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Research Report

Kinetics of hematoma expansion in murine warfarin-associated intracerebral hemorrhage

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ABSTRACT

Background and purpose: The burden of intracerebral hemorrhage associated with oral anticoagulants (OAC-ICH) is growing. However, little is known about the pathophysiology of W-ICH. Herein, we refine a mouse model of OAC-ICH using repetitive T2* MRI to describe kinetics of hematoma enlargement, and establish a benchside point of care INR assay (PoC) for assessment of anticoagulation. *Methods:* C57/BL6 mice drank warfarin (0.4 mg/kg/24 h) in their water. ICH was induced by stereotactic injection of collagenase type VII (0.045 U) into the left striatum. Hemorrhagic blood volume was quantified by MRI T2* images and on cryosections 48 h after ICH induction. Kinetics of hematoma expansion were compared in strongly, moderately, and non-anticoagulated mice using repeated MRI T2* imaging. The PoC INR technique was validated against standard laboratory INR, and tail vein bleeding time (TVBT). *Results:* PoC INR correlated with central laboratory measurements ($r=0.989$; $p<0.0001$) and with TVBT ($r=0.982$; $p<0.0001$). Hematoma volume was 21.2±6.7 mm^3 in heavily (PoC INR 4–5), 12.3±4.8 in moderately (INR 2–3), and 8.6±3.3 in non-anticoagulated mice (INR<1.2). Hematoma volume determined from cryosections and T2* MRI correlated well ($r=0.922$). Strength of anticoagulation was associated with neurologic outcome. Hematoma enlargement occurred mainly during the first 3 h in anticoagulated mice. *Conclusions:* PoC allows repeated benchside INR measurements in individual mice which reflect the level of anticoagulation. Stronger anticoagulation results in larger hematoma volumes. As hematoma enlargement occurs mainly during the first hours, potential hemostatic therapies should be tested early in this OAC-ICH model.

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Abbreviations: ICH, Intracerebral hemorrhage; OAC-ICH, Oral anticoagulants associated intracerebral hemorrhage; INR, International normalized ratio; PCC, Prothrombin concentrate; PoC, Point of care INR assay; TVBT, Tail vein bleeding time

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1. Introduction

Intracerebral hemorrhage (ICH) represents about 15% of all strokes in the industrialized countries and its mortality rate exceeds that of other strokes considerably (Sjoblom et al., 2001). Currently, 10–18% of all ICH are associated with the use of oral anticoagulants (OAC-ICH) but the incidence of OAC-ICH is expected to increase considerably in parallel to the rising prevalence of atrial fibrillation, the most frequent indication for long-term therapy with vitamin K antagonists (Broderick et al., 2007). Intracranial hemorrhage is the most serious and lethal complication of antithrombotic therapy as it causes about 90% of deaths and most of the permanent disability from warfarin-associated bleeding (Fang et al., 2007). Depending on the intensity of anticoagulation, vitamin K antagonists increase the risk of ICH two- to five-fold (Hart et al., 2005). The degree of anticoagulation, expressed by the international normalized ratio (INR) for prolongation of the prothrombin time at the time of admission correlates with initial hematoma size (Rosand et al., 2004), progressive hematoma enlargement after admission (Flaherty et al., 2008), functional outcome (Berwaerts et al., 2000) and mortality (Yasaka et al., 2003). One of the most important independent predictors of mortality and poor outcome is the hematoma growth (Davis et al., 2006). Current therapeutic guidelines for OAC-ICH recommend the administration of coagulation factors such as prothrombin concentrate (PCC) and fresh frozen plasma in order to reverse the anticoagulant effect of OAC and stop the hematoma enlargement (Broderick et al., 2007).

Until recently, preclinical research regarding the pathophysiology and therapy of OAC-ICH was difficult because no adequate experimental model was available. In an elegant pioneer study, Foerch et al. established a mouse model of OAC-ICH in which collagenase was injected into the striatum of warfarin pre-treated mice (Foerch et al., 2008). Additional experiments showed that PCC administration 45 min after ICH induction prevented hematoma enlargement in this model (Foerch et al., 2009). While these reports laid the ground for subsequent experimental studies, important aspects remained to be resolved. Crucial issues encompass the kinetics of hematoma expansion in individual animals in relation to the effective dose of the oral anticoagulant, identification of the best time for hemostatic interventions, and establishment of a technique that allows *in vivo* assessment of coagulation status in individual anticoagulated mice during ICH induction.

