

# **Reducing Hemorrhagic Complication by Dabigatran via Neurovascular Protection after Recanalization with tPA in Ischemic Stroke of Rat**

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Running title: Reducing Hemorrhage by Dabigatran

## **Abstract**

This study aimed to assess the risk and benefit of tPA treatment under oral anticoagulation with dabigatran compared to warfarin or vehicle control in transient middle cerebral artery occlusion (tMCAO). After pretreatment with warfarin (0.2 mg/kg/day), dabigatran (20 mg/kg/day), or vehicle (0.5% carboxymethyl cellulose sodium salt) for 7 days, tMCAO was induced for 120 min followed by reperfusion and tPA (10 mg/kg/10 ml). Clinical parameters, including cerebral infarction volume, hemorrhagic volume, and blood coagulation, were examined. At 24 h after reperfusion, markers for the neurovascular unit at the periischemic lesion were immunohistochemically examined in brain sections, and MMP-9 activity was measured by zymography. Paraparesis and intracerebral hemorrhage volume were significantly improved in the dabigatran-pretreated group than in the warfarin-pretreated group. A marked dissociation between astrocyte foot processes and the basal lamina or pericyte was observed in the warfarin-pretreated group, which was greatly improved in the dabigatran-pretreated group. Furthermore, a remarkable activation of MMP-9 in the ipsilateral warfarin-pretreated rat brain was greatly reduced in dabigatran-pretreated rats. The present study reveals that the mechanism of intracerebral hemorrhage with warfarin-pretreatment plus tPA in ischemic stroke rats is the dissociation of the neurovascular unit, including the pericyte. Neurovascular protection by dabigatran, which was first shown in this study, could partly explain the reduction in hemorrhagic complication by dabigatran reported in the clinical study.

**Key Words:** dabigatran; hemorrhagic complication; neurovascular unit; pericyte; thrombolysis; tPA

## Introduction

As the world's population is progressively aging in most countries, so too is the number of patients who are suffering from stroke also rapidly increasing. Half of all strokes occur in people who are over 70 years old, and a quarter occurs in patients who are > 85 years of age (Bamford et al., 1988; Brown et al., 1996). Since atrial fibrillation (AF) is an age-dependent incident that is more common in the elderly, cardiogenic cerebral embolic stroke is the major cause of increasing strokes among the elderly.

The new oral anticoagulant (NOAC) dabigatran is a direct thrombin inhibitor that was approved by the US Food and Drug Administration in October 2010 based upon data from the Randomized Evaluation of Long-term Anticoagulant Therapy With Dabigatran Etexilate (RE-LY) study which demonstrated it to be as safe as, safer than or at least as effective as warfarin (Connolly et al., 2009). A notable benefit of dabigatran, unlike warfarin, is that it does not need the international normalized ratio (INR) to be monitored nor the diet to be restricted. Therefore, the number of dabigatran-treated patients having an ischemic stroke is increasing around the world.

Upon ischemic stroke, a patient that was pretreated with warfarin can still receive tissue plasminogen activator (tPA) if their INR is  $\leq 1.7$ , which could increase the risk

of hemorrhagic complication. The National Institute of Neurological Disorders and Stroke (NINDS) tPA study (The National Institute of Neurological Disorders and Stroke rtPA Stroke Study Group, 1995), the European Cooperative Acute Stroke Study trials (ECASS I-III) (Hacke et al., 1995; Hacke et al., 1998; Hacke et al., 2008), and the Safe Implementation of Thrombolysis in Stroke Monitoring Study (SITS-MOST) (Wahlgren et al., 2007) excluded patients receiving oral anticoagulant treatment, regardless of the INR. In the Japan post-Marketing Alteplase Registration Study (J-MARS) (Nakagawara et al., 2010), 3.5% of patients had symptomatic intracranial hemorrhage, but there is limited data on the safety of tPA in warfarin-pretreated patients. In addition, there is no guideline whether dabigatran-pretreated patients within 4.5 h with acute ischemic stroke can be considered eligible for tPA treatment or not. The aim of this study was to assess the risk and benefit of tPA treatment under oral anticoagulation with dabigatran compared to warfarin or a placebo control.

