Hemostatic Therapy in Experimental Intracerebral Hemorrhage Associated With Rivaroxaban

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Background and Purpose—Rivaroxaban has recently been approved for stroke prevention in atrial fibrillation. However, lack of an effective antidote represents a major concern in the event of intracerebral hemorrhage (ICH). The aims of the present study were to establish a murine model of ICH associated with rivaroxaban, and to examine the effectiveness of different hemostatic factors in preventing excess hematoma expansion.

Methods—In C57BL/6 mice receiving 10 or 30 mg/kg rivaroxaban by gastric gavage, plasma concentration, prothrombin time, and coagulation factor activities were measured repeatedly. Thirty minutes after inducing ICH by intrastriatal collagenase-injection, mice received an intravenous injection of either saline, prothrombin complex concentrate (100 U/kg), murine fresh frozen plasma (200 μL), or recombinant human Factor VIIa (1 mg/kg). ICH volume was quantified on brain cryosections and using hemoglobin spectrophotometry 24 hours later.

Results—Rivaroxaban in 30 mg/kg dose substantially increased the hematoma volume in ICH induced by 0.060 U collagenase. Prothrombin complex concentrate, fresh frozen plasma, or Factor VIIa prevented excess hematoma expansion caused by anticoagulation. Prevention of hematoma expansion by prothrombin complex concentrate was dose-dependent. None of the 3 agents completely corrected the prolonged prothrombin time, although they restored the activities of deficient FII and X.

Conclusions—Prothrombin complex concentrate, Factor VIIa, and fresh frozen plasma prevent excess intracerebral hematoma expansion in a murine ICH model associated with rivaroxaban. The efficacy and safety of this reversal strategy must be further evaluated in clinical studies. (Stroke. 2013;44:771-778.)

Key Words: anticoagulation ■ factor VIIa ■ fresh frozen plasma ■ prothrombin complex concentrate ■ stroke

Thromboembolism in atrial fibrillation is a major cause of stroke. Oral anticoagulation (OAC) with the vitamin K antagonist warfarin or one of the new direct oral anticoagulants (nOAC) is highly effective for stroke prevention in atrial fibrillation. The most severe complication of long-term anticoagulation is intracranial hemorrhage. Indeed, intracerebral hemorrhage (ICH) during therapy with vitamin K antagonist is responsible for 88% of all hemorrhage-associated deaths caused by anticoagulation. Moreover, vitamin K antagonist-associated ICH expands more frequently and over a longer time period than spontaneous ICH. To prevent hematoma expansion—a major therapeutic goal in ICH therapy—replacement of coagulation factors is recommended.

Although nOAC appear to carry a substantially lower risk of ICH compared with warfarin, ICH during nOAC therapy remains a life-threatening complication. Currently, no specific antidote is available, and the effect of administering hemostatic coagulation factors is dubious. Recently, an experimental murine model of ICH during OAC has been established in which ICH is induced by injection of collagenase into the striatum during anticoagulation. This model shows early hematoma expansion exceeding that of nonanticoagulated mice, during anticoagulation with either warfarin or a high dose of the direct thrombin inhibitor dabigatran. Excess hematoma expansion in this model could be prevented most effectively by infusing prothrombin complex concentrate (PCC), whereas the effect of recombinant human factor VIIa (FVIIa) and fresh frozen plasma (FFP) was less consistent. Rivaroxaban is another nOAC that directly inhibits the central coagulation factor Xa. Its widespread clinical use beyond stroke prevention in atrial fibrillation is expected because it has been proven effective and safe for additional indications, including acute coronary syndrome and venous thromboembolism. So far, no specific antidote for rivaroxaban is available, and the efficacy of hemostatic factors in the setting of ICH is unknown.

The purpose of the present study was to establish a murine model of ICH associated with rivaroxaban, and to evaluate the effect of different hemostatic agents on excess hematoma expansion.
growth caused by anticoagulation. We also aimed to evaluate coagulation tests for monitoring of reversal of anticoagulation with rivaroxaban.

Material and Methods

The study was conducted in accordance with national and international guidelines for the use of experimental animals. The protocols were approved by the local and governmental committees for animal care and use (Regierungspräsidium Karlsruhe, Germany). In the present work, 378 sexually mature male mice were used (C57BL/6, Charles River Laboratories, 10–12 weeks of age, body weight 22–26 g).

