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Anticoagulation With the Oral Direct Thrombin Inhibitor Dabigatran Does Not Enlarge Hematoma Volume in Experimental Intracerebral Hemorrhage

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Background—The direct thrombin inhibitor dabigatran etexilate (DE) may constitute a future replacement of vitamin K antagonists for long-term anticoagulation. Whereas warfarin pretreatment is associated with greater hematoma expansion after intracerebral hemorrhage (ICH), it remains unclear what effect direct thrombin inhibitors would have. Using different experimental models of ICH, this study compared hematoma volume among DE-treated mice, warfarin-treated mice, and controls.

Methods and Results—CD-1 mice were fed with DE or warfarin. Sham-treated mice served as controls. At the time point of ICH induction, DE mice revealed an increased activated partial thromboplastin time compared with controls (mean±SD 46.1 ± 5.0 versus 18.0 ± 1.5 seconds; $P=0.022$), whereas warfarin pretreatment resulted in a prothrombin time prolongation (51.4 ± 17.9 versus 10.4 ± 0.3 seconds; $P<0.001$). Twenty-four hours after collagenase-induced ICH formation, hematoma volume was 3.8 ± 2.9 μL in controls, 4.8 ± 2.7 μL in DE mice, and 14.5 ± 11.8 μL in warfarin mice (n=16; Welch ANOVA between-group differences $P=0.007$; posthoc analysis with the Dunnett method: DE versus controls, $P=0.899$; warfarin versus controls, $P<0.001$; DE versus warfarin, $P=0.001$). In addition, a model of laser-induced cerebral microhemorrhage was applied, and the distances that red blood cells and blood plasma were pushed into the brain were quantified. Warfarin mice showed enlarged red blood cell and blood plasma diameters compared to controls, but no difference was found between DE mice and controls.

Conclusions—In contrast with warfarin, pretreatment with DE did not increase hematoma volume in 2 different experimental models of ICH. In terms of safety, this observation may represent a potential advantage of anticoagulation with DE over warfarin. (***Circulation***. 2011;124:00-00.)

Key Words: anticoagulants ■ cerebral hemorrhage ■ intracerebral hemorrhage ■ warfarin ■ dabigatran ■ stroke

For decades, oral anticoagulation with vitamin K antagonists (eg, warfarin) has been the gold standard for treatment and prophylaxis of thrombotic and thrombembolic disorders. Although highly effective in, for example, reducing the risk of cardioembolic brain infarction in patients with atrial fibrillation, the use of warfarin is associated with several shortcomings, such as a narrow therapeutic window, the need for regular coagulation monitoring, and critical food and drug interactions.[1,2] Furthermore, intracerebral hemorrhage (ICH) is the most feared complication of long-term anticoagulation with vitamin K antagonists. Both clinical and experimental studies revealed that anticoagulation associated ICH is a particularly severe type of stroke with short-term mortality rates exceeding 50% because of increased hematoma volumes and prolonged bleeding.[3–6]

Clinical Perspective on p ●●●

More recently, the search for alternative strategies for long-term anticoagulation was intensified in order to overcome the problems associated with vitamin K antagonists.[7,8] Parenteral direct thrombin inhibitors, such as lepirudin, were shown to be at least as effective as heparin for treatment of patients with arterial and venous thrombosis.[9] They bind to both the active site and the exosite 1 (bivalent binding) of the thrombin molecule, resulting in an irreversible thrombin inhibition. This irreversible inhibition is likely responsible for the increased bleeding risk associated with lepirudin compared with heparin seen in several clinical trials.[10] In contrast, the oral direct thrombin inhibitor dabigatran etexilate (DE) binds reversibly only to the active site of the thrombin molecule. In the Randomized Evaluation of Long-Term

Anticoagulation Therapy (RE-LY) trial, DE showed significantly reduced rates of stroke along with a favorable risk–benefit profile compared with warfarin in patients with nonvalvular atrial fibrillation.[11] Dabigatran etexilate has also been evaluated for the prophylaxis of thrombotic complications in patients undergoing total hip replacement.[12] It is under investigation for the treatment of acute symptomatic venous thromboembolism[2] and long-term secondary prevention of venous thromboembolism.[13]

Very limited information is available on the characteristics of ICH occurring during treatment with direct thrombin inhibitors.[14] For both doses of DE used in the RE-LY trial, the incidence of intracranial bleeding was significantly lower than in the warfarin group.[11] Still, bleeding risk increased dose dependently. The influence of DE pretreatment on hematoma expansion and prognosis of ICH compared with warfarin remains undetermined. This study uses well characterized animal models of ICH to investigate the influence of direct thrombin inhibition on hematoma volume and functional outcome.[4,5,15]

Methods

Animals
All experiments were conducted in accordance with the guide from the National Institute of Health for the care and use of laboratory animals. For the entire study, male CD-1 mice 12 to 16 weeks of age (mean body weight ±SD: 39.6±2.5 g) were used. Mice were subjected to antithrombotic pretreatment with DE, warfarin, lepirudin, heparin, or fondaparinux or received saline (as control). Eighty-nine mice were used for coagulation parameter determination, 146 mice underwent ICH induction by collagenase injection, and 9 animals were subjected to the procedure of laser-mediated rupture of blood vessels to produce cortical microhemorrhages.

Pretreatment With Antithrombotic Medication
A DE tablet (110 mg, Pradaxa, Boehringer Ingelheim, Ingelheim, Germany) was dissolved with 1% dimethylsulfoxide (DMSO) in saline solution. Solutions with 3 different DE concentrations were prepared: 10 mg/mL, 20 mg/mL, and 30 mg/mL. DE mice were fed 3 times orally using a gastric tube with intervals of 8 hours. Each feeding consisted of 0.15 mL of the respective solution, resulting in a dose of 37.5 mg/kg, 75 mg/kg, or 112.5 mg/kg body weight per feeding for a 40 g mouse. Oral gavages of comparable dosages of DE were previously shown to cause significant activated partial thromboplastin time (aPTT) prolongation in rats.[16] Control mice were fed 3 times with 0.15 mL saline following the same modus of application. Determination of coagulation parameters or ICH induction by collagenase injection and laser-induced vessel rupture, respectively, was performed 0.5 hours after the last oral gavage of either DE or saline. In a subset of mice, multiple coagulation parameter analyses over time were performed in order to determine the kinetics of DE anticoagulation. Furthermore, the effect of the solvent DMSO alone on coagulation parameters and ICH volume was determined.