Accordingly, the purpose of the present study was to further characterize and refine the murine OAC-ICH model. Specifically, we aimed to describe the individual kinetics of hematoma expansion using repetitive multimodal MRI scanning. We also established a simple benchside coagulometric test that allowed repetitive measurements of the INR in individual mice.

2. Results

2.1. Calibration of point of care INR measurements

Feeding 14 naive mice with warfarin for various periods of time led to a wide range of INR values as measured with the PoC device. Central laboratory INR measurements of the same samples yielded different absolute INR values but the correlation between both measurements was excellent ($r=0.989$; $p<0.0001$; Fig. 1-B). Moreover, comparison of PoC INR values with the tail vein bleeding time of a second set of animals ($n=12$) revealed good correspondence of these parameters. The TVBT value of non-anticoagulated mice was 120 s with an excellent correlation with the PoC INR in anticoagulated and non-anticoagulated mice ($r=0.982$; $p<0.0001$; Fig. 1-C). Thus, PoC INR measurements reflected a corresponding effect on the coagulation system in mice.

2.2. Clinical outcome

Mean neurologic outcome score at 48 h was 2.0±1.2 in the strongly anticoagulated group, 1.3±0.6 in the moderately anticoagulated group, and 0.6±0.4 in the non-anticoagulated group (ANOVA; $p=0.041$). The mean body weight was 20.3±0.8 g in strongly, 21.7±1.3 in moderately, and 23.0±1.3 in non-anticoagulated mice ($p=0.0089$). No significant differences of rectal temperatures were found within 48 h after ICH induction (strong: 36.9±0.5; moderate: 37.1±0.6; no anticoagulation: 37.4±0.3; ANOVA $p=0.34$)

Mortality in the heavy-anticoagulated mice group was 25% (2/8) at 48 h and 0% in the mild anticoagulation group (0/8) and non-anticoagulated group (0/10), respectively.

2.3. Hematoma size on cryosections and T2* MRI sequence

Forty-eight hours after ICH induction, the mean size of the hematoma, measured by the T2* hyperintensity area was 21.2±6.7 mm² in the strongly, 12.3±4.8 in the moderately, and 8.6±3.3 in the non-anticoagulated group (ANOVA $p=0.0028$) (Fig. 2). The mean volume of the hematoma, measured in the cryosections was 20.3±6.3 mm^3 in the strongly, 11.7±5.8 mm^3 in the moderately, and 7.6±3.2 mm^3 in the non-anticoagulated mice. (ANOVA $p=0.0055$). Overall the two methods of hematoma volume measurement correlated very well ($r=0.922$) ($p<0.0001$). Also, using the abc/2 formula for hematoma size estimation in the MR images we found a hematoma volume of 20.6 mm^3±6.9 in the strongly, 13.7 mm^3±8.5 in the moderately, and 7.4±4.2 mm^3 in the non-anticoagulated group (ANOVA $p=0.043$). Thus, there was a good correlation between the 2 volumetric techniques($r=0.906$; $p<0.0001$).

2.4. MRI hematoma expansion curve

In the strongly anticoagulated group (PoC INR 4–6) the maximal hematoma size was reached already 6 h after the collagenase injection (18.41±6.7 mm^3), while in the non-anticoagulated group it was obtained 24 h after the ICH induction (9.49±2.2 mm^3). In the anticoagulated group 69.8% of the total volume was achieved during the 1st h after the ICH induction and 95.0% during 3 h. In the non-anticoagulated group the volume measure at 1, 3 and 6 h was 52.2%, 75.5% and 80.0% of the total volume (Fig. 3).

2.5. Kinetics of INR depends on concomitant anesthesia

In the anticoagulated group ($n=15$) mean PoC INR value was 5.3±1.5 at the time of collagenase injection, 4.9±1.1 after 1 h, 6.8±1.0 after 3 h, 6.6±0.9 after 6 h, 2.4±2.4 after 24 h and 1.2±0.3 after 48 h.