## **Materials and Methods**

### **Experimental Model**

Male Wistar rats (SLC, Shizuoka, Japan) 11 weeks old (body weight 240–270 g) were divided into 3 groups: vehicle-treated (0.5% carboxymethyl cellulose sodium salt;

V+tissue plasminogen activator (tPA)) group, warfarin-treated (0.2 mg/kg/day; W+tPA) group, and dabigatran-treated (20 mg/kg/day; D+tPA) group, with n=9 in each group. For each drug, the dose and interval between the last intake of drug and the induction of cerebral ischemia were determined so as to inhibit clot formation by 70% in the rat venous thromboembolism model (Toomey et al., 2006; Wienen et al., 2007). Each drug was administered orally for 7 days starting from when rats were 11 weeks old. Warfarin was administered once a day and dabigatran twice a day. Thrombus formation was reduced by 91% with 10 mg/kg of dabigatran after 30 min of administration and reduced by 70% after 1 h of administration according to Wienen et al. Thrombus formation was also reduced by 70% with 0.2 mg/kg of warfarin after 1 h of administration according to Toomey et al. Thus, the last intake of both drugs was 1 h before the induction of cerebral ischemia. Body weight and blood pressure were measured twice before the first and last administration. Blood was drawn from the left femoral vein prior to and 1 h after the last administration of each drug, and prothrombin time (PT), activated prothrombin time (aPTT), and thrombin-antithrombin complex (TAT) were measured.

At 7 days of daily administration of the vehicle (12 w of age), warfarin or dabigatran, the rats were anesthetized with a mixture of nitrous oxide/oxygen/isoflurane

(69: 30: 1) during surgical preparation with an inhalation mask. Body temperature was monitored and maintained at  $37 \pm 0.3$  °C using a heating pad during the surgical procedure. The right middle cerebral artery (MCA) was occluded by inserting a 4–0 surgical nylon thread with silicon coating through the common carotid artery as described previously (Abe et al., 1992). After 120 min of transient MCA occlusion (tMCAO), the nylon thread was gently removed to restore blood flow in the MCA territory and was treated with tissue plasminogen activator (tPA; Grtpa, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan, intravenous bolus, 10 mg/kg/10 ml). 24 h after reperfusion, blood pressure was measured and behavior was analyzed.

For histological examinations, the rats (n=9 each) were transcardially perfused with heparinized saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2). The whole brain was removed and immersed in the same fixation for 12 h at 4°C. After washing with PBS (pH 7.2), the tissues were transferred into a 10%, 20%, and 30% (w/v) sucrose gradient and then embedded in powdered dry ice and stored at -80°C. Coronal brain sections 20 µm thick were prepared using a cryostat at -18°C and mounted on a silane-coated glass slide.

For gelatin zymography, a different set of rats (each group, n=5) were treated as above. 24 h after reperfusion, rats were anesthetized by intraperitoneal injection of

pentobarbital (40 mg/kg) and transcardially perfused with chilled heparin (5 U/ml in PBS; pH 7.2). Brains were removed quickly and divided into ipsilateral-periischemic and contralateral-nonischemic hemispheres. Each hemispheric brain was frozen immediately in dry ice and stored at -80°C until use.

All experimental procedures were approved by the Animals Committee of the Graduate School of Medicine and Dentistry, Okayama University.

### **Behavioral Analysis**

Before cerebral ischemia and 24 h after reperfusion, the rats were tested for behavioral activities and scored according to the Zhang et al. (Zhang et al., 2002) corner test with a minor modification by calculating the difference in the numbers of turning right (the paraparesis score).

### **Histology and immunohistochemistry**

To determine the area of ischemic lesions, sections were stained with hematoxylin-eosin (HE) and examined under a light microscope (Olympus SZX-12; Olympus Optical Co.). The sections were made at 2, 0, -2, -4, and -6 mm from the Bregma. The infarct area was measured at these five sections by counting pixels using

Photoshop CS5 and infarct volume was calculated by multiplying the infarct area by 2 mm thickness (Kawai et al., 2011). To analyze brain hemorrhage, iron staining was performed using an enhanced Perl's reaction. Brain sections were incubated with Perl's solution (5% potassium ferrocyanide and 5% HCl, 1:1) for 45 min, washed in distilled water, and incubated again in 0.5% diamine benzidine tetrahydrochloride with nickel for 60 min, as described by Wu et al. (Wu et al., 2003).

For immunohistochemistry, the following primary antibodies were used: rabbit anti-MMP-9 antibody (1:200; Abcam); rabbit anti-collagenIV antibody (1:200; Novotec); mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:1000; Chemicon); and rabbit anti-platelet-derived growth factor (PDGF) receptor beta antibody (1:500; Abcam). To detect vascular endothelial cells, N-acetylglucosamine oligomer (NAGO) was used as the specific endothelial cell marker (Augustin et al., 1995). Brain sections were washed with PBS (pH 7.4) and then incubated in 0.3% hydrogen peroxidase/methanol for 10 min to block endogenous peroxidase activity and incubated with bovine serum albumin for 1 h. Then they were incubated overnight at 4 °C with mouse anti-GFAP antibody and rabbit anti-collagenIV antibody or rabbit anti-PDGF receptor beta antibody, and with biotinylated *Lycopersicon esculentum* lectin (1:500; Vector Laboratories), which binds NAGO and rabbit anti-PDGF receptor



beta antibody. On the next day, the slices were washed in PBS (pH 7.4) and incubated for 2 h at room temperature with fluorochrome-coupled secondary antibody (1:500; Alexa Fluor™, Molecular Probes, A21424, A21429, and A11034). The sections were then rinsed 3 times in PBS (pH 7.4) and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, H1200). A confocal microscope equipped with argon and HeNe1 lasers (Zeiss, LSM 510) was used to capture fluorescent images.