Warfarin was applied via drinking water following a previously established protocol.[4,15] For mice with a body weight of 40 g, a daily water consumption of 15 mL/100 g provided an estimated warfarin intake of 0.1 mg (2.5 mg/kg) within a 30-hour feeding period. Determination of coagulation parameters or ICH induction, respectively, was done at the end of the warfarin feeding period.

In addition, we treated mice with lepirudin (1.5 mg/kg), heparin (80IU/kg), or fondaparinux (0.1 mg/kg). Heparin inhibits thrombin indirectly by catalyzing its inactivation by antithrombin III. In addition, it inhibits factor Xa in complex with antithrombin III. As a selective factor Xa inhibitor, fondaparinux does not have direct effects on thrombin.[17] Each agent was dissolved in saline (total volume 0.1 mL) and administered by a single retro-orbital intravenous injection, immediately followed by a single subcutaneous injection of the same dose and volume. These doses were chosen according to the literature.[18–20] Determination of coagulation parameters or ICH induction by collagenase injection, respectively, was done 0.5 hours after the subcutaneous application.

Ex Vivo Clotting Times and Quantitative Factor Assays
Whole blood (0.45 mL) was collected from deeply anesthetized mice by cardiac puncture with a 19-gauge needle. Blood was transferred to plastic tubes containing 0.05 mL of 3.2% citrate and was gently mixed. Specimens were centrifuged to obtain platelet-poor plasma. Measurements of prothrombin time (PT) and aPTT were performed on an MDA coagulation analyzer (Trinity Biotech, Berkeley Heights, NJ) using MDA Simplastin HTF (Trinity Biotech) for the PT determination and MDA Platelin L (Trinity Biotech) for the aPTT measurements. For the diluted thrombin time (dTT), 100 μL of MDA thrombin (Trinity Biotech) was added to 100 μL of mouse plasma diluted 1:4 in normal human plasma. Activities of coagulation factors II, VII, IX, and X were determined using human factor-deficient plasmas (Precision Biological, Dartmouth, Nova Scotia, Canada) with an aPTT reagent (Platelin L, Trinity Biotech) for factor IX and Simplastin HTF for the other factors on an MDA coagulation analyzer. Anti-Xa activity was determined with Stachrom Heparin Assays (Diagnostic Stago) using a calibration curve for fondaparinux. All coagulation parameter measurements were performed in the coagulation laboratory at the Massachusetts General Hospital.

ICH Induction by Collagenase Injection
All procedures were performed in a blinded fashion. Animals were anesthetized with isoflurane (1.5% to 2.0%). To maintain similar circulation conditions, the deepness of anesthesia was adjusted by achieving a reduction of 50% in respiratory rate (ie, to 80–100 breaths per minute). After drilling a small burr hole, a 32-gauge 0.5 μL injection needle (Hamilton 7000 series, Hamilton, Reno, NV) was slowly lowered into the right striatum (stereotactic coordinates in relation to bregma: 0.0 mm anterior, 2 mm lateral, 3.5 mm deep). Thereafter, 0.5 μL of saline containing 0.2 or 0.25 U of collagenase VII-S (Sigma-Aldrich, St Louis, MO) was administered over a period of 5 minutes. The needle was left in place for 10 minutes before it was slowly removed over a period of 5 minutes. The burr hole was sealed with bone wax and the scalp closed. The whole surgical procedure lasted ≈35 minutes.[4,15] Body temperature was maintained using a heat lamp. Thereafter, animals were allowed to recover in their cages.

We calculated the required sample size for a 2-tailed t test on the basis of the experience of previous studies using the same model.[4] For DE, an effect size (ie, increase in ICH volume) of 25% versus controls was assumed (8.8±1.9 versus 7.0±1.5 μL). For heparin, lepirudin, and fondaparinux, an effect size of 35% versus controls was assumed (9.5±2.0 versus 7.0±1.5 μL). Sixteen and 10 animals per group are required to detect this difference with a power (1–β) of 0.8 and a level of acceptability of a false-positive result (α) of 0.05, respectively.[21]

Outcome Assessment
Twenty-four hours after ICH induction, neurological deficits were blindly rated on a 5-point scale (0: no apparent deficit, 1: slight instability while walking without circling, 2: circling toward the right with some straight movement, 3: heavy circling toward the right without straight movement or no movement at all, 4: deceased). In addition, a standard hanging wire test was performed. Mice were gently placed on the wire until they had achieved a firm grip with their paws. The period of time to falloff was recorded. A maximum of 60 seconds of hanging was allowed, and the test was repeated 3 times for every mouse. No presurgical training was performed.[4,15]

Quantification of ICH volume

After outcome assessment, mice underwent transcardial perfusion with 30 mL of PBS under deep (5%) isoflurane anesthesia. After removal and separation into left and right hemispheres, brains were placed into glass tubes containing 3 mL PBS. Hematoma volume was quantified using a photometric assay.[4,15] Mice that died within 24 hours of ICH induction could not undergo transcardial perfusion before measurements. We determined 1.95 ± 0.26 μL to be the mean difference in intracerebral blood volume between 3 unperfused and 3 perfused brains (data not shown). Therefore, we subtracted 1.95 μL from the total hematoma volume that was calculated for the unperfused brains of the dead mice.