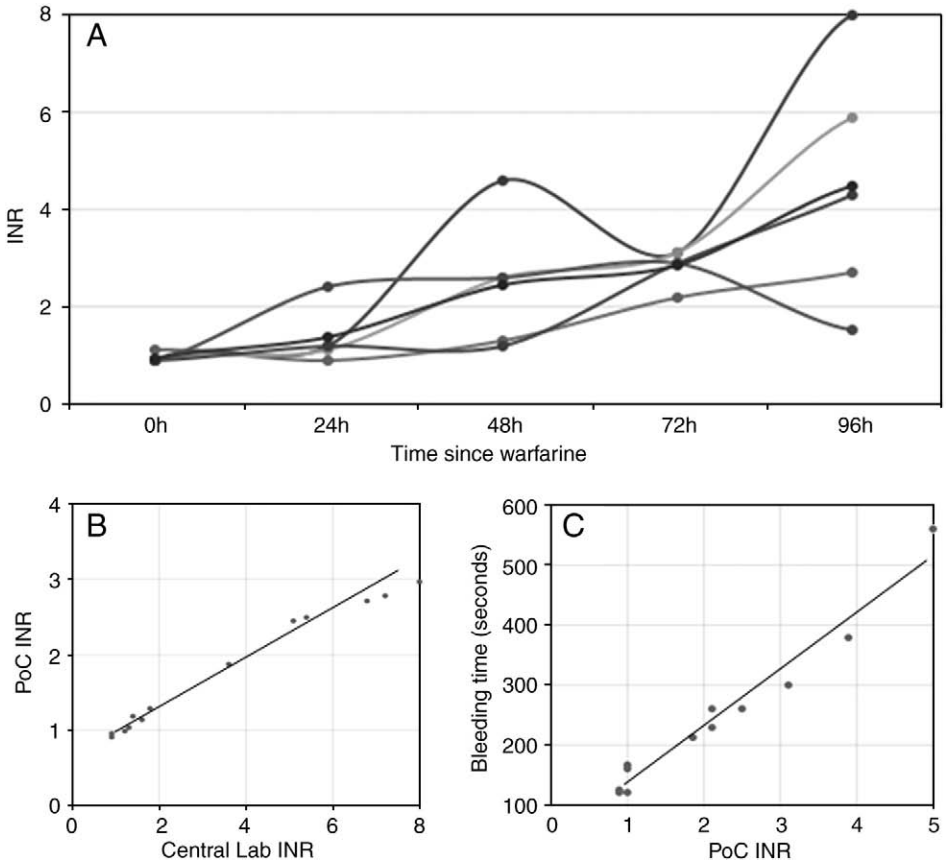

Fig. 1 – INR. A. PoC INR after 5 days of treatment with warfarin 0.4 mg/kg/24 h. Every line represents INR values for a single mouse at 1, 2, 3, 4 and 5 days. B. A plot chart of PoC INR value versus standard central laboratory measurement. ($r = 0.989$, $p < 0.0001$). C. A plot chart of PoC INR value versus tail vein bleeding time measurement. ($r = 0.982$, $p < 0.0001$).

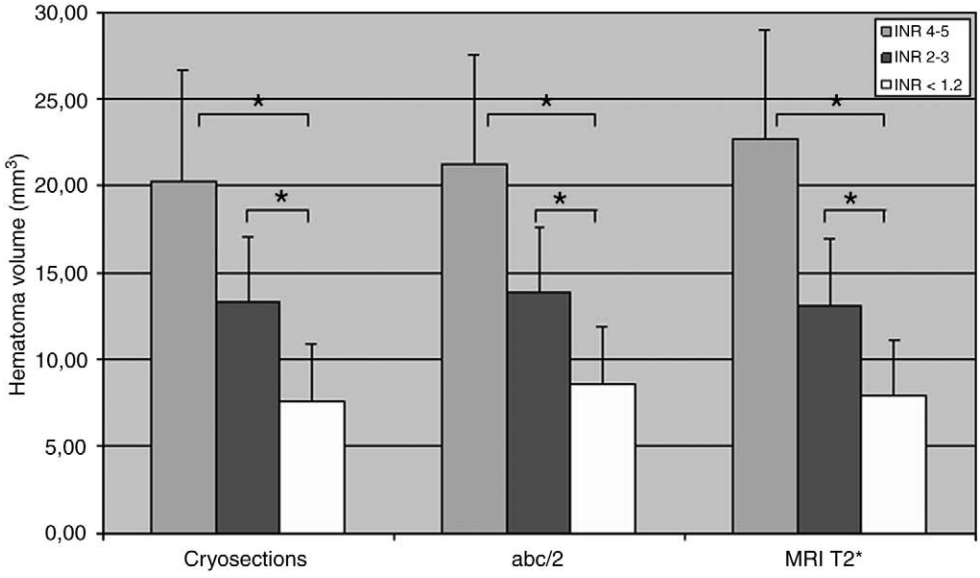

Fig. 2 – Hematoma size. Hematoma size, expressed in mm^3, in strongly (PoC INR 4–5, gray bars), moderately (INR 2–3, black bars) and non-anticoagulated mice (INR < 1.2, white bars) measured by three different methods: unstained cryosections, abc/2 method from brain MRI and MRI volumetry (details see text).