Establishing Effective Systemic Anticoagulation With Rivaroxaban in Mice

Rivaroxaban (BAY 59–7939, Bayer HealthCare AG, Wuppertal, Germany) was dissolved in a vehicle solution containing 10% ethanol, 40% Solutol HS 15 (Sigma, Germany), and 50% water for injection. Different doses of rivaroxaban (3, 10, 30 mg/kg) were administered by gastric gavage. The plasma concentration and prothrombin time (PT) were measured before, 30 minutes, 1 hour, 2 hours, and 4 hours after rivaroxaban administration to evaluate the effects on the systemic coagulation (n=3/dose and time point). The nonanticoagulated controls received vehicle solution (10 mL/kg) only.

Rivaroxaban plasma concentrations were determined using a high-performance liquid chromatography system, similarly as described (see the online-only Data Supplemental methods).

PT was measured in the same mice, which were involved in plasma concentration determination, as described (see the online-only Data Supplemental methods).

In separate mice anticoagulated with 30 mg/kg rivaroxaban, the activities of factors II, VII, IX, X, and Protein C and S were measured in the central laboratory of the University Heidelberg (see the online-only Data Supplemental methods).

Model of ICH Associated With Rivaroxaban

Because 30 mg/kg rivaroxaban led to systemic anticoagulation that results in 2- to 3-fold prolongation of PT compared with baseline value for more than 4 hours, we chose this dose in the further experiments to compare with the 20 mg and even higher dose given in clinical studies.12,13 Mice were anticoagulated with 30 mg/kg rivaroxaban per os 1 hour before intrastratal injection of 0.5 mL of saline containing either 0.030 U, 0.045 U, 0.060 U, or 0.075 U collagenase type VII (Sigma, Germany) to induce hematoma (see the online-only Data Supplemental methods).

Twenty-four hours after ICH induction, hematoma size in surviving mice was measured by a rater, who was blind to the group allocation, as previously described.14

Effect of Hemostatic Agents on Hematoma Size

In mice pretreated with 30 mg/kg rivaroxaban, the effect of different hemostatic agents on intracerebral hematoma volume was studied. Mice were randomly allocated to 1 of 5 groups: (1) nonanticoagulated controls, (2) mice anticoagulated with rivaroxaban, (3) anticoagulated mice receiving PCC (Beriplex P/N 500, 100 U/kg, CSL Behring, Germany), (4) FFP (200 mL, produced by centrifugation of fresh murine blood in EDTA-coated tube at 1500 rpm for 10 minutes, as described),15 and (5) recombinant FVIIa (Novo Seven, 1 mg/kg, NovoNordisk, Denmark).

Groups 1 and 2 received 200 mL saline via the left femoral vein over 5 minutes to match the infused volume of 200 mL for groups 3 to 5 receiving hemostatic/coagulation factors. Saline and hemostatic factors, respectively, were injected 30 minutes after striatal collagenase injection (0.060 U) because the majority of hematoma expansion takes place within the first 1 to 3 hours in this model.14,16

Efficiency of the hemostatic agents was assessed by measuring hematoma volume on cryosections and blood content in the hemorrhagic hemisphere using hemoglobin spectrophotometry (see the online-only Data Supplemental methods) 24 hours after ICH induction.

Dose-Dependency of Reversal of Anticoagulation by PCC

Additional experiments were performed in anticoagulated mice, in which the effect of lower doses of PCC (25 and 50 U/kg, respectively) on hematoma volume and neurological deficits were examined. ICH-induction in anticoagulated animals was followed by intravenous injection of PCC (see above). Twenty-four hours later, the neurological deficit was assessed by an examiner, who was blind to the group assignment using the corner test, as described (see the online-only Data Supplemental methods).22 To minimize the influence of the surgical procedure on the behavioral performance, sham controls (n=4) were also measured which had been operated according to the same surgical protocol, but without receiving either collagenase or PCC.

After neurological assessment, mice were euthanized to assess the effect of the different PCC doses on intracerebral hematoma.

Effect of Hemostatic Factors in Assays Assessing Systemic Coagulation

The reversal effect of the different hemostatic agents induced by 30 mg/kg rivaroxaban on the systemic coagulation was assessed using the same in vitro assays as above (in separate animals, n=5/group). The effect of the hemostatic therapies was measured 90 minutes after administration of rivaroxaban and 30 minutes after administration of hemostatic agents, respectively. We measured the plasma concentration, PT, and the plasma activities of the coagulation factors II, VII, IX, X, and Protein C and S.