Laser-Mediated Rupture of Cerebral Blood Vessels Under In Vivo Imaging

Bilateral long-term cranial windows ($\approx$3-mm radius with center at stereotactic coordinates in relation to bregma: 3 mm posterior, 0 mm lateral) were implanted in mice (n=9). Ten days later, animals were blindly and randomly assigned to receive pretreatment with DE (dosage 75 mg/kg, n=3), warfarin (n=3), or saline (control, n=3) as described above.

In preparation to induce a cortical microhemorrhage, mice were put under isofluorene anesthesia ($\approx$2.0%). Body temperature was maintained at 37.5°C with a heat blanket and a thermometer. A retro-orbital intravenous injection of 0.1 mL of 5% (weight/volume) Texas-Red dextran (70 kDa) fluorescent dye in physiological saline was given to label the vasculature. We imaged into the brain using in vivo, 2-photon, excited fluorescence microscopy (excitation source: 1045 nm, 1-MHz, 350-fs pulse train from a Yb-fiber oscillator/amplifier system; μJewel FCPA, IMRA America Inc; detection filter: 645/65 nm Chroma filter). Bleeding was induced in 2 to 5 targeted penetrating arterioles per mouse by injuring the endothelium with tightly focused femtosecond laser pulses as described elsewhere.[22] The laser only causes damage at the focus ($\approx$1 μm^2) leaving the surrounding regions intact. Microhemorrhage depth varied between 50 and 120 μm beneath the cortical surface. The bleedings were separated from each other by at least 1 mm. Before and immediately after ($\approx$5 minutes) ICH induction, a stack of images was taken at the hemorrhage site with 2 μm step size from brain surface to a depth of 150 μm. The laser-induced hemorrhages are characterized by a core region filled with red blood cells (RBC) and a surrounding region where blood plasma penetrates into the parenchymal tissue. The RBC core appeared black whereas blood plasma is bright, labeled by the Texas Red dye. To determine hemorrhage size, we measured the diameters of the RBC-filled microhemorrhage core and of the surrounding blood plasma–filled region from a 20 μm z-projection of the stack images centered on the hemorrhage.

Statistical Analysis

We used SPSS version 15.0 (SPSS Inc, Chicago, IL), Matlab (The Mathworks Inc), and JMP version 8.0 (SAS Institute Inc) for statistical analysis. Lilliefors and Levene tests were used to analyze data distribution and equality of variances. Comparison of coagulation parameters, hematoma volumes, and diameters of the microhemorrhage regions between groups were performed using Welch ANOVA and the Dunnett method. Statistical analysis of ordinal data (functional outcome) was performed using the Kruskal-Wallis test with the Dunn correction.

Results

Ex Vivo Clotting Times and Quantitative Factor Assays

Control animals (n=4) revealed a mean$\pm$SD aPTT of 18.0 ± 1.5 seconds. Dabigatran etexilate pretreatment led to significant aPTT prolongation (46.1 ± 5.0 seconds in the 37.5 mg/kg DE group; 55.7 ± 19.6 seconds in the 75 mg/kg DE group; 85.8 ± 20.0 seconds in the 112.5 mg/kg DE group;
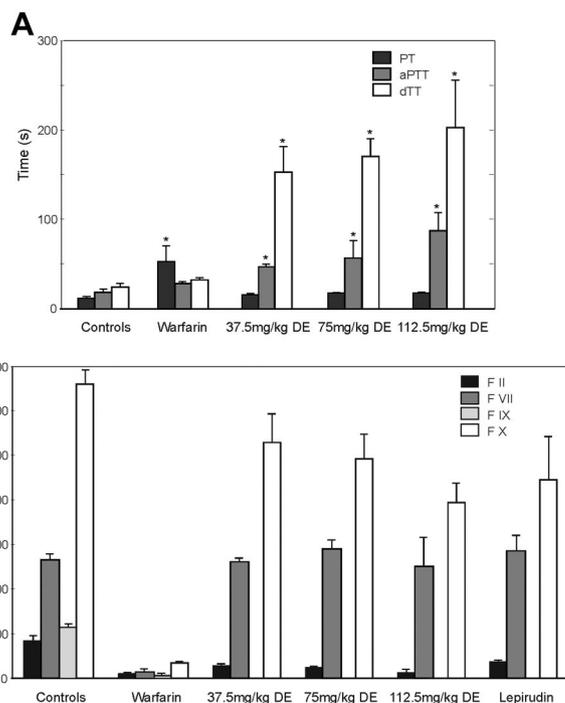
Figure 1. A, Mean PT, aPTT, and dTT values at the time point of ICH induction in controls as well as in warfarin-pretreated and in DE-pretreated mice (3 different dosages). *$P<0.05$ compared to controls; error bars indicate SD. **B,** Mean coagulation factor activity (FII, FVII, FIX, FX; measured with human factor-deficient plasma) for control mice as well as for mice pretreated with warfarin, DE (3 different dosages), or lepirudin. Factor IX activity was not determined in animals treated with DE or lepirudin. PT indicates prothrombin time; aPTT, activated partial thromboplastin time; dTT, diluted thrombin time; and DE, dabigatran etexilate.