### **Vascular dissociation index**

To assess the detachment of astrocyte endfeet from the basement membrane in the GFAP/collagenIV double-labeled sections or from the pericyte in the GFAP/PDGF receptor beta double-labeled sections, and to assess the detachment of the pericyte from vascular endothelial cells in the PDGF receptor beta/NAGO, 3 levels of the caudate putamen (1.2, 0.7, and 0.2 mm rostral to the bregma) (Paxinos and Watson, 1982) of each animal, and 4 areas in the ipsilateral peri-infarcted cortex in each section were chosen randomly and captured at ×100 magnification with a confocal laser microscope. We confirmed the border between the ischemic core and peri-infarct lesion through cresyl violet staining of adjacent sections according to a previous method (Omori et al., 2002), and measured the area between astrocyte endfeet and the basement membrane of

each blood vessel, as well as the length of each blood vessel. Then, the area to length ratio was calculated as the ‘vascular dissociation index’ (Yamashita et al., 2009). In the same way, the area between astrocyte endfeet and pericyte, as well as the area between pericyte and vascular endothelial cells were measured, and the area to length ratio was calculated in each blood vessel.

### **Gelatin Zymography**

Gelatin zymography was performed using frozen brain tissue from the cerebral cortex. Frozen brain samples were homogenized in 10× volume lysis buffer (150 mM NaCl, 1% SDS, 0.1% deoxycholic acid and 50 mM Tris-HCl, pH 7.4) containing protease inhibitors. After centrifugation at  $9,000 \times g$  for 15 min at 4°C, the supernatant was collected. Total protein concentration of each supernatant was spectrophotometrically determined using the Bradford assay (Ultrospec 3100 Pro; GE Healthcare, Tokyo, Japan). The activity of MMP-9 in each sample was measured using a gelatin-zymography kit (Primary Cell, Sapporo, Japan) according to the manufacturer’s instructions. In brief, each sample containing 20 µg protein was diluted with the homogenizing buffer in the kit, mixed with an equal volume of sample buffer,

and loaded for electrophoresis for 2 h. The gels were washed and incubated for 24 h in incubation buffer at 37°C, then stained with Coomassie blue and scanned. Quantitative densitometric analysis was performed in Image J software.

### **Statistical Analysis**

All data are presented as the mean  $\pm$  SD. Statistical analyses were performed using 1-factor analysis of variance followed by Tukey–Kramer’s postcomparison test. Differences with a probability value of  $p < 0.05$  were considered statistically significant.

## **Results**

Mean body weight and systolic and diastolic blood pressure were not significantly different among the three groups (Table 1). The paraparesis score was significantly improved in the D+tPA group ( $3.7 \pm 2.3$ ,  $*p < 0.05$ ) than in the W+tPA group ( $6.5 \pm 2.1$ ) (Fig. 2A). Infarction volume was not different among the three groups (Fig. 2B). Intracerebral hemorrhage volume was significantly larger in the W+tPA group than in V+tPA or D+tPA groups (Fig. 2C,  $*p < 0.05$ ). Significant PT prolongation was observed in the W+tPA group compared to the baseline (Fig. 2Da,  $\star p < 0.05$ ), in D+tPA groups compared to the baseline (Fig. 2Da,  $\star\star p < 0.01$ ), and in the V+tPA

group (Fig. 2Da, \* $p < 0.05$ ). Significant aPTT prolongation was found only in the D+tPA group compared to the baseline (Fig. 2Db, ★★ $p < 0.01$ ), in the V+tPA group (Fig. 2Db, \* $p < 0.05$ ), and in the W+tPA group (Fig. 2Db, # $p < 0.05$ ). Although TAT was significantly reduced in both W+tPA and D+tPA groups compared to the V+tPA group (Fig. 2Dc, \*\* $p < 0.01$ ), and in the D+tPA group compared to the baseline (Fig. 2Dc, ★ $p < 0.05$ ), there was no difference between W+tPA and D+tPA groups, indicating that the antithrombotic effect was almost the same in both groups. Intracerebral hemorrhage was sometimes observed on the surface (Fig. 3A, arrowheads) and in the coronal section (Fig. 3B, arrowheads) of the brain in W+tPA and D+tPA groups, but was more evident in the W+tPA group.