Statistical Analysis

All values are expressed as mean±SD. Mean values were compared using Student t test for comparison between 2 groups and ANOVA with post hoc Bonferroni test for multiple-group comparisons. The correlation analysis between hematoma volume and hemoglobin spectrophotometry, and with the corner test, respectively, was performed using Pearson correlation test. All analyses were performed using SPSS 13.0 software. A probability value of <0.05 was considered as statistically significant.

Results

Systemic Anticoagulation Induced by Different Doses of Rivaroxaban

Rivaroxaban plasma concentrations reached their peak already 1 hour after gastric gavage in all dose groups and were not detectable at 4 hours in the 3 mg/kg and 10 mg/kg groups. In the 30 mg/kg group, a mean peak plasma level of 0.88±0.05 µg/mL was measured at 1 hour and a level of 0.39±0.14 µg/mL at 4 hours (Figure 1A).

PT was substantially prolonged at 1 hour after oral administration in all 3 dose groups and gradually approached the normal range (nonanticoagulated controls: 16.4±6.4 seconds) within 4 hours in the 3 mg/kg and 10 mg/kg groups. In contrast, the 30 mg/kg group still showed a prolonged PT (35.4±4.1 seconds) at 4 hours (Figure 1B).

Effect of Rivaroxaban on Hematoma Volume Depends on Collagenase Dose

In the subsequent experiments, we chose 30 mg/kg rivaroxaban as our working dose to assure persistent effective anticoagulation during the 24-hour experimental period.

Intrastrial injection of 0.030 U or 0.045 U collagenase did not induce excess hematoma expansion in anticoagulated compared with nonanticoagulated mice (0.030 U collagenase: 17.2±3.8 mm³ versus 15.0±3.1 mm³; 0.045 U collagenase:...
27.0±7.4 mm³ versus 24.7±5.6 mm³; P>0.05; n=13/group; Figure 2A). In contrast, induction of ICH with either 0.060 U or 0.075 U of collagenase resulted in significantly larger hematoma volumes in mice treated with rivaroxaban compared with nonanticoagulated control (0.06 U: 32.2±8.2 mm³ versus 19.7±6.8 mm³; 0.075 U: 35.2±11.8 mm³ versus 29.2±8.3 mm³; P<0.05; n=20/group; Figure 2B). Although mortality was <10% in the experiments using 0.060 U of collagenase, 0.075 U collagenase resulted in a 24-hour mortality rate of 31% in the rivaroxaban group and 17% in the nonanticoagulated controls (Figure 2C).

Hemostatic Agents Prevent Excess Hematoma Expansion

Following the above experiments, we studied the effect of hemostatic factors in ICH induced by 0.060 U of collagenase after gastric administration of 30 mg/kg of rivaroxaban. 24 hours after ICH induction, the mean hematoma size was 18.4±6.1 mm³ in nonanticoagulated controls receiving vehicle solution only. Anticoagulation with 30 mg/kg rivaroxaban increased the hematoma volume to 32.4±7.9 mm³. The excess hematoma expansion could be effectively prevented by intravenous administration of any of the 3 hemostatic agents (FVIIa: 22.9±8.4 mm³, FFP: 16.3±5.7 mm³, and PCC: 20.4±6.4 mm³; P<0.05; Figure 3C). The blood content in the hemorrhagic hemispheres as assessed by hemoglobin spectrophotometry correlated linearly with the hematoma volumetry on cryosections (the online-only Data Supplemental Figure I).

Prevention of Excess Hematoma Expansion by PCC Is Dose-Dependent

We decided to study the effect of PCC in more detail because it is immediately available in the emergency situation in patients, can be rapidly infused over minutes, and causes less thromboembolic complications than FVIIa. Similar to the 100 U/kg dose, 50 U/kg of PCC significantly reduced the hematoma size in mice anticoagulated with 30 mg/kg rivaroxaban (20.7±6.1 mm³ versus 30.0±9.7 mm³; P<0.05). The results were verified by the spectrophotometric hemoglobin assay (Figure 4A). The 25 U/kg dose of PCC was not quite as effective as 50 U/kg in preventing excess hematoma expansion in anticoagulated mice (22.9±7.9 mm³ versus 30.0±9.7 mm³; P>0.05), but it also substantially improved the neurological deficits resulting from OAC–ICH (corner test: 25 U/kg: 41.4±24.2% right-turn; 50 U/kg: 38.5±12.8% right-turn; anticoagulated controls: 72.4±12.8% right-turn; P<0.05; n=13/group; Figure 4B).