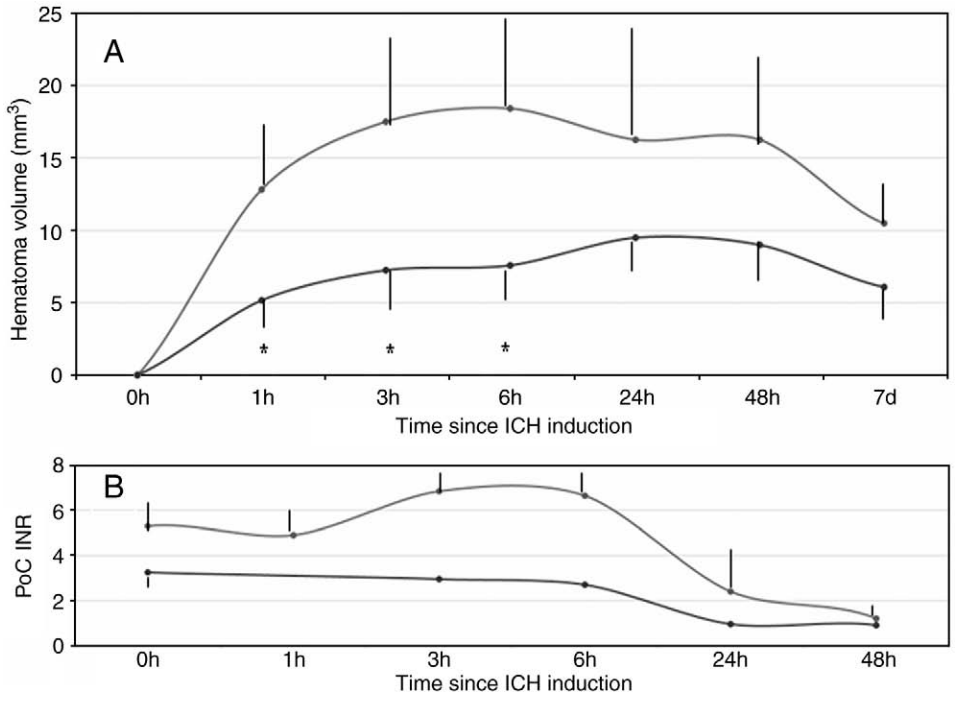

Fig. 3 – Hematoma volume and PoC INR kinetic curves. A: Hematoma volume in strongly anticoagulated (PoC INR 4–5; superior line) and non-anticoagulated mice (inferior line), measured at 1 h, 3 h, 6 h, 24 h, 48 h and 7 days after collagenase injection. B: PoC INR in mice anesthetized more than 2 times during the first 6 h after ICH induction (superior line) and mice without further anesthesia after the collagenase injection (inferior line).

Anticoagulated mice which weren't operated, scanned or anesthetized during the first 24 h ($n=5$) had mean INR values at 0, 1, 3, 6, 24 and 48 h of 3.2, 2.9, 2.7, 2.4, 0.9 and 0.9 respectively (Fig. 3-B).

3. Discussion

Although the management of OAC-ICH is an issue of increasing clinical relevance, an experimental murine model has only recently been established. The major new findings of our study are that: (1) a PoC assay for benchside measurement of the INR in individual anticoagulated mice was established and the corresponding anticoagulatory effects of INR measurements were validated in mice. (2) A clear association of higher PoC INR values with larger final hemorrhage volume, more extensive hematoma enlargement, and worse neurological outcome was established. (3) MRI T2* scans adequately reflect hematoma volume, and allow the monitoring of the individual kinetics of hematoma expansion during the first 7 days after ICH induction in mice (Fig. 4). (4) Hematoma expansion in this OAC-ICH model occurs predominantly in the first 1–3 h. (5) Repetitive anesthesia with halothane prolongs the anticoagulatory effect of warfarin in mice.