In the V+tPA group, little dissociation of the neurovascular unit was found in the periischemic lesion (Fig. 4Aa, left panels). In contrast, a marked dissociation of the basal lamina (collagen IV) and astrocyte foot processes (GFAP) was observed in the periischemic lesion of the W+tPA group (Fig. 4Aa, middle panels, arrowheads, Fig. 4Ab, \*\* $p < 0.01$ ), which was dramatically improved in the D+tPA group (Fig. 4Aa, right panels, Fig. 4Ab, \*\* $p < 0.01$ ). Dissociation of pericyte (PDGFR $\beta$ ) and astrocyte foot processes (GFAP) was significantly larger in the W+tPA group (Fig. 4Ba, middle panels, arrowheads) than in the V+tPA or D+tPA groups. The vascular dissociation

index revealed a larger dissociation in the W+tPA group than in V+tPA or D+tPA groups (Fig. 4Bb,  $**p < 0.01$ ). On the other hand, there was no difference among the three groups in terms of dissociation between pericyte (PDGFR $\beta$ ) and vascular endothelial cells (NAGO) (Fig. 4Ca), with no quantitative vascular dissociation index among the three groups (Fig. 4Cb).

Gelatin zymography indicated that there was no activation and no difference in contralateral MMP-9 activities among the three groups (Fig. 5A, 4B). In contrast, the ipsilateral brain showed considerable activation of MMP-9 in the W+tPA group compared to contralateral V+tPA (Fig. 5B,  $**p < 0.01$ ), and contralateral W+tPA (Fig. 5B,  $##p < 0.01$ ). This ipsilateral activation of the W+tPA group was greatly reduced in the D+tPA group (Fig. 5A, 5B,  $\blacklozenge p < 0.05$ ).

## Discussion

In the present study, pretreatment with dabigatran greatly improved the clinical score (Fig. 2A) and intracerebral hemorrhage (Fig. 2C, 2E, 2F) than warfarin-pretreated rats after thrombolytic therapy with tPA. In the Randomized Evaluation of Longfrom Term Anticoagulant Therapy With Dabigatran Etexilate (RE-LY) study, both 110 and 150 mg doses of dabigatran lowered intracranial

bleeding with a similar or lower rate of stroke than warfarin (Connolly et al., 2009). Among six previous reports on the dabigatran+tPA combination in acute stroke patients, there was only one case of fatal intracerebral hemorrhage, and one case of asymptomatic arm ecchymosis (Smedt et al., 2010; Matute et al., 2011; Naranjo et al., 2011; Marrone and Marrone, 2012; Lee et al., 2012; Sangha et al., 2012). Although the above fatal intracerebral hemorrhage patient carried other risks of intracerebral hemorrhage such as large infarct volume (>2/3 of the MCA area) and diabetes mellitus, the last intake of dabigatran was the shortest of the six cases (6 h before tPA), suggesting that a shorter time interval from dabigatran to tPA is an important risk factor of intracerebral hemorrhage. However, the interval between the last dabigatran intake and tPA treatment is different between these clinical reports and the present study. Thus, we may not be able to directly compare our findings with these clinical reports.

In the present study, macroscopic intracerebral hemorrhage was observed in 66.7% of the warfarin-pretreated rats, 44.4% of the dabigatran-pretreated rats, and 44.4% of the vehicle rats (Table 1,  $p=0.55$ ), which suggests a lower risk of occurrence of macroscopic intracerebral hemorrhage with dabigatran+tPA. Hemorrhagic volume was much lower in the D+tPA group (Fig. 2C). Thus, dabigatran reduced not only

hemorrhagic incidence but reduced hemorrhagic volume even more (Table 1, Fig. 2C).

At a higher plasma level of dabigatran, the risk of severe intracerebral hemorrhage may still rise (Pfeilschifter et al., 2012), although there is no common marker for anticoagulant activity by dabigatran at emergency. In the present study, significant prolongation of PT was observed in W+tPA and D+tPA groups (Fig. 2Da), and significant aPTT prolongation was found only in the D+tPA group (Fig. 2Db). The TAT complex was significantly reduced in both W+tPA and D+tPA groups compared to the vehicle group (Fig. 2Dc,  $**p < 0.01$ ), indicating a similar antithrombotic effect in both groups with each dose for obtaining 70% inhibition of clot formation in the rat venous thromboembolism model (Toomey et al., 2006; Wienen et al., 2007).