Welch ANOVA between-group differences $P<0.001$; posthoc controls versus DE 37.5 mg/kg, $P=0.022$; controls versus DE 75 mg/kg, $P=0.002$; controls versus DE 112.5 mg/kg, $P<0.001$; n=4 per group; Figure 1A). Figure 2 shows the kinetics of aPTT elevation over time for mice treated with different DE concentrations and controls (n=3 per group and time point). Prothrombin time was only marginally influenced by DE pretreatment (10.4 ± 0.3 seconds in controls; 14.6 ± 1.2 seconds in the 37.5 mg/kg DE group; 16.1 ± 0.8 seconds in the 75 mg/kg DE group; 17.0 ± 0.9 seconds in the 112.5 mg/kg DE group; n=3 per group; Figure 1A). Also, DE pretreatment resulted in a pronounced dTT increase (22.9 ± 3.9 seconds in controls; 150.6 ± 19.0 seconds in the 37.5 mg/kg DE group; 168.1 ± 20.2 seconds in the 75 mg/kg DE group; 210.0 ± 46.9 seconds in the 112.5 mg/kg DE group; n=3 per group; Welch ANOVA between-group differences $P=0.002$; posthoc controls versus DE 37.5 mg/kg, $P=0.001$; controls versus DE 75 mg/kg, $P<0.001$; controls versus DE 112.5 mg/kg, $P<0.001$; Figure 1A). Administering the solvent (1% DMSO in saline) alone did not significantly alter aPTT (18.7 ± 1.9 seconds, n=4) or PT (10.3 ± 0.7 seconds, n=4) compared to saline controls.

Warfarin pretreatment led to significant PT prolongation (51.4 ± 17.9 seconds, Welch ANOVA between-group differences $P<0.001$; posthoc controls versus warfarin, $P<0.001$; n=4 per group) whereas aPTT was only modestly increased
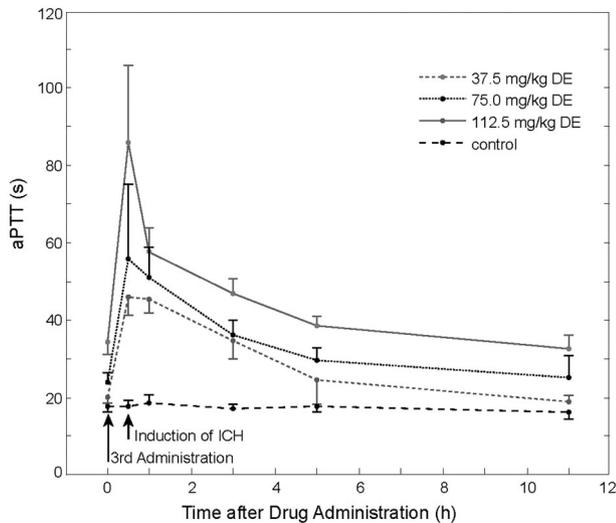
Figure 2. Mean aPTT prolongation over time in DE-treated animals (3 different dosages) and in controls. Error bars indicate SD. DE indicates dabigatran etexilate; aPTT, activated partial thromboplastin time; and ICH, intracerebral hemorrhage.

(26.8±4.6 seconds; Figure 1A). After parenteral pretreatment with the irreversible thrombin inhibitor lepirudin, aPTT was prolonged to 40.4±4.7 seconds whereas heparin application led to an aPTT prolongation of 80.1±18.9 seconds (n=3 per group; Welch ANOVA between-group differences $P<0.001$; posthoc controls versus lepirudin, $P=0.019$; controls versus heparin, $P<0.001$). Anti-Xa activity after administering the selective factor Xa inhibitor fondaparinux was increased compared to controls (1.20±0.10 mg/mL versus 0.11±0.01 mg/mL; controls versus fondaparinux, $P=0.001$; n=3 per group).

Figure 1B shows the results of quantitative coagulation factor assays (n=3 per group). Pretreatment with direct thrombin inhibitors (ie, lepirudin, DE) reduced the activity of factor II whereas factor VII and X remained largely unaffected. In warfarin-pretreated animals, all 4 vitamin K–dependent factors were significantly reduced.

Hematoma Volume 24 Hours After ICH Induction

Twenty-four hours after ICH induction using 0.2 U collagenase, mean hematoma volume was 3.8±2.9 μL in controls, 4.8±2.7 μL in the 37.5 mg/kg DE group and 14.5±11.8 μL in the group of animals that received warfarin treatment (n=16 per group; Welch ANOVA between-group differences $P=0.007$). Posthoc analysis revealed no differences in ICH volume between DE-treated mice and controls ($P=0.899$), but warfarin animals had significantly larger bleeds than both DE mice ($P=0.001$) and controls ($P<0.001$; Figure 3A). We repeated the study using a higher concentration of DE (112.5 mg/kg) and an increased dosage of collagenase (0.25 U). Now, mean hematoma volume was found to be 5.9±1.2 μL in controls, 8.8±4.9 μL in DE mice, and 14.2±8.0 μL in warfarin mice (n=16 per group; Welch ANOVA between-group differences $P<0.001$). Again, posthoc analysis did not show significant differences between DE mice and controls ($P=0.237$), but warfarin mice had significantly larger ICH volumes than both controls ($P<0.001$) and DE-treated mice
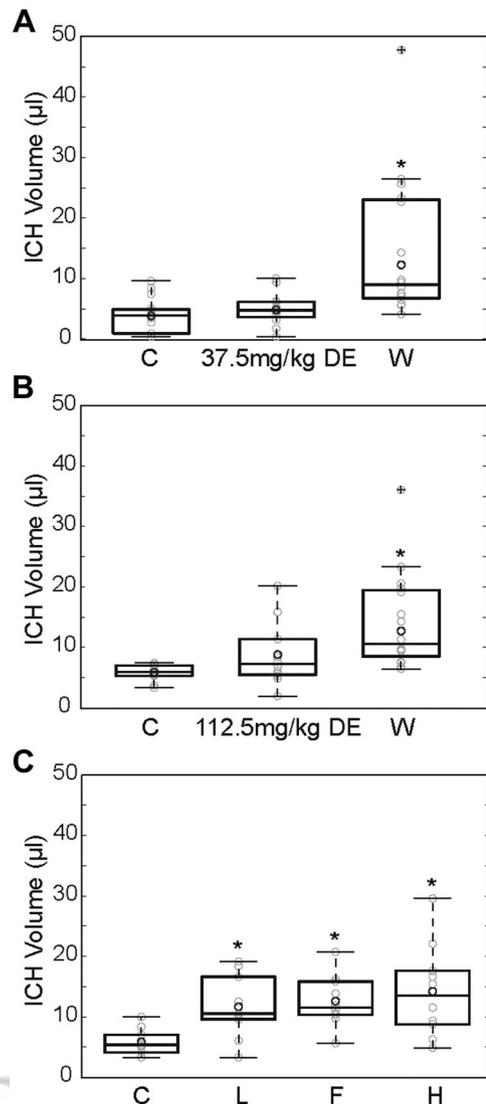
Figure 3. A, Hematoma volume 24 hours after ICH induction by collagenase injection in controls and in mice pretreated with DE (37.5 mg/kg) or warfarin (n=16 per group). The collagenase dosage in this experimental series was 0.2 U. Data are displayed using box plots with the box indicating the median and the 25th and 75th percentile, respectively. Whiskers extend to the maximum and minimum values that are not outliers. Circles in light gray represent individual data points. Outliers have a cross imbedded in the circle. The dark gray circle represents mean without outliers. *$P<0.05$ compared to controls. **B,** Same as in panel **A** but the DE dosage was increased to 112.5 mg/kg and the collagenase dosage was increased to 0.25 U. **C,** Hematoma volume 24 hours after ICH induction by collagenase injection in controls and in mice pretreated with lepirudin, fondaparinux, or heparin (n=10 per group). ICH indicates intracerebral hemorrhage; C, controls; DE, dabigatran etexilate; W, warfarin; L, lepirudin; F, fondaparinux; and H, heparin.