Several modifications of the OAC-ICH recently introduced model (Foerch et al., 2008) were performed. First, the daily dose of warfarin was lowered resulting in a lower mortality rate. Also, we injected only 0.045 U of collagenase type VII instead of 0.075 U, the dose used in the majority of previous studies in non-anticoagulated mice (James et al., 2008), because this modification resulted in smaller circumscribed hematomas in the anticoagulated group in contrast to the massive hemispheric hemorrhage described previously in this model (Foerch et al., 2008). Although the T2* MRI sequence is a powerful approach for visualizing hemorrhage in acute stroke in patients (von Kummer, 2002), it has not been used for experimental investigations in intracerebral hemorrhage to our knowledge. Comparison of hematoma volume measured from MRI T2* sequences either by planometry or by the abc/2 formula frequently used in the clinical setting (Newmann, 2007) corresponded closely with measurements based on cryosections.

Importantly, administering warfarin dissolved in drinking water (0.4 mg/kg per day) for 3 days resulted in a broad spectrum of PoC INR values. While Foerch et al. performed INR measurements only in a separate set of animals (i.e. not those in which ICH was induced), the PoC INR device used in our study allows simple, repetitive INR measurements from a small amount of blood in individual experimental animals. Comparative measurements with standard prothrombin time coagulometry in the central laboratory revealed an excellent correlation of both methods. Since both coagulometric methods were developed for human blood analysis, and none of them uses murine thromboplastin as a trigger of the extrinsic coagulation pathway, the anticoagulatory effect of the INR measured in mice cannot be expected to be equivalent to the human setting. However, the correlation of higher PoC INR values with larger ICH volumes, longer TVBT, and bigger standard INR values underscores that higher PoC INR values reflect a stronger anticoagulatory effect. Because we were able to measure INR in individual mice, a clear relationship could be shown between the level of anticoagulation and the resulting hematoma volume. Compared with non-anticoagulated mice,

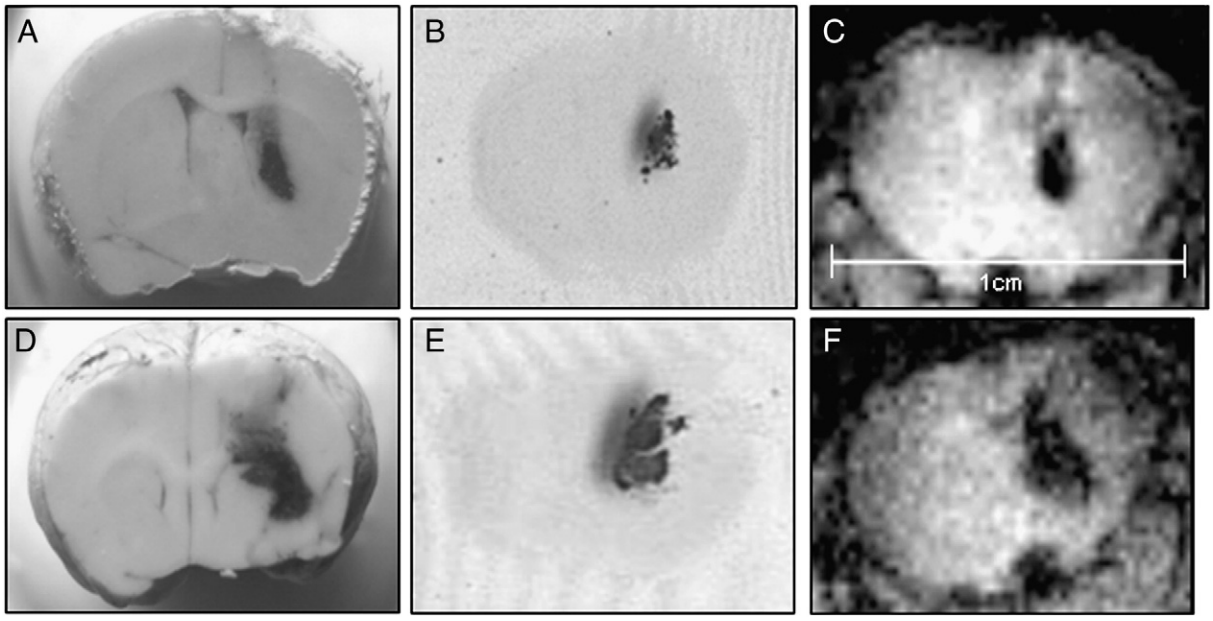

Fig. 4 – Corresponding ICH in sections (A, D), brain histology on 40 μL thick non-staining cryosections (B, E) and T2* MRI (D, F) for a non-anticoagulated mouse (superior row) and strongly anticoagulated mouse (bottom row).