Various proinflammatory mediators (MMPs, thrombin, vascular endothelial growth factor, and bradykinin) increase in the ischemic brain (Aschner et al., 1997; Kamiya et al., 1993; Rosenberg GA, 2002; Suarez and Ballmer-Hofer, 2001), accompanied by brain edema, endothelial cell death (Maier et al., 2006), disruption of tight junctions, and loss of the basal lamina/extracellular matrix (collagenIV, laminin-1, and fibronectin). Any of these changes could promote intracerebral hemorrhage associated with tPA treatment (Zoppo and Mabuchi, 2003). We previously reported that MMP-9 activation after tMCAO induced the dissociation between the vascular

basal lamina and the astrocyte endfeet in the ischemic rat brain treated with tPA (Yamashita et al., 2009), which was confirmed in the present study (Fig. 4A, 4). A marked dissociation between astrocyte foot processes and the basal lamina was observed on the periischemic lesion of warfarin-pretreated rats in association with MMP-9 activation (Fig. 4A, 4), which improved dramatically in the dabigatran-pretreated group (Fig. 4A, 4).

The mechanism in which warfarin activates MMP-9 while dabigatran inhibits the activation, remains obscure, but several inferences can be drawn from other reports. Factor VII (FVII) forms a complex with the cell surface co-factor, tissue factor (TF), which appears after vascular injury, and the FVII and TF complex initiates the coagulation cascade (Vadivel and Bajaj, 2012). Warfarin inhibits FVII (Sakata et al., 1995) and the coagulation cascade does not start, even after endothelial cells are injured. Therefore, the inability to repair endothelial cells and asymptomatic microbleeding may occur recurrently. tPA causes disruption of the neurovascular matrix through MMP-9 upregulation when it leaks into the parenchyma (Goto et al., 2007). We speculate that recurrent injury and the inability to repair endothelial cells by warfarin-pretreatment may facilitate the leakage of tPA outside the vessel, leading to the upregulation of MMP-9 activity after ischemia and tPA treatment.



In the present study, we focused on the role of pericytes which encircle capillary vessels, and are important for the maturation and stabilization of the capillary vessels during angiogenesis. After cerebral ischemia, pericytes increased neurotrophin-3 production, which potentiated the secretion of nerve growth factor (NGF) from astrocytes (Ishitsuka et al., 2012). Ischemia and reperfusion-induced injury to pericytes may impair microcirculatory reflow and negatively affect survival (Yemisci et al., 2009). Our present study showed a marked dissociation between astrocyte foot processes and pericytes in the periischemic lesion of warfarin-pretreated rats, which was dramatically improved in the dabigatran-pretreated group (Fig. 4B). The dissociation between astrocyte foot processes and pericytes could also allow the development of a neurovascular unit for intracerebral hemorrhage (Fig. 2-4).

In summary, the present data suggests a lower risk of intracerebral hemorrhage after tPA in ischemic stroke rats with pretreated dabigatran compared to pretreated warfarin. A remarkable activation of MMP-9 with warfarin caused a marked dissociation of the neurovascular unit raising the risk of intracerebral hemorrhage after tPA, which was greatly ameliorated by replacing with dabigatran. Thus, this study is the first evidence of neurovascular protection by dabigatran which could partly explain the mechanism of reducing hemorrhagic complications by dabigatran reported in the

RE-LY clinical study.

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## Figure legends

Fig. 1) Experimental groups including V (vehicle) +tPA group, W (warfarin) +tPA group, and D (dabigatran) +tPA group. tMCAO, transient middle cerebral artery occlusion; tPA, tissue plasminogen activator. At 24 h after 2 h tMCAO, rats were sacrificed.

Fig. 2) Clinical, serum chemical and hemorrhagic complications in animal groups. (A) The paraparesis score was significantly improved in the D+tPA group (\*p < 0.05) than in the W+tPA group. (B) Infarction volume was not different among the three groups. (C) Intracerebral hemorrhage volume was significantly larger in the W+tPA group than in V+tPA or D+tPA groups (\*p < 0.05). (D) Significant PT prolongation was observed in W+tPA and D+tPA groups compared to baseline (Da), and significant aPTT prolongation was found only in the D+tPA group compared to the baseline (Db). Although TAT was significantly reduced in both W+tPA and D+tPA groups compared to the V+tPA group, there was no difference between W+tPA and D+tPA groups (Dc). ★p < 0.05, ★★p < 0.01 versus baseline, \*p < 0.05, \*\*p < 0.01 versus V+tPA, #p < 0.05 versus W+tPA.

Fig. 3) Intracerebral hemorrhage was sometimes observed on the surface (A) and coronal section (B) of the brain in W+tPA and D+tPA groups (arrowheads), but was more evident in the W+tPA group.

Fig. 4) Double immunofluorescent analysis of collagen IV+GFAP (A), PDGFR $\beta$ +GFAP (B), and NAGO+PDGFR $\beta$  (C). A marked dissociation between astrocyte foot processes and basal lamina (A) or pericyte (B) was observed on the periischemic lesion of warfarin-pretreated rats (Bar = 20  $\mu$ m) but there was no dissociation between pericyte and vascular endothelial cells among the three groups (bar = 100  $\mu$ m) (C). The vascular dissociation index estimates the space between each construction (\*\*p < 0.01) (Ab to Cb).