Effect of Hemostatic Agents on the Systemic Coagulopathy Associated With Rivaroxaban Was Assessed Using In vitro Assays

As expected, intravenous administration of hemostatic agents had no significant influence on the plasma concentration of rivaroxaban (Figure 5B). Compared with the PT of anticoagulated and nonanticoagulated controls (38.6±5.5 seconds and 14.6±2.5 seconds, respectively), none of the hemostatic factors normalized the PT. Indeed, 1 mg/kg of FVIIa was the only agent that significantly reduced the PT compared with anticoagulated mice not receiving any hemostatic factor (FVIIa: 28.1±1.8 seconds; P<0.05; FFP: 35.7±5.0 seconds and PCC: 40.7±5.1 seconds; P>0.05; Figure 5C). In addition, we measured the plasma activities of various coagulation factors. Compared with the values of nonanticoagulated controls, anticoagulation with 30 mg/kg rivaroxaban reduced the coagulant activity of FII by 34% and FX by 40%, respectively, in anticoagulated mice. Administering any of the 3 hemostatic agents corrected the factor-deficiency after anticoagulation. Moreover, FVIIa increased the FVII-coagulant activity at least 2-fold above normal, FFP elevated the activities of Protein C and S 3-fold, and PCC increased the plasma activities of all factors, except FVII, 2- to 8-fold (Figure 5D).

Discussion

The present study yielded 4 major new findings: (1) A murine model of OAC–ICH associated with rivaroxaban was established based on previous OAC–ICH models. (2) High doses of rivaroxaban were required to enlarge intracerebral hematoma volume in this model. (3) Intravenous administration of either PCC, FVIIa, or FFP prevented excess intracerebral hematoma expansion. (4) Prevention of excess hematoma expansion by PCC was dose-dependent.
expansion in anticoagulated mice. (4) Administration of PCC, FFP, or FVIIa had heterogeneous effects on prolonged PT in anticoagulated mice, although they restored or even increased the activities of plasma coagulation factors.

The murine OAC–ICH model in the present study is based on the widely used ICH model induced by intrastriatal injection of collagenase, and is a modification of the previously established experimental models of OAC–ICH associated with warfarin or dabigatran. In these models, hematoma enlargement beyond that occurring in nonanticoagulated animals can be induced by oral anticoagulants allowing to study preventive reversal strategies. Prevention of hematoma expansion is an important early therapeutic goal in ICH because hematoma volume is a major prognostic factor in ICH.

Effective systemic anticoagulation after oral administration of a single dose of rivaroxaban was verified using well-established in vitro coagulation assays. The 30 mg/kg rivaroxaban resulted in high peak plasma concentrations exceeding those

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**Figure 2.** Induction of rivaroxaban-related intracerebral hemorrhage (ICH) using different doses collagenase. Rivaroxaban in 30 mg/kg dose exacerbated ICH expansion only in high dose (0.06 U and 0.075 U) collagenase. A and B, Hematoma volume was measured on cryosections at 24 hours after collagenase-injection (n=13 per group for 0.03 U and 0.045 U collagenase, and n=20 per group for 0.06 U and 0.075 U collagenase). *indicates P<0.05. C, Twenty-four hours mortality after intrastrial injection of high-dose collagenase (0.06 U and 0.075 U).
of patients in the ROCKET-AF trial 3- to 4-fold. Moreover, peak plasma concentrations were reached faster in anticoagulated mice (30–60 minutes) compared with patients (3 hours). The rivaroxaban dose examined in the present study also prolonged the PT 2- to 3-fold compared with nonanticoagulated control mice for at least 4 hours.

Similar to previous studies in dabigatran, a high dose of rivaroxaban was necessary to induce hematoma enlargement exceeding that of nonanticoagulated mice. In contrast, dosing of warfarin resulting in the therapeutic international normalized ratio range of 2 to 3.5 in humans suffices to enlarge the intracerebral hematoma in this model. Although both warfarin and new OAC result in effective systemic anticoagulation in patients, in this model, there is a striking discrepancy regarding the effect on intracranial bleeding. The mechanisms underlying the lower incidence of ICH in patients as well as the particularly high dose of rivaroxaban necessary for hematoma enlargement in the present experimental study remain to be elucidated.