there was a mean 1.8-fold increase of hematoma size in moderately anticoagulated animals, and a 2.7-fold increase in mice with strong anticoagulation. In addition to larger hematoma volumes, higher INR values were associated with higher mortality and a worse neurologic outcome.

An important goal of establishing an OAC-ICH model is to test potential hemostatic approaches to prevent hematoma expansion (Foerch et al., 2009). A prerequisite for the timing of the administration of an experimental hemostatic substance is to know the kinetics of OAC-ICH expansion. In the clinical setting, orally anticoagulated patients more frequently suffer prolonged or repetitive hemorrhage compared to non-anticoagulated patients (Aguilar et al., 2007). In contrast, in the present OAC-ICH model, hematoma enlargement mainly occurred within the first hour, and 95% of maximal hematoma value was reached already 3 h after collagenase injection (Fig. 5). Consequently, any hemostatic therapy can only be effective in this experimental model when treatment is initiated within the first 3 h, and ideally within the first hour after ICH induction.

Warfarin is cleared rapidly in mice (Ryan et al., 1969) but anesthetics can modify the clearance. Our data suggest a complete reversal of the anticoagulatory effect of warfarin already 24 h after discontinuation of warfarin feeding in mice undergoing only 2 halothane anesthesias (at the beginning and end of the experiment, respectively). In contrast, in mice that were anesthetized several times with halothane for repetitive MRI scanning, normalization of INR took at least 48 h. Indeed, halothane can potentiate the pharmacological effect of warfarin by the effect of its metabolite trifluoroacetic acid on the binding of warfarin to serum proteins (Calvo et al., 1989).

In conclusion, our study substantially refines a recently introduced murine experimental model of OAC-ICH. In particular, better *in vivo* characterization of the kinetics of hematoma enlargement and the establishment of a benchside INR assay will allow evaluation of potential hemostatic therapies in future studies.

4. Experimental procedures

The study was conducted in accordance with national guidelines for the use of experimental animals. The protocols were approved by the governmental committees for animal care and use (Regierungspraesidium Karlsruhe, Germany). In all experiments sexually mature male mice (C57BL/6, Charles River Laboratories, 10–12 weeks of age, body weight 20–27 g, $n=92$) were used.

4.1. *Warfarin administration*

Warfarin is a water soluble molecule and is completely absorbed after oral administration (Ansell et al., 2008). In our initial experiments, 5 mg of warfarin (Coumadin®, Bristol-Myers-Squibb) was dissolved in 375 mL of drinking water for 24 h as reported previously (Foerch et al., 2008). However, 3 of the 5 mice pre-treated with that concentration were dead after 24 h and the remainder were very strongly anticoagulated (POC INR>8). Therefore, 2.5 mg of warfarin was dissolved in 800 mL of drinking water in subsequent experiments, assuming that the normal water intake of a C57BL/6 mouse weighing 25 g is 3.25 g/24 h (Fox et al., 1980). Accordingly, mice received 0.01 mg of warfarin per day (0.4 mg/kg) resulting in a range of INR measurements after 72 h of warfarin administration (Fig. 1-A).

4.2. *Calibration of point of care INR measurements*

One set of naive warfarin-treated animals ($n=14$) was used for calibration of INR measurements. The INR measurements were performed on the same venous blood sample drawn from the