($P=0.024$; Figure 3B). Pretreatment with the solvent (1% DMSO in saline) alone did not significantly affect ICH volume compared to saline-treated controls (4.9±2.2 μL versus 5.1±2.8 μL; n=5 per group).

Mice that were pretreated with lepirudin, heparin, or fondaparinux showed significantly enlarged ICH volumes compared to controls (lepirudin: 11.7±5.1 μL, fondaparinux: 12.6±4.2 μL, heparin: 14.2±7.7 μL, and controls: 5.9±2.1
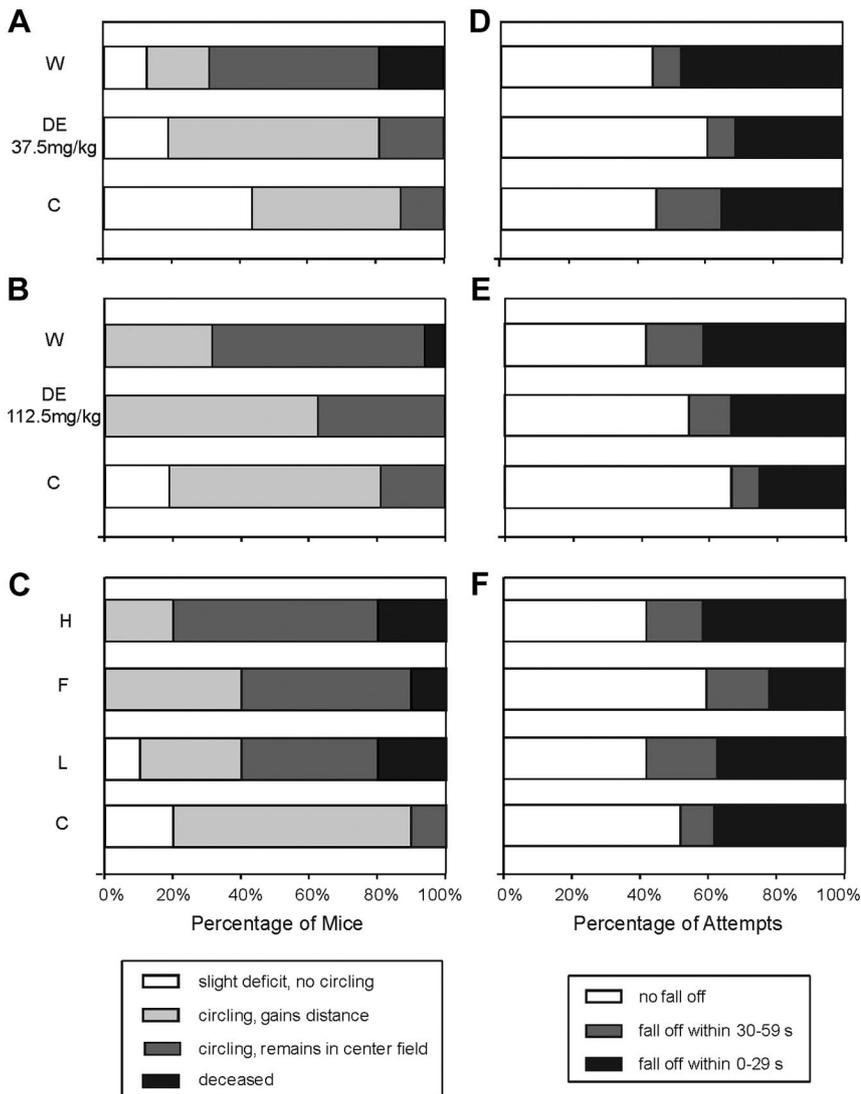
Figure 4. A, Functional outcome 24 hours after ICH induction using collagenase (0.2 U) injection assessed using an ordinal neurological scale for controls and for mice treated with DE (37.5 mg/kg) or warfarin (n=16 per group). B, Same setting as in A but the DE dosage was increased to 112.5 mg/kg and the collagenase dosage was increased to 0.25 U. C, Functional outcome in controls and in lepirudin-, fondaparinux- or heparin-pretreated mice. D through F, Same treatments as in A through C, respectively. Results of the hanging wire test are presented. The time period to falloff was recorded (3 attempts per mouse), and a maximum of 60 seconds of hanging was allowed. W indicates warfarin; DE, dabigatran etexilate; C, controls; H, heparin; F, fondaparinux; and L, lepirudin.