The absence of a specific antidote is a major concern in case of severe hemorrhage during anticoagulation with nOAC. The main purpose of our study was to identify an appropriate strategy to prevent hematoma enlargement in case of ICH. All tested hemostatic agents prevented the excess intracerebral hematoma expansion induced by anticoagulation effectively. Comparison of 100 U/kg with 50 U/kg and 25 U/kg PCC showed similar or only slightly less efficacy of lower doses, in accordance with our previous study in experimental ICH associated with dabigatran. Prevention of hematoma enlargement was paralleled by a better neurological outcome.

Monitoring the effectiveness of reversal of anticoagulation would be desirable in patients with life-threatening or critical organ bleeding. In the present study, effectiveness of prevention of excess intracerebral hematoma enlargement was only variably mirrored in the effect of hemostatic factors on the coagulation assays. Interestingly, despite increasing the activities of FII, IX, X, and Protein C and S, PCC failed to reduce the prolongation of the PT induced by rivaroxaban. In contrast, Eerenberg and colleagues reported recently the rapid and complete reversal of the effect of rivaroxaban on the PT after administration of 50 U/kg PCC (Cofact). However, in this study, the PT was only slightly prolonged after anticoagulation with rivaroxaban. In our study, PT was assessed using Neoplastine Plus, which has been shown to be the most sensitive thromboplastin reagent of PT for monitoring anticoagulation with rivaroxaban. We found a 2- to 3-fold prolongation of PT in the anticoagulated mice, which is consistent with previous findings in rats and baboons. Considering the higher plasma
concentration of rivaroxaban in our anticoagulated mice and different sensitivities of the applied PT reagents among studies, a direct comparison is difficult. An alternative explanation is that the activation status of coagulation factors affects in vitro coagulation tests. PCC contains human FII, VII, IX, X in an inactivated form. Agents containing activated FVII (eg, rFVIIa, Novo Seven; aPCC, FEIBA) accelerate the generation of FII, and therefore exhibit a more pronounced effect on the correction of prolonged PT. In essence, our findings support the notion that monitoring of the reversal of anticoagulation using standard coagulation assays such as the PT to predict the effect on remote critical organ bleeding is complex and potentially misleading.

Our study has several limitations. First, we examined only a single high dose instead of repetitive longer term administration of rivaroxaban. However, the single dose of rivaroxaban produced a profound anticoagulatory effect during the experimental period. Second, considering that the blood volume required for arterial blood gases is great enough to induce hypovolemia in mice, we only monitored rectal body temperature during and after surgical procedures. Third, FVIIa has been shown to attenuate hematoma enlargement in collagenase-induced ICH, and therefore we could not exclude the direct inhibitory effect of coagulation factors on the collagenase enzymatic activity. Finally, our study was not designed to understand the mechanisms underlying the lower incidence of ICH associated with rivaroxaban compared with ICH during warfarin therapy. The high dose of rivaroxaban required to enlarge the hematoma may suggest a lower risk of this complication during anticoagulation with new OAC.

In conclusion, administration of any of the hemostatic agents PCC, FFP, or FVIIa can prevent excess hematoma expansion in a murine OAC–ICH model associated with rivaroxaban. Because the pathophysiology of ICH in patients is only partially reflected in current experimental ICH models, the efficacy and safety of this strategy must be further evaluated in the clinical setting.
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Disclosures

The study was investigator-initiated. It was planned and performed independently of Bayer Pharma AG except for determining rivaroxaban plasma concentration and prothrombin time, which were measured by Drs Bütehorn, Heitmeier, and Perzborn.

Dr Veltkamp has been an investigator in the ROCKETF-AF and the RELY trials. He has received speaker’s honoraria, travel support, and consulting fees from Bayer, Boehringer Ingelheim, and BMS Pfizer. Drs Bütehorn, Heitmeier, and Perzborn are employees of Bayer Pharma.

All other authors have no conflict of interest to declare.