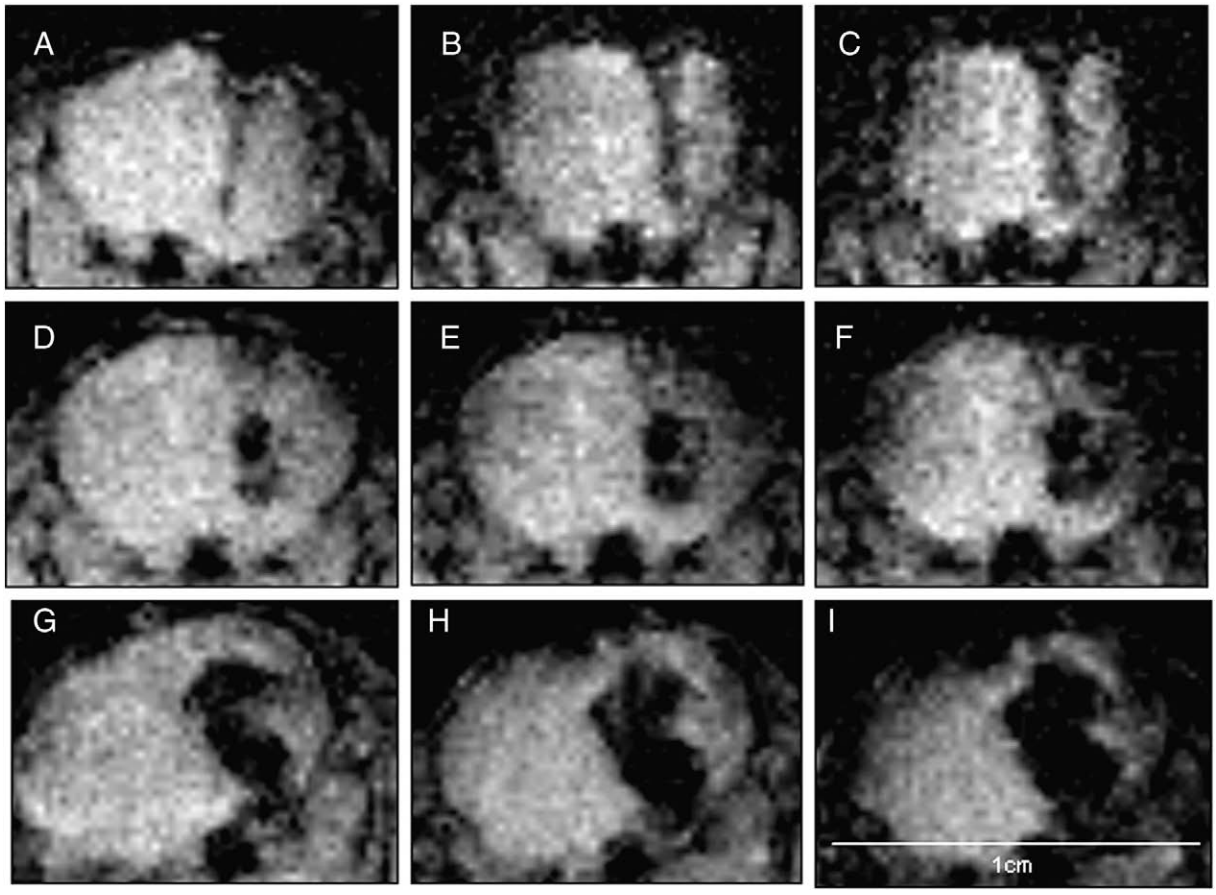

Fig. 5 – Brain T2*MRI images 1 h (A, D, G), 3 h (B, E, H) and 6 h (C, F, I) after ICH induction in a non-anticoagulated mouse (superior row), moderate-anticoagulated (middle row) and strongly anticoagulated mouse (inferior row).

orbital sinus by a commercially available point of care coagumeter (PoC; Coaguchek XS®, Roche, Mannheim, Germany) and the standard coagulometric technique used by the central laboratory of the University Hospital Heidelberg (Innovin® clotting assay in a CA-7000, Siemens Healthcare Diagnostics). While the PoC device uses a recombinant human thromboplastin on a chip requiring only about 10 μL of blood, the standard central laboratory technique requires 0.5–1 mL of blood which prevents repetitive INR measurements in the same mouse.

To better characterize the physiological meaning of a given PoC INR value in a mouse, individual INR values were compared to the tail vein bleeding time (TVBT) (Bronze et al., 2001) in anticoagulated ($n=8$) and non-anticoagulated ($n=4$) mice. Briefly, in anesthetized mice the tail was cut 5 mm from the distal tip, and immediately submerged in 0.9 % saline at 37 °C. The time until the bleeding stopped for 20 s was recorded.