μL; Welch ANOVA between-group differences $P<0.001$, posthoc controls versus lepirudin, $P=0.043$; controls versus fondaparinux, $P=0.017$; and controls versus heparin, $P=0.003$; n=10 per group; Figure 3C).

Functional Outcome 24 Hours After ICH Induction

Twenty-four hours after ICH induction using 0.2 U collagenase, 2 out of 16 animals in the control group showed a worse functional outcome (score 3 or 4; ie, heavy circling or death). This compares to 6 out of 16 mice in the DE group and to 11 out of 16 mice in the warfarin group (Figure 4A). Repeating this experiment using the increased collagenase dosage and the higher DE concentration resulted in 3 out of 16 control mice, 6 out of 16 DE mice, and 11 out of 16 warfarin mice having a worse functional outcome (for both collagenase dosages: Kruskal Wallis between-group differences $P<0.01$, posthoc controls versus warfarin $P<0.05$, and controls versus DE not significant; Figure 4B). A higher rate of a worse functional outcome was observed in mice pretreated with lepirudin (6/10), fondaparinux (6/10), or heparin (8/10), respectively, compared to controls (1/10; Figure 4C). Figure

4D through 4F shows the results of the hanging wire tests. The proportion of warfarin-treated mice that fell off the wire within 29 seconds was significantly higher compared with control animals (for both collagenase dosages: Kruskal Wallis between-group differences $P<0.05$, posthoc controls versus warfarin $P<0.05$). For the DE groups, there were no significant differences compared to controls.

Laser-Mediated Rupture of Cerebral Blood Vessels Under In Vivo Imaging

A total number of 12 (control), 13 (DE treatment), and 13 (warfarin treatment) microhemorrhages were induced by rupturing targeted penetrating arterioles with tightly focused femtosecond laser pulses (Figure 5, n=3 mice per group, 2–5 microhemorrhages per mouse). None of the animals died during this procedure. Both the diameter of the RBC core and the diameter of the blood plasma–filled region were larger for warfarin-pretreated mice than for controls and DE-treated animals (controls: 116.2 ± 38.0 μm and 327.8 ± 80.4 μm; DE mice: 108.1 ± 35.2 μm and 331.6 ± 51.2 μm; warfarin mice: 192.8 ± 101.4 μm and 467.5 ± 113.6 μm, respectively; Figure 6A and 6B). The Welch ANOVA between-group differences
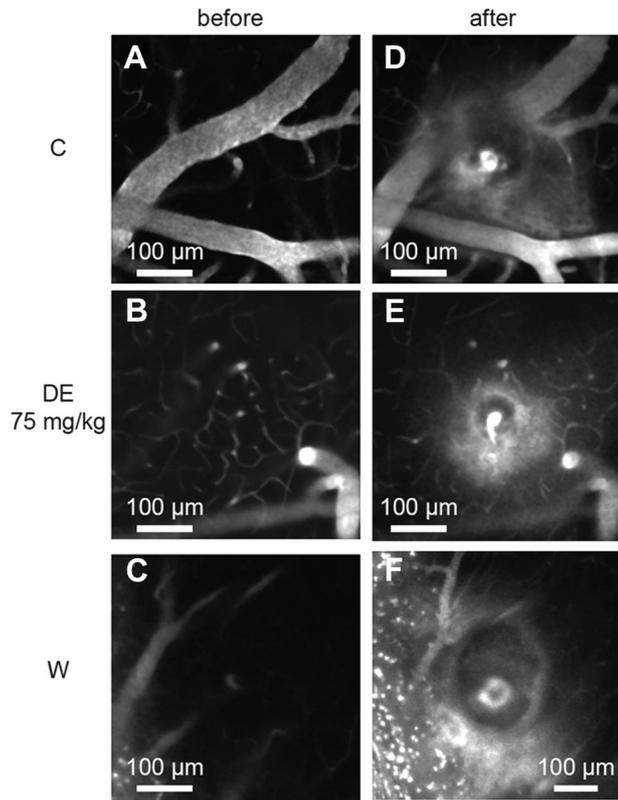
Figure 5. Projections of in vivo 2-photon excited fluorescence image stacks of fluorescently labeled blood plasma spanning a 20-μm depth centered at the microhemorrhage origin. Images are shown before (**A** through **C**) and after (**D** through **F**) rupturing the wall of a single penetrating arteriole using tightly focused femtosecond laser pulses. Representative examples from the 3 differently treated groups (saline-treated controls (**A, D**); DE 75 mg/kg (**B, E**); and warfarin (**C, F**)) are shown. Extravasated plasma is visualized by diffuse fluorescence and can be seen in the posthemorrhage images in a halo surrounding the target vessel. The dark core immediately adjacent to the target vessel is filled with RBCs. C indicates control; DE, dabigatran etexilate; and W, warfarin.

Figure 6. A, Diameter of RBC-filled core after laser-induced microhemorrhage in controls and in mice pretreated with DE (75 mg/kg) or warfarin (n=12–13 microhemorrhages per group). **B**, Diameter of plasma extravasation volume after laser-induced microhemorrhage for the lesions shown in A. RBC, red blood cell; hem, hemorrhage; C, controls; DE, dabigatran etexilate; and W, warfarin.

for RBC and blood plasma diameters were found to be significant ($P=0.035$ and $P=0.003$, respectively). Posthoc analysis revealed no differences in RBC and blood plasma diameters between DE-treated mice and controls ($P=0.935$ and $P=0.991$, respectively), but warfarin animals had significantly larger RBC and plasma diameters than both DE mice and controls (warfarin versus DE $P=0.008$ and $P<0.001$, respectively; control versus warfarin $P=0.013$ and $P<0.001$, respectively).

Discussion

We investigated the effects of pretreatment with the direct thrombin inhibitor DE on hematoma volume and functional outcome using 3 different dosages in 2 different experimental models of ICH. As a positive control, the vitamin-K antagonist warfarin was also tested. Whereas warfarin anticoagulation led to increased hematoma volumes, DE mice did not differ from sham-treated animals. Our study suggests that compared to warfarin anticoagulation, the effects of DE anticoagulation on intracerebral bleeding were less severe.