References


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Supplementary Methods

**Rivaroxaban plasma concentration:** Rivaroxaban plasma concentrations were determined using a high-performance liquid chromatography (HPLC) system coupled to a tandem mass spectrometer in selected reaction monitoring mode with a turbo ion spray (Agilent system 1100 coupled with MS/MS API 4000; AB Sciex, USA). Stable isotope-labelled rivaroxaban was used as internal standard ($^{15}$N,D$_5$; Bayer Healthcare AG, Wuppertal, Germany). After protein precipitation with acetonitrile containing the internal standard, plasma samples were injected into the HPLC-MSMS system. Rivaroxaban eluted from the HPLC column with a retention time of approximately 3min. The ions monitored were 436$\rightarrow$145 (rivaroxaban) and 442$\rightarrow$145 (internal standard). The method was validated by assaying quality control samples of blank plasma spiked with known concentrations of rivaroxaban. Rivaroxaban concentrations above the lower limit of quantification (0.5-1µg/L) were determined with a precision of 1.7–3.2% and an accuracy of 97.7–106.5%.

**Prothrombin time (PT):** PT was assessed in the same mice, which were involved in plasma concentration determination, by mixing 50µL platelet-poor-plasma at 37°C with freeze-dried thromboplastin from rabbit brain with an international sensitivity index of 1.23 (Neoplastin®, Diagnostica Stago, Asnières, France). PT was measured in a ball coagulometer KC 10 (Amelung, Lemgo, Germany) according to the manufacturer's instructions.

**Assessment of the activities of coagulant factors:** In separate mice anticoagulated with 30mg/kg rivaroxaban, the activities of the coagulation factors II, VII, IX, X, Protein C and S were measured in the central laboratory of the University Heidelberg. FII, VII, X were measured using specific factor-deficient human plasma samples and FIX were measured using FIX-deficient human plasma samples and a partial activated thromboplastin (Siemens Healthcare Diagnostics). For assessing the activity of FII, FVII, FX, coagulation was initiated with a high-sensitivity thromboplastin reagent (Siemens Healthcare Diagnostics) based on recombinant human tissue factor, and for FIX, coagulation was started with CaCl$_2$. The beginning of the coagulation was detected by a turbidimetric method. Protein C and S were measured using specific chromogenic assay (Siemens Healthcare Diagnostics).

**Surgical induction of intracerebral haemorrhage:** Spontaneously breathing mice were anesthetized with halothane (1% - 1.5%) in an oxygen/air mixture as described$^1$. Animals were placed in a stereotactic frame (Model 51650, Stoelting®, USA) with a mouse adaptor (Model 51625, Stoelting®, USA). A borehole was drilled (0.5mm anterior and 2mm left to bregma), and a 10 µL-needle (SGE®, Austria) was placed into the left striatum at 3.5mm depth from the skull. Then 0.5 µL of saline containing either 0.030U, 0.045U, 0.060U or 0.075U collagenase type VII (Sigma, Germany) were injected into different groups of animals. The needle stayed in place for another 10min. After withdrawal of the needle, the borehole was sealed with bone wax, and the scalp was sutured. The surgical procedure lasted 20min.

**Measurement of hematoma size:** Twenty-four hours after inducing ICH, mice were anesthetized again and perfused transcardially with saline. After decapitation, brains were collected and frozen in isopentane at -20°C. Every 400µm, 40-µm-thick coronal cryosections were cut. Unstained sections were scanned at 300dpi. Hematoma area was encircled and measured by a rater, who was blind to the group allocation, using a public domain image analysis program (ImageJ®) as previously described$^1$. Total hematoma volume was determined by integrating measured areas and distance between sections.
Spectrophotometric hemoglobin assay: the blood content of the hemorrhagic hemisphere was quantified using a spectrophotometric hemoglobin assay as previously described with some modification\textsuperscript{2}. Briefly, the materials were collected from the hemorrhagic hemisphere of the 40µm-thick serial cryosections for hematoma volumeetry and homogenized in 100µL PBS at 4ºC. After being centrifuged at 13000r.p.m. for 30min, the hemoglobin-containing supernatants were collected and mixed 1:4 (v/v) with Drabkin’s reagent (Sigma-aldrich, München, Germany, K\textsubscript{3}Fe(CN)\textsubscript{6} 200mg/L, KCN 50mg/L, NaHCO\textsubscript{3} 1g/L, pH 8.6). The mixture was allowed to stand for 15min to convert hemoglobin to cyanomethemoglobin and then the optical density was measured at a wavelength of 540nm with a spectrophotometer (Synergy™ 2 Multi-Detection Microplate Reader, BioTec, USA). The standard absorbance curve was generated by adding incremental aliquots of murine blood (1, 2, 4, 6, 8 and 10µL) into the same PBS/Drabkin’s solution. The blood content (µL) of hemorrhagic hemisphere was calculated as following: The blood content of hemisphere (µL) =the blood content of sample (µL) x (distance between the serial cryosections (400µm)/ thickness of the sections (40µm)).