Fig. 5) Gelatin zymography (A) showing considerable activation of MMP-9 in the ipsilateral warfarin-pretreated rat brain but which was greatly reduced in dabigatran-pretreated rats (A). (B) Densitometric analysis of zymography. \*\*p < 0.01 versus contralateral hemisphere of V+tPA, ##p < 0.01 versus contralateral hemisphere of W+tPA, ♦p < 0.05 versus ipsilateral hemisphere of W+tPA.

Table 1. Physiological Parameter in 3 experimental groups

		V+tPA (n=9)	W+tPA (n=9)	D+tPA (n=9)
Body weight	(g)	282.6 ± 15.4	282.6 ± 11.5	284.3 ± 13.0
Before tMCAO				
Systolic BP	(mmHg)	145.4 ± 36.0	152.6 ± 29.7	132.9 ± 28.3
Diastolic BP	(mmHg)	88.4 ± 8.1	93.3 ± 12.5	88.7 ± 5.9
24 h after tMCAO				
Systolic BP	(mmHg)	165.5 ± 33.2	166.6 ± 29.1	152.0 ± 32.4
Diastolic BP	(mmHg)	106.4 ± 23.1	95.3 ± 15.9	95.2 ± 13.2
Intracerebral hemorrhage occurrence	(%)	44.4	66.7	44.4 (p=0.55)