Part of our investigation used the well-established mouse model of collagenase-induced ICH. Using this model, we have previously demonstrated that warfarin anticoagulation targeting international normalized ratio values between 2 and 4 leads to significantly larger hematoma volumes, prolonged bleeding, and a worse functional outcome.[4] Moreover, the rapid reversal of anticoagulation using prothrombin complex concentrates containing high amounts of coagulation factor II, VII, IX, and X was shown to prevent excessive hematoma formation.[15] The collagenase model of ICH is often criticized for not properly reflecting the arteriolar rupture that typically underlies human ICH although the pathological changes surrounding the collagenase-induced bleeding are comparable to those described in human ICH.[23] In order to verify that our results were not an artifact related to the collagenase model itself, we repeated our study in a pathophysiologically different ICH model based on the laser-mediated (ie, collagenase-independent) rupture of cerebral blood vessels under in vivo imaging.[22] In contrast to the basal ganglia (deep) hematoma induced by collagenase application, the laser model provokes cerebral microhemorrhages around arterioles penetrating the cortex (lobar).

We tried to carefully monitor the status of anticoagulation after application of different doses of DE to mice via a gastric tube. Peak aPTT prolongation was determined to occur 0.5 hours after the third DE feeding, and the aPTT was prolonged over a period of several hours. Because hematoma formation in the collagenase model occurs within the first 3 hours after ICH induction,[5] the aPTT prolongation extends over this

period of time. The same is true for the laser model where microhemorrhage formation occurs within a minute after vessel rupture and the 2 to 5 microhemorrhages induced in each mouse can be completed within 2 hours. Despite a considerable effect on aPTT, ranging from 2.6-fold prolongation in the 37.5 mg/kg DE group to a 4.8-fold prolongation in the 112.5 mg/kg DE group, only a minor effect of DE on the PT was detectable. This has been observed both in humans and in animals and confirms that PT is not appropriate for monitoring anticoagulant effects of DE.[8,16] Although aPTT exhibits a rather flattened dose–response curve at higher DE concentrations,[8] it shows a linear relationship with the square root of the DE plasma concentration in studies investigating pharmacokinetics and pharmacodynamics of DE.[24] Furthermore, the aPTT was reported to correlate well with DE-mediated antithrombotic activity in a rabbit model.[16] The observed prolongation in our study up to a mean of 85.8 seconds is greater than the mean aPTT determined in patients receiving 150 mg DE twice daily.[24] This was the highest dose examined in the RE-LY trial.[11] Thus, we consider our study to mimic both the therapeutic range used in humans and supratherapeutic dosages. Because the thrombin-clotting-time test directly assesses the activity of thrombin in the plasma sample, it is frequently considered to be superior to aPTT measurements for assessing the level of anticoagulation after DE therapy because of a linear dose–response curve.[8,24] With dTT values up to 9.2 fold baseline, it was confirmed that our mouse model well reflects both moderate and strong DE anticoagulation.[25] Relative to warfarin anticoagulation, oral feeding with warfarin via bottled drinking water led to a 4.9-fold PT prolongation. All 4 vitamin K–dependent coagulation factors were found decreased at the time point of hematoma induction, mimicking full warfarin anticoagulation.[15] The PT prolongation lies above the therapeutic range in humans. However, previous studies demonstrated that both therapeutic and supratherapeutic dosages of warfarin led to significantly enlarged hematoma volumes compared to control animals whereas we could not demonstrate hematoma enlargement after DE pretreatment in both therapeutic and supratherapeutic ranges.[4,5,15]

What might be the reasons why warfarin but not dabigatran anticoagulation leads to enlarged hematoma volumes in case of ICH? In vitro investigations revealed that deficiencies of the coagulation factors II, VII, and X cause delayed clot initiation and affect clot propagation and clot strength. However, the presence of a small amount of factor II activity already resulted in clot initiation values similar to those in control plasma whereas nearly every decrease of the factors VII and X further increased time to clot.[26] In our in vivo experiments, both the inactivation of factor II in combination with factor X after heparin treatment and the isolated inhibition of factor Xa with fondaparinux increased hematoma volumes after ICH induction compared to control animals. After the propagation phase of coagulation, the secondary thrombin burst leads to the activation of thrombin-activatable fibrinolysis inhibitor, leading to down-regulation of fibrinolysis. Direct thrombin inhibitors fail to inhibit thrombin-activatable fibrinolysis inhibitor generation whereas drugs that target factor Xa (fondaparinux, heparin, and warfarin)

enhance plasma fibrinolytic potential.[27] This may contribute to their increased bleeding potential.

On the other hand, when we compared the isolated inhibition of factor II by the direct thrombin inhibitors DE and lepirudin, respectively, enlarged hematoma volumes were found only in the lepirudin-treated group. This implies that the modus of thrombin inhibition may be of crucial importance in this context. The thrombin molecule has 3 binding sites for the interaction with thrombin inhibitors, the active site and 2 exosites.[7] Lepirudin as a bivalent direct thrombin inhibitor forms an irreversible complex with thrombin by binding to the active site and exosite 1. Both exosites are involved in promoting thrombin-mediated platelet activation.[28] Assigned to the group of univalent direct thrombin inhibitors, DE blocks only the active site (but not the exosites) in a reversible manner. Thus, DE-inhibited thrombin is still able to contribute to platelet activation and aggregation.[29] Furthermore, even with an inhibited active site, exosite 1 still allows thrombin to enhance fibrin polymerization by bridging between fibrinogen molecules.[30] This effect is diminished by the bivalent binding of hirudines.[31]