Corner test: Mice were positioned to walk towards a V-shaped corner with a 30° angle. Left or right turns involving full rearing along the wall were counted. The measurement was repeated for 15 times with at least a 30sec interval in between. The percentage of right turns was calculated.

The a priori sample size calculations: For the a priori sample size calculations for the experiments examining the effect of hemostatic agents we assumed a difference of the mean hematoma volume between rivaroxaban pretreated versus nonanticoagulated /antagonized animals to be 12mm\textsuperscript{3} (30 mm\textsuperscript{3} vs 18 mm\textsuperscript{3}). The power was set to 90%, a 2-sided \( p \)-value < 0.05 was regarded as significant. Assuming a standard deviation of 7mm\textsuperscript{3} in each group and 5 experimental groups, a sample size of 13/group was calculated.

Supplementary figures

![Supplementary figure 1](image_url) Correlation of the results of hematoma volumetry and blood content of the hemorrhagic hemisphere assessed using hemoglobin spetrophotometry. Peason’s correlation coefficient: \( r=0.73, \ p<0.001, \ n=117 \)
Supplementary figure 2 Correlation of the results of hematoma volumetry and neurological deficit assessed using corner-test. Pearson's correlation coefficient; \( r=0.86, \ p<0.001, \ n=56 \)

Supplementary figure 3 Correlation of blood content assessed by hemoglobin spectrophotometry and neurological deficit assessed by the corner-test. Pearson's correlation coefficient; \( r=0.72, \ p<0.001, \ n=56 \)

Supplementary reference

リバーロキサバンに関連する実験的脳内出血における止血治
療
Hemostatic Therapy in Experimental Intracerebral Hemorrhage Associated With Rivaroxaban

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Abstract

背景および目的: リバーロキサバンは、心房細動患者における脳卒中予防薬として近年承認された。しかし、効果的な拮抗薬がないことが、脳内出血 (ICH) が生じた場合の大きな懸念となっている。本研究の目的は、リバーロキサバンに関連する ICH マウスモデルを確立し、過剰な血腫拡大予防における異なる止血因子の有効性を検討することであった。

方法: 10 mg/kg または 30 mg/kg のリバーロキサバンを経胃管的に投与した C57BL/6 系マウスにおいて、血漿中濃度、プロトロンビン時間および凝固因子活性を繰り返し測定した。線条体内にコラゲナーゼを注入し、ICH を誘発させから 30 分後に、生理食塩水、プロトロンビン複合体濃縮製剤 (100 U/kg), マウスの新鮮凍結血漿 (200 μL), または組換え型ヒト第 VIIa 因子製剤 (1 mg/kg) のいずれかを静脈内投与した。24 時間後、ヘモグロビン分光光度法を用いて、脳凍結切片にて ICH の容積を定量化した。

結果: コラゲナーゼ 0.060 U により ICH を誘発したマウスにおいて、リバーロキサバン 30 mg/kg 投与群で、血腫容積が大幅に増加した。プロトロンビン複合体濃縮製剤、新鮮凍結血漿、または第 VIIa 因子製剤は、抗凝固作用による過剰な血腫拡大を予防していた。プロトロンビン複合体濃縮製剤による血腫拡大の予防は、用量依存的であった。

第 II 因子および第 X 因子の活性欠損は回復していたものの、3 剤のうちでプロトロンビン時間延長を完全に正常化した薬剤はなかった。

結論: プロトロンビン複合体濃縮製剤、第 VIIa 因子製剤、新鮮凍結血漿は、ICH マウスモデルにおけるリバーロキサバンに関連する過剰な脳内血腫の拡大を予防していた。本研究における補充療法の有効性および安全性に関しては、臨床試験におけるさらなる評価が必要である。

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図 5

リバーロキサバン抗凝固薬に関連する凝固異常に対する各止血剤の効果

D. リバーロキサバン投与またはリバーロキサバンのみ投与の対照群、およびリバーロキサバンによる抗凝固療法を受けた。止血剤投与マウス群における各凝固因子の活性（各群 n = 5）。結果は、ヒト健康被験者の標準値と比較した活性化の割合で示す。FVIIa：活性化第 VIIa 因子製剤、FFP：新鮮凍結血漿、PCC：プロトロンビン複合体濃縮製剤

(Stroke 誌の図を一部省略して掲載)