Fig.1

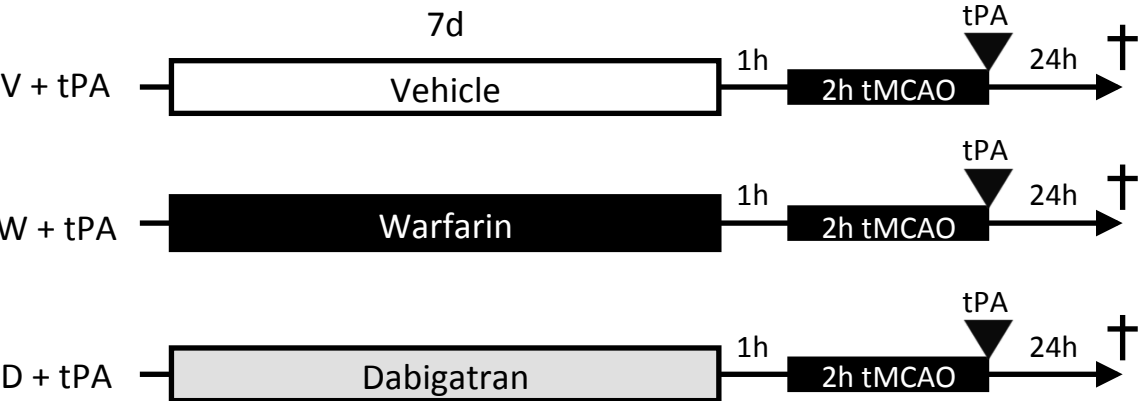


Fig.2

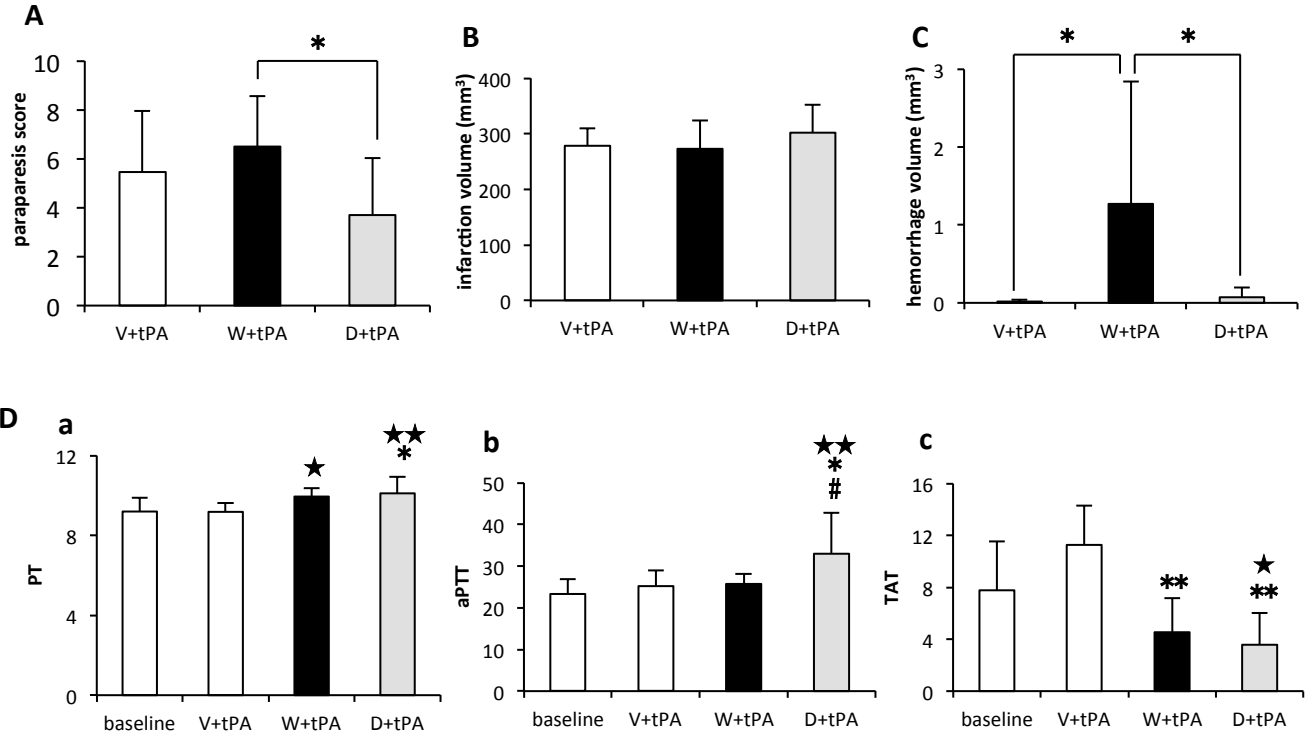


Fig.3

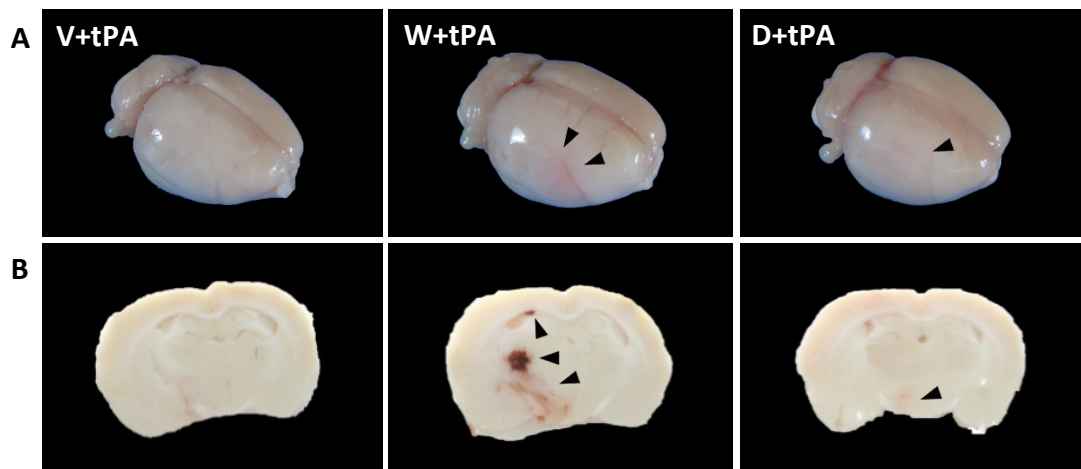


Fig.4

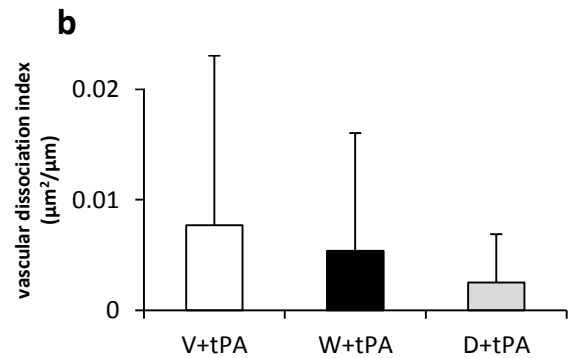