In contrast, bivalent binding to both the active site and the exosite 1 of the thrombin molecule results in an irreversible bond. In ex vivo experimental investigations, lepirudin treatment strongly delayed the lag phase of thrombin generation and thus the initiation of coagulation. This was not observed with argatroban, another reversible thrombin inhibitor, which mainly affected the propagation phase by reducing the endogenous thrombin potential.[32] Determination of thrombin activity after induction of thrombin generation by tissue factor revealed a similar delaying effect of lepirudin therapy, whereas argatroban increased the peak levels of thrombin. Thrombin, reversibly released from its complex with argatroban in the subsampling procedure, has been assumed to contribute to this finding.[33]

Our results indicate that an isolated decrease in factor-II activity by DE-mediated, univalent, reversible thrombin inhibition may still result in sufficient hemostasis in the scenario of ICH. Our pathophysiological hypothesis is supported by other animal studies that observed a bleeding-time prolongation after DE therapy only at supratherapeutic doses.[16] In addition, a dissociation between antithrombotic efficacy and absence of bleeding-time prolongation was reported in studies using different oral thrombin inhibitors.[34–36]

In the RE-LY trial, incidence rates of ICH in DE-treated patients were found to be reduced compared with those in warfarin-treated patients despite a similar rate of antithrombotic efficiency. It was speculated that this finding is linked to the decrease of other vitamin K–dependent coagulation factors (other than factor II) in the case of warfarin anticoagulation and their contribution to hemostasis whereas DE does not directly affect parameters other than factor II.[11] Our experimental study investigating the behavior of laser-induced cerebral microbleeds after DE and warfarin pretreatment provides a more detailed insight: Microhemorrhages induced in warfarin-treated mice more often expand toward having increased RBC and blood plasma diameters whereas microbleeds in DE mice do not differ from controls. Thus, we may speculate that in the RE-LY trial, the absolute number of

cerebral micobleeds occurring under oral anticoagulants was similar in the warfarin and the dabigatran groups but that microhemorrhages under warfarin more often expanded toward symptomatic ICH.[37,38]

Our findings have several clinical implications. Intracerebral bleeding occurring during warfarin treatment is particularly severe, with short-term mortality rates of 50%.[3] Clinical and experimental data have shown that hematoma expansion is prolonged in the case of warfarin anticoagulation, thus leading to larger hematoma volumes and a worse functional outcome.[3–6] In warfarin-associated ICH, measures to rapidly reverse anticoagulation may prevent such prolonged bleeding and may improve functional outcome.[3,6,15] Our data suggest that DE anticoagulation does not facilitate ongoing bleeding and extensive hematoma growth. It is likely that this is mirrored by better prognosis, as well. As a caveat, one may assume that factor II activity decreases with increasing DE dosages. Thus, there may be insufficient hemostasis above a critical dose. Because DE is primarily cleared renally, an impaired renal function may lead to drug accumulation and to supratherapeutic concentrations.[7] Indeed, increased numbers of bleeding events have been observed in patients with impaired renal function in a dose-escalation study of DE.[39]

Some important shortcomings of the present study should be mentioned. First, the murine coagulation system has been reported to be similar to the human coagulation system, both physiologically and with regard to the coagulation tests that are used.[40] However, there are differences in the molecular structure between mouse and human coagulation factors, and the impact of these differences in the setting of the present study is not yet defined.[41] Thus, one must be cautious in translating our findings into the human setting, and clinical data may be warranted. However, in this context it is important to mention that an animal model is most likely the only way to reasonably test in a randomized design whether DE pretreatment increases ICH volume compared to controls. Among >12 000 patients receiving DE in the RE-LY trial, only 0.1% per year developed ICH. Considering the large number of patients needed to sufficiently adjust for confounding variables, it appears practically impossible to address this question in a clinical trial.[11,42] Second, although all antithrombotic drug treatments led to reproducible effects on coagulation measures, these tests were not performed in the same animals that underwent ICH induction. So we were not able to directly correlate the results of the coagulation measurements with ICH volume and functional outcome. In addition, it is not possible to directly compare the level of anticoagulation after DE and warfarin pretreatment by means of coagulation parameters because coagulation tests respond differently to these drugs. Third, we used 1% DMSO for dissolving the DE tablet. Although the resulting DE solution produced prolonged aPTT and dTT, we cannot completely rule out an interaction between DMSO and DE that may have influenced our results. However, we found no effects of a 1% DMSO gavage alone on both coagulation parameters and ICH volumes.

In summary, our experimental study suggests that cerebral hemorrhages occurring during DE treatment are smaller and less harmful than those under warfarin anticoagulation. In terms of safety, this may represent a potential advantage of the direct thrombin inhibitor DE over warfarin.

Sources of Funding

Disclosures

None.

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CLINICAL PERSPECTIVE

The direct thrombin inhibitor dabigatran was recently approved for long-term prophylaxis of thrombembolic events in patients with atrial fibrillation. For this indication, the Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial revealed a favorable benefit–risk profile for dabigatran compared with that of the gold standard, warfarin. Intracerebral hemorrhage (ICH) is the most feared complication of long-term anticoagulation. Whereas warfarin pretreatment leads to largely increased hematoma volumes and higher mortality rates compared to those of ICH occurring in nonanticoagulated patients, no such data are available for dabigatran anticoagulation. In 2 animal models of ICH, we found no differences in terms of hematoma volume between dabigatran-treated mice and controls, whereas warfarin anticoagulation dramatically worsened ICH volume. On a molecular level, warfarin vastly reduced activity levels of coagulation factors II, VII, IX, and X, but dabigatran reversibly inhibited the active site of factor II only, still allowing sufficient coagulation induction to prevent extensive hematoma enlargement. If confirmed in humans, our findings may represent a significant safety advantage of dabigatran anticoagulation over that of warfarin. Further study is warranted to determine if rapid anticoagulation reversal (eg, by means of prothrombin complex concentrates) is not necessary for ICH occurring during dabigatran treatment.