30-day mortality [2, 4, 10, 11, 17]. The time course for progression of ICH in humans is controversial. Herbstein and Schaumburg [14] injected $^{51}$Cr-labeled erythrocytes into 11 patients with hypertensive ICH between 1 to 2 and 4 to 5 hours after onset. Postmortem examination revealed no significant radioactivity in the primary hematoma, suggesting that bleeding had ceased within at least 2 to 5 hours after onset [14]. On the other hand, cerebral angiography performed 1.5 to 7 hours after onset showed extravasation of the contrast medium from perforating arteries in 7 patients with ICH [27]. An association between early hematoma growth and irregular clot morphology, which is presumably the result of multifocal bleeding, has also been reported [18]. Initial bleeding has been thought to be completed quickly in many cases as a result of clotting and tamponade by surrounding brain tissue. Hematoma growth has been assumed to result from rebleeding from the initial site of arterial or arteriolar rupture. Although this may be true in many cases, there have been several case reports in which active bleeding seemed to last more than 6 hours after onset of ICH. Recent studies suggest that early hematoma growth may also result from secondary bleeding into perilesional tissue in the periphery of the initial clot [24, 25]. Simultaneous CT and single-photon emission CT studies have demonstrated instances in which ICH growth results from the addition of discrete hemorrhages within the no-flow zone around the existing clot [24, 25]. These clinical data suggest that early hematoma growth is a dynamic process in acute ICH and in some cases may result from bleeding into a "penumbra" of damaged and congested brain tissue immediately surrounding a hematoma.

*Collagenase-induced ICH model in rats*

In the collagenase-induced ICH model, histopathological studies have shown that erythrocytes appear around blood vessels at the needle puncture site within the first hour and there is an extensive hematoma 4 hours after collagenase infusion with tissue disruption by extravasated erythrocytes [29]. Enlargement of the hematoma was observed for up to 4 hours after collagenase injection in this model of ICH using magnetic resonance imaging and examining comparable histological sections [6]. We have slightly modified the procedure reported by Rosenberg *et al.* [29] by increasing the amount of solution and decreasing the concentration of collagenase in the solution. In our model, there was already a small amount of hemorrhage in the striatum spreading along the external capsule 2 hours after collagenase injection. The relatively large amount of injection solution used in this study readily spread through the white matter. By 4 hours, the extravasated blood in the striatum was almost contiguous with the external capsule and became roughly spherical, occupying almost the entire area of the striatum, extending to the ventricular wall medially and corpus callosum superiorly. ICH gradually increased in size and extended to the thalamus posteriorly by 24 hours after collagenase injection. Our modification has the benefit of producing a slow-growing ICH of uniform shape and reproducible size in the basal ganglia. In this model, the effects might be caused by tissue compression and ''infusion edema'' because of the relatively large amount of solution injection. However, rats that received 10 μL of vehicle (saline) injection did not exhibit an increase in brain water content in the basal ganglia and overlying cortex (data not shown).

*Effects of rFVIIa on hematoma enlargement*

Because hematoma growth is a dynamic process during an acute ICH, intervention with ultra-early hemostatic therapy could minimize and possibly even prevent early hematoma growth. FVIIa is an important natural initiator of hemostasis exerting its primary effects locally in regions of endothelial disruption and vascular injury [13]. Factor VII forms a complex with exposed tissue factor at local bleeding sites, activating the hemostatic cascade locally to form a hemostatic plug. A pharmacological dose of rFVIIa amplifies this process. Long-term clinical use for the treatment of hemophilic patients with inhibitors, rFVIIa has been associated with low risk of systemic coagulation and thromboembolic complications and has shown good clinical results for treating intracranial hemorrhage [1, 22, 31]. Clinical trials also indicate that rFVIIa promotes hemostasis in neurosurgical patients with normal coagulation activity [15, 28]. Clinical studies are currently ongoing to evaluate its efficacy for reducing the frequency of early hematoma growth in patients with acute ICH [8, 23, 24]. Arrest of early hematoma growth might reduce the frequency of neurological deterioration by preventing early worsening related to hematoma growth, as well as late deterioration from perihematomal edema and mass effect.

The ideal animal model has not been established for early hematoma growth and prevention of hematoma enlargement using pharmacological intervention. While our model does not faithfully mimic the complex and dynamic nature of human ICH, it does resemble human ICH dynamics and enables evaluation of the effect of rFVIIa on early hematoma growth. As a clotted hematoma forms, plasma rich in thrombin quickly seeps into surrounding tissue [32]. Thrombin causes blood-brain barrier disruption and enhances brain edema formation in rats [20, 21]. In spite of reduced hematoma volume by rFVIIa administration, there was no reduction in the brain water content in the overlying cortex between the rFVIIa-treated and vehicle-treated groups in our experiment. A pharmacological dose of rFVIIa amplifies thrombin formation at the clot-brain interface and may exacerbate brain edema formation in rFVIIa-treated animals. However, results from phase II trials have shown that there was no dose-related effect of rFVIIa on edema-to-ICH volume ratio and even a high dose of rFVIIa (160 μg/kg) did not exacerbate brain edema formation around ICH [26]. A recent study has shown that the thrombin inhibitor argatroban can reduce ICH-induced edema formation in rats [19]. Further studies are necessary to evaluate the effect of combined treatment using rFVIIa and argatroban on ICH growth and perihematomal brain edema formation in rats. While we administered the rFVIIa immediately after collagenase injection into the brain, studies of delayed treatments of rFVIIa on hematoma growth should also be performed before endorsing the clinical use of rFVIIa in patients with acute ICH.

In conclusion, the present experimental study indicates that treatment with rFVIIa may be useful in reducing the frequency of early hematoma growth in patients with ICH. Our experimental results further

justify the ongoing clinical trial of rFVIIa on human ICH.

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