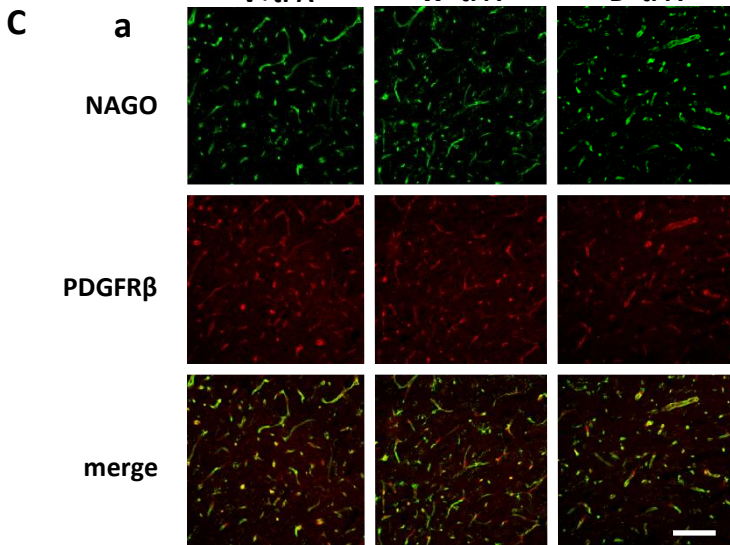
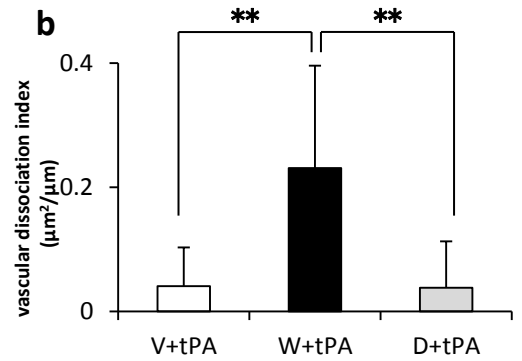
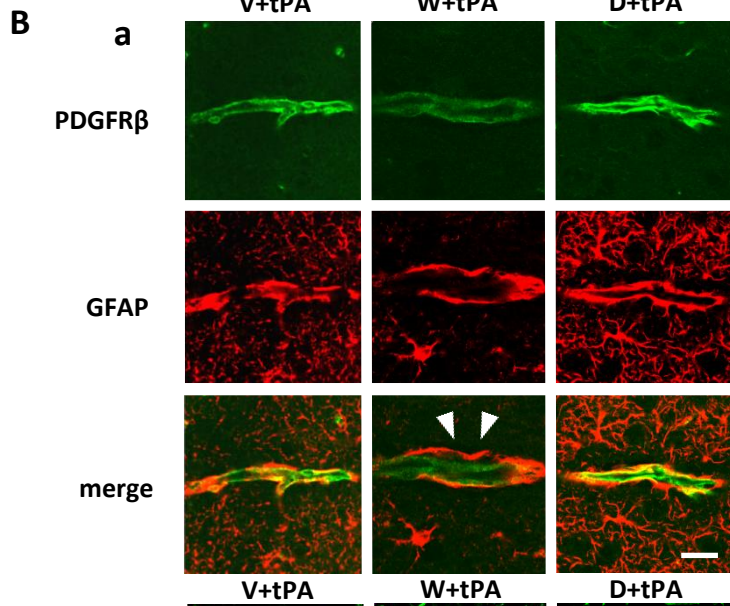
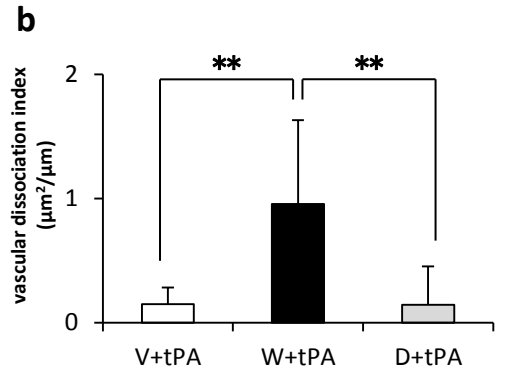
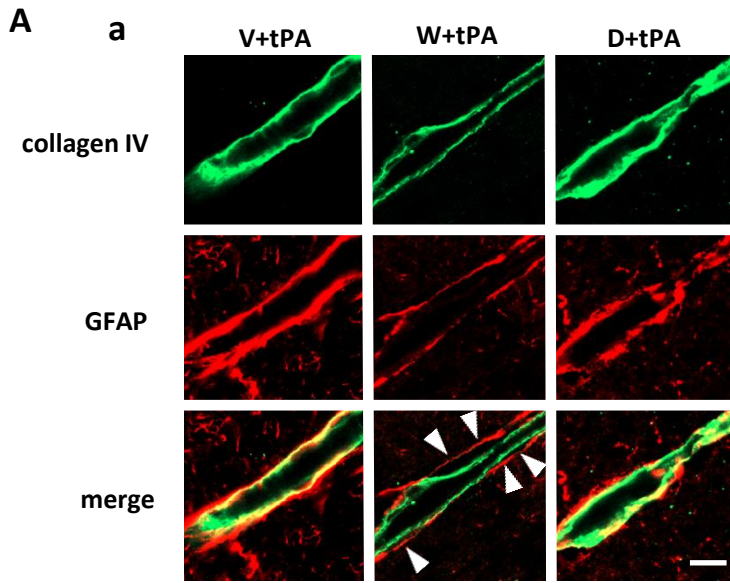


Fig.5