4.3. *Induction of intracerebral hemorrhage*

Spontaneously breathing mice were anesthetized with halothane (1.5%–2%) in an oxygen/air mixture. Animals were placed in a stereotactic frame (Model 51650, Stoelting®, USA) with a mouse adaptor (Model 51625, Stoelting®, USA). A borehole was drilled (0.5 mm anterior and 2 mm lateral to bregma), and a 10 μL needle (Hamilton) was placed into the left striatum (3.5 mm depth from the scull). Then, 0.3 μL of saline containing 0.045 U collagenase type VII-S (Sigma®) was injected. After 5 min, the needle was withdrawn, the borehole was sealed with bone wax, and the scalp was sutured. The surgical procedure lasted 15–20 min. A heat lamp was used to maintain animal body temperature after the operation.

4.4. *Measuring hematoma size in cryosections and by T2* MRI*

The animals were examined in a 2.35 T scanner (Biospec 24/40, Bruker Medizintechnik, Germany). A resonator cage with a 40 mm inner diameter was used as the radiofrequency coil. Mice were deeply anesthetized with halothane 1.5–2% and placed into the MRI scanner. T2* images of 1 mm thickness were obtained every 1.5 mm. T2*-weighted imaging was achieved using the MGE 8 echo sequence (TR 1500 ms, TE 16.5 ms, FOV 5.5 × 5.5 cm, matrix 256 × 256, 6 slices). Image data were subsequently transferred to a SUN Sparcstation 10 (SUN Microsystems) and measured by an examiner blinded to treatment allocation.

Immediately after the final MRI, mice were deeply anesthetized with tribromoethanol and perfused transcardially with saline. Brains were removed and frozen: 40 μm thick coronal cryosections were cut every 400 μm. Sections were scanned at 600 dpi; the hematoma area was encircled and measured using a public domain image analysis program (Scion Image). The total hematoma volume was obtained by integrating measured areas and distance between sections. Also, we examined a

frequently used method in the clinical setting for hematoma size estimation in the MR images assuming that the hematoma shape is an ellipsoid (abc/2) (Broderick et al., 1993).

4.5. *Neurological outcome*

We used a 5-point outcome scale (Foerch et al., 2008) to assess the neurological deficits 48 h after ICH induction. Briefly, mice were placed at the center of a glass jar and their spontaneous motor activity was videotaped for 60 s. If mice stopped moving during the observation period or did not move at all we repeated the test after a few minutes. Videotapes were rated for neurological status by a person blinded to group assignment using a 5 point scale; 0: no apparent deficit; 1: slight deficit during walking, no circling; 2: circling to the right with at least some straight movements and some covering of distance; 3: heavy circling to the right without straight movements or no movements at all; and 4: death or complete immobility.

4.6. *Relationship between hematoma size and strength of anticoagulation*

ICH was induced in three different groups depending on the level of anticoagulation at the time of collagenase injection: (1) strongly anticoagulated mice (PoC INR 4–5, $n=8$), (2) moderately anticoagulated mice (PoC INR 2–3, $n=8$), and (3) non-anticoagulated mice (PoC INR <1.2, $n=10$). Forty-eight hours after ICH induction, animals underwent neurological examination and subsequent MRI. Thereafter, anesthetized mice were euthanized and brains were frozen in isopentane at –20 °C.

4.7. *Hematoma expansion kinetics using repeated MRI T2* imaging*

For these experiments, MRI scans were performed at 1, 3, 6, 24, 48 h, and 7 days after the ICH induction. At these time points, 10 μL of blood was drawn from the periorbital sinus with a capillary tube. INR measurements were performed with the PoC. Two groups of animals were studied. The first set of animals ($n=15$) had been pre-treated with warfarin (INR at the time of the collagenase injection 4–6). The second group (non-anticoagulated mice, INR <1.2, $n=15$) served as control.

4.8. *PoC INR kinetic curve*

PoC INR was measured every time before an anesthetized mouse underwent an MRI scan in order to obtain an INR kinetic curve as well. To examine the effect of repeated halothane anesthesia, a separate set of nonoperated mice ($n=5$) were pre-treated with warfarin without repeated anesthesia. The PoC INR values were measured 1, 3, 6, 24 and 48 h after withdrawal of warfarin.

4.9. *Statistical analysis*

All values are expressed as mean±standard deviation (SD). Comparison of mean values was performed by ANOVA for multiple comparisons with posthoc Tukey test using PSPP analysis open source software. Comparison of ordinal variables was performed by X^2. A p value of 0.05 was considered statistically significant.